

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
20 January 2011 (20.01.2011)

(10) International Publication Number  
**WO 2011/008974 A2**

PCT

(51) International Patent Classification:  
*A61K 39/155* (2006.01)

(21) International Application Number:  
PCT/US2010/042161

(22) International Filing Date:  
15 July 2010 (15.07.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
61/225,805 15 July 2009 (15.07.2009) US  
61/294,426 12 January 2010 (12.01.2010) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: RSV F PROTEIN COMPOSITIONS AND METHODS FOR MAKING SAME

(57) Abstract: The present invention relates to immunogenic compositions comprising RSV F protein, methods for preparing compositions that contain RSV F protein ecto-domain polypeptides, and to certain engineered RSV F proteins and nucleic acids that encode the engineered RSV F proteins. Compositions prepared using the methods can contain RSV F protein ecto-domain polypeptides in a predominant or single desired form and conformation. The invention also relates to methods for inducing an immune response to RSV F.



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## RSV F PROTEIN COMPOSITIONS AND METHODS FOR MAKING SAME

### RELATED APPLICATIONS

This application claims the benefit of U.S. Patent Application No. 61/225,805, filed on July 15, 2009, and U.S. Patent Application No. 61/294,426, filed on January 12, 2010. The entire teachings of the above applications are incorporated herein by reference.

### BACKGROUND OF THE INVENTION

Respiratory syncytial virus (RSV) is an enveloped non-segmented negative-strand RNA virus in the family *Paramyxoviridae*, genus *Pneumovirus*. It is the most common cause of bronchiolitis and pneumonia among children in their first year of life. RSV also causes repeated infections including severe lower respiratory tract disease, which may occur at any age, especially among the elderly or those with compromised cardiac, pulmonary, or immune systems.

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

The RSV F protein is translated from mRNA into an approximately 574 amino acid protein designated F<sub>0</sub>. Post-translational processing of F<sub>0</sub> includes removal of an N-terminal signal peptide by a signal peptidase in the endoplasmic reticulum. F<sub>0</sub> is also cleaved at two sites (approximately 109/110 and approximately 136/137) by cellular proteases (in particular furin) in the trans-Golgi. This cleavage results in the removal of a short intervening sequence and generates two subunits designated F<sub>1</sub> (~50 kDa; C-terminal; approximately residues 137-574) and F<sub>2</sub> (~20 kDa; N-terminal; approximately residues 1-109) that remain associated with each other. F<sub>1</sub> contains a

hydrophobic fusion peptide at its N-terminus and also two amphipathic heptad-repeat regions (HRA and HRB). HRA is near the fusion peptide and HRB is near the transmembrane domain. Three F<sub>1</sub>-F<sub>2</sub> heterodimers are assembled as homotrimers of F<sub>1</sub>-F<sub>2</sub> in the virion.

5           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 protein. However, for this approach it is desirable that the purified RSV F protein is in a single form and conformation that is stable over time, consistent between vaccine lots, and conveniently purified.

10           The RSV F protein can be truncated, for example by deletion of the transmembrane domain and cytoplasmic tail, to permit its expression as an ectodomain, which may be soluble. In addition, although RSV F protein is initially translated as a monomer, the monomers are cleaved and assemble into trimers. When RSV F protein is in the form of cleaved trimers, the hydrophobic fusion peptide is exposed. The  
15       exposed hydrophobic fusion peptides on different trimers, e.g., soluble ecto-domain trimers, can associate with each other, resulting in the formation of rosettes. The hydrophobic fusion peptides can also associate with lipids and lipoproteins, for example from cells that are used to express recombinant soluble RSV F protein. Due to the complexity of RSV F protein processing, structure and refolding, purified,  
20       homogeneous, immunogenic preparations are difficult to obtain.

          Thus, there is a need for improved RSV F protein compositions and methods for making RSV F protein compositions.

#### SUMMARY OF THE INVENTION

          The invention relates to immunogenic compositions that contain one or more  
25       RSV F polypeptides, and to certain engineered RSV F proteins and nucleic acids that encode the engineered RSV F proteins.

          In one aspect the RSV F protein is soluble. For example, the RSV F protein can have the transmembrane region and cytoplasmic tail deleted. In some aspects, the soluble RSV F contains one or more of 1) one or more mutations to one or both furin-  
30       cleavage sites, 2) one or more mutations to the fusion peptide, 3) one or more mutations to the p27 linker, 4) contains an added oligomerization sequence, and 5) contains an

added amino acid sequence that provides a protease cleavage site. In additional or alternative aspects, the RSV F protein is a monomer, a trimer, or a combination of monomers and trimers. The trimer can be monodispersed or in the form of a rosette. In further additional or alternative aspects, the RSV F protein can be in a prefusion conformation, an intermediate conformation or a postfusion conformation.

In one aspect, the immunogenic composition contains one or more respiratory syncytial virus F (RSV F) polypeptides in which amino acids 100-150 are replaced with the amino acid sequence of SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7; SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:91 or SEQ ID NO:92. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

In another aspect, the immunogenic composition contains an RSV F polypeptide in which amino acids 100-150 of the RSV F are replaced with the amino acid sequence of SEQ ID NO:12. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

In yet another aspect, the immunogenic composition contains an RSV F polypeptide in which amino acids 100-150 of the RSV F are replaced with the amino acid sequence of SEQ ID NO:9, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7; SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:92. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

In another aspect, the immunogenic composition contains an RSV F polypeptide in which amino acids 100-150 are replaced with the amino acid sequence of SEQ ID NO:9. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

In another aspect, the immunogenic composition contains an RSV F polypeptide in which RSV F contains amino acids 23-99 and 151-524 of SEQ ID NO:1 or SEQ ID NO:2. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

In one aspect, the immunogenic composition contains a polypeptide selected from the group consisting of SEQ ID NO:49, SEQ ID NO:68, SEQ ID NO:71, SEQ ID NO: 25, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID



NO:48, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:69, SEQ ID NO:70, SEQ ID  
5 NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, and SEQ ID NO:93. In some embodiments, the signal peptide and/or HIS tag is omitted. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

In one aspect, the immunogenic composition contains SEQ ID NO:68 or alternatively, SEQ ID NO:68 in which the signal peptide, and optionally the HIS tag, is  
10 omitted.

In another aspect, the immunogenic composition contains a polypeptide selected from the group consisting of SEQ ID NO:49, SEQ ID NO:71, and any of the foregoing sequences in which the signal peptide, and optionally the HIS tag, is omitted. In some  
embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

15 In preferred embodiments, the immunogenic composition will include an adjuvant. The adjuvant is preferably an aluminum salt, a squalene-in-water emulsion (such as MF59), a benzonaphthyridine compound, a phospholipid compound (such as E6020), a small molecule immunopotentiator or a combination of any two or more of any of the foregoing.

20 Yet another aspect of the invention includes recombinant RSV F polypeptides. The RSV F may be in the form of a monomer, trimer, rosette of trimers, or combination of monomers and trimers. The recombinant polypeptide may include a heterologous oligomerization domain, an epitope or a signal peptide. The heterologous oligomerization domain is preferably a trimerization domain from influenza  
25 hemagglutinin, from SARS spike, or from HIV gp41, NadA, modified GCN4, or ATCase.

In one aspect, the recombinant RSV F polypeptide has amino acids 100-150 replaced with the amino acid sequence of SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8,  
30 SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:91 or SEQ ID NO:92. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

In another aspect, the recombinant RSV F polypeptide has amino acids 100-150 of the RSV F replaced with the amino acid sequence of SEQ ID NO:12. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

5 In another aspect, the recombinant RSV F polypeptide has amino acids 100-150 of the RSV F replaced with the amino acid sequence of SEQ ID NO:9, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7; SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:92. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

10 In yet another aspect, the recombinant RSV F polypeptide has amino acids 100-150 of the RSV F replaced with the amino acid sequence of SEQ ID NO:9. In some embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

In one aspect, the recombinant polypeptide is selected from the group consisting of SEQ ID NO:49, SEQ ID NO:68, SEQ ID NO:71, SEQ ID NO: 25, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:47, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:93, and any combinations thereof. Optionally, the signal peptide and/or HIS tag is omitted. In some  
20 embodiments the RSV F polypeptide is soluble (e.g., an ectodomain).

Still another aspect includes nucleic acids encoding any of the foregoing  
25 polypeptides. The nucleic acid may be a self-replicating RNA molecule.

Another aspect of the invention is an immunogenic composition comprising a self-replicating RNA that encodes an RSV F polypeptide. The immunogenic composition can include a delivery system.

Another aspect of the invention includes methods of inducing an immune  
30 response to RSV F by administering any of the immunogenic compositions.

The invention relates to methods for preparing compositions and to compositions that contain RSV F protein, such as soluble RSV F ecto-domain

polypeptides, including immunogenic compositions. The RSV F ecto-domain polypeptides can be in a single form, such as uncleaved monomers, uncleaved trimers, cleaved trimers, or rosettes of cleaved trimers. The RSV F ectodomain polypeptides can also be in two or more forms, for example two or more forms that exist in  
5 equilibrium, such as equilibrium between uncleaved monomers and uncleaved trimers. The invention provides several advantages. For example, the presence of a single desired form of RSV F in an immunogenic composition provides a more predictable immune response when the composition is administered to a subject, and more consistent stability and other physical and chemical characteristics when formulated  
10 into a vaccine.

In one aspect, the invention is a method for producing a composition comprising cleaved RSV F protein ecto-domain polypeptides. The method includes a) providing uncleaved RSV F protein ecto-domain polypeptides containing one or more protease cleavage sites that, when cleaved, produce F<sub>1</sub> and F<sub>2</sub> fragments, and b) cleaving the  
15 uncleaved RSV F protein ecto-domain polypeptides with a protease or proteases that recognize the protease cleavage site or sites. In general, the amino acid sequence of the uncleaved RSV F protein ecto-domain polypeptides contains altered furin cleavage sites, and the RSV F protein ecto-domain polypeptides are secreted from a host cell that produces them uncleaved at a position from amino acid 101 to amino acid 161, (e.g., is  
20 not cleaved at the furin cleavage sites at positions 106-109 and 131-136). In some embodiments, the uncleaved RSV F protein ecto-domain polypeptides provided in a) are purified.

The uncleaved RSV F protein ecto-domain polypeptides provided in a) can comprise an intact fusion peptide or an altered fusion peptide (e.g., a deleted fusion  
25 peptide or mutated fusion peptide). When the uncleaved RSV F protein ecto-domain polypeptides provided in a) contain an intact fusion peptide, the cleaving in step b) results in the formation of rosettes of trimers. When the uncleaved RSV F protein ecto-domain polypeptides provided in a) comprise an altered fusion peptide, the cleaving in step b) results in the formation of trimers.

30 The method can further comprise the optional step of purifying the rosettes or trimers produced by cleaving the uncleaved RSV F protein ecto-domain polypeptides.

In preferred embodiments, the cleaved RSV F protein ecto-domain polypeptides produced according to the method are substantially free of lipids and lipoproteins.

In another aspect, the invention is a method for producing a composition comprising uncleaved RSV F protein ecto-domain polypeptide monomers, trimers or a  
5 combination of monomers and trimers. The method includes a) providing a biological material that contains uncleaved RSV F protein ecto-domain polypeptides; and b) purifying uncleaved RSV F protein ecto-domain polypeptide monomers or trimers from the biological material. In general, the amino acid sequence of the uncleaved RSV F  
10 protein ecto-domain polypeptides contains altered furin cleavage sites, and the RSV F protein ecto-domain polypeptides are secreted from a host cell that produces them uncleaved at a position from amino acid 101 to amino acid 161, (e.g., is not cleaved at the furin cleavage sites at positions 106-109 and 131-136). In some embodiments, the amino acid sequence of the uncleaved RSV F protein ecto-domain polypeptides further  
15 contain altered trypsin cleavage sites, and the RSV F protein ecto-domain polypeptides are not cleaved by trypsin at a site between amino acid 101 and amino acid 161. In other embodiments, the amino acid sequence of the uncleaved RSV F protein ecto-domain polypeptides further contain an altered fusion peptide.

In some embodiments, uncleaved RSV F protein ecto-domain polypeptide trimers are purified. In other embodiments, uncleaved RSV F protein ecto-domain  
20 polypeptide monomers are purified. In still other embodiments a mixture of uncleaved RSV F protein ecto-domain monomers and trimers, which may be in a dynamic equilibrium, are purified. In preferred embodiments, the cleaved RSV F protein ecto-domain polypeptides produced according to the method are substantially free of lipids and lipoproteins.

25 In another aspect, the invention is a method for producing a composition comprising cleaved RSV F protein ecto-domain polypeptide monomers, trimers or a combination of monomers and trimers. The method includes a) providing a biological material that contains cleaved RSV F protein ecto-domain polypeptides that contain an altered fusion peptide; and b) purifying cleaved RSV F protein ecto-domain  
30 polypeptides from the biological material.

In some embodiments, cleaved RSV F protein ecto-domain polypeptide trimers are purified. In other embodiments, cleaved RSV F protein ecto-domain polypeptide

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monomers are purified. In still other embodiments a mixture of cleaved RSV F protein ecto-domain monomers and trimers, which may be in a dynamic equilibrium, are purified. In preferred embodiments, the cleaved RSV F protein ecto-domain polypeptides produced according to the method are preferably substantially free of lipids and lipoproteins. In still another embodiment, a cleaved RSV F protein ectodomain trimer containing an altered fusion peptide is purified.

In other aspects, the invention provides compositions, including immunogenic compositions, produced using the methods of the invention.

## 10 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the schematic of the wild type RSV F (FIG 1A) and of a ectodomain construct in which the transmembrane domain and cytoplasmic tail have been removed and an optional HIS<sub>6</sub>-tag has been added to the C-terminus (FIG 1B). For clarity, residue numbering is related to the wild type A2 strain RSV F beginning at the N-terminal signal peptide and is not altered in constructs containing amino acid deletions. Labeled in the schematics is the signal sequence or signal peptide (sp). FIG. 1A is a schematic of RSV F protein showing the signal sequence or signal peptide (SP), p27 linker region, fusion peptide (FP), HRA domain (HRA), HRB domain (HRB), transmembrane region (TM), and cytoplasmic tail (CT). The C-terminal bounds of the ectodomain can vary. FIG 1B is a general schematic of the RSV F ectodomain construct depicting the shared features with the schematics in FIG. 1A and including an optional HIS<sub>6</sub>-tag (HIS TAG). Furin cleavage sites are present at amino acid positions 109/110 and 136/137. FIG. 1C also shows the amino acid sequence of amino acids 100 – 150 of RSV F (wild type) (SEQ ID NO:108) and several proteins (Furmt-SEQ ID NO:3; Furdel-SEQ ID NO:4; Furx-SEQ ID NO:6; Furx R113Q, K123N, K124N-SEQ ID NO:5; Furx R113Q, K123Q, K124Q-SEQ ID NO:92; Delp21 furx-SEQ ID NO:7; Delp23 furx-SEQ ID NO:8; Delp23 furdel-SEQ ID NO:9; N-Term Furin-SEQ ID NO:10; C-term Furin-SEQ ID NO:11; Fusion Peptide Deletion1-SEQ ID NO:12; and Factor Xa-SEQ ID NO:13) in which the one or both furin cleavage sites and/or fusion peptide region were mutated or deleted. In FIG. 1C, the symbol “-” indicates that the amino acid at that position is deleted.

FIG. 2 shows the amino acid sequence of the carboxy terminus from amino acid position 488 to the start of the TM region of RSV F (wild type) (SEQ ID NO:94) and several proteins (SEQ ID NOS:95-100) that contain added protease cleavage sites. In FIG. 2, the symbol “-” indicates that there is no amino acid at that position.

5           FIG. 3 is a chromatogram and image of an electrophoresis gel showing the purification of RSV F monomers (3) using size exclusion chromatography.

FIGS. 4A-4F shows the nucleotide sequence (SEQ ID NO:101) of the plasmid encoding the pT7-TC83R-FL.RSVF (A317) self-replicating RNA molecule which encodes the respiratory syncytial virus F glycoprotein (RSV-F). The nucleotide  
10           sequence encoding RSV-F is underlined.

FIG. 5 is an alignment of the amino acid sequences of F proteins from several strains of RSV. The alignment was prepared using the algorithm disclosed by Corpet, Nucleic Acids Research, 1998, 16(22):10881-10890, using default parameters (Blossum 62 symbol comparison table, gap open penalty: 12, gap extension penalty:  
15           A2, F protein of the strain A2 (accession number AF035006) (SEQ ID NO:102); CP52, F protein of the CP52 strain (accession number AF013255) (SEQ ID NO:103); B, F protein of the B strain (accession number AF013254) (SEQ ID NO:104); long, F protein of the long strain (accession number AY911262) strain (SEQ ID NO:105), and 18537strain, F protein of the 18537 strain (accession number Swiss Prot P13843) (SEQ  
20           ID NO:106). A consensus of F protein sequences is also shown (SEQ ID NO:107)

FIG. 6 shows relevant regions of size exclusion (SEC) chromatograms from select RSV F antigen purifications. The principle peak containing the indicated antigen is indicated by an asterisk with the retention time of the Superdex P200 16/60 column (GE Healthcare) is indicated in milliliters. On a calibrated column, the approximate  
25           retention times of 47 mls, 65 mls and 77 mls correspond to the column void volume, the retention of the RSV F trimer and the retention of the RSV F monomer, respectively. In FIG. 6A, the uncleaved Delp23 Furdel ( $\Delta$ p23 Furdel) construct is purified from the monomer peak at approximately 77 mls. When the uncleaved Delp23 Furdel RSV F antigen is treated with trypsin, the protein can form rosettes, which now migrate on  
30           SEC in the void volume at approximately 47 mls (FIG. 6B). The cleaved trimer species of RSV F fusion peptide deletion is purified from the trimer peak at approximately 65

mls retention time (FIG. 6C) while the uncleaved Delp21 Furx construct ( $\Delta p21$  Furx) is purified from the monomer peak at approximately 77 mls (FIG. 6D).

FIG. 7 shows representative EM images of select RSV F antigens. FIG. 7A shows an EM image of RSV F  $\Delta p23$  (Delp23) before trypsin treatment. The crutch shapes in FIG. 7A, consistent with a postfusion trimer conformation, are not always observed in the uncleaved  $\Delta p23$  (Delp23) Furdel construct. When the  $\Delta p23$  (Delp23) Furdel construct is treated with trypsin and purified from the void volume of an SEC column and observed by EM the proteins are found to have adopted rosette conformations (FIG. 7B). When the RSV F fusion peptide deletion construct is purified from the trimer peak on an SEC column a monodispersed crutch shape is observed, consistent with the a postfusion trimer (FIG. 7C). Shown in FIG. 7D are three preparations of either  $\Delta p21$  (Delp21) furx RSV F (labeled Monomer), Fusion peptide deletion RSV F (lanes labeled Trimer) and purified RSV F rosettes (labeled Rosettes). The gel contains several lanes of GE Full Range Standard (molecular weights standard are labeled to the left of the gel) while approximate retention times of RSV F fragments are indicated on the right of the gel.

FIGS. 8A-8C are graphs showing that monomers (uncleaved  $\Delta p21$  (Delp21) furx), rosettes of trimers (cleaved  $\Delta p23$  (Delp23) Furdel), and trimers (fusion peptide deletion) of RSV F ecto-domain polypeptides are immunogenic in cotton rats. Serum titers of anti-RSV F IgG and neutralizing anti-RSV antibodies were measured 2 weeks after the 1<sup>st</sup> vaccination (2wp1), 3 weeks after the 1<sup>st</sup> vaccination (3wp1), and/or 2 weeks after the 2<sup>nd</sup> vaccination (2wp2).

#### DETAILED DESCRIPTION OF THE INVENTION

The invention relates to respiratory syncytial virus F (RSV F) polypeptides and/or proteins, immunogenic compositions comprising RSV F polypeptides and/or proteins, methods for producing RSV F polypeptides and/or proteins and compositions comprising RSV F polypeptides and/or proteins, and nucleic acids that encode RSV F polypeptides and/or proteins.

In general, the immunogenic compositions comprise RSV F polypeptides and/or proteins that contain mutations (e.g., amino acid replacements, deletions or additions) which provide beneficial characteristics, such as one or more of 1) stabilized prefusion

or intermediate (non-post fusion) conformation, 2) reduced or eliminated exposure of the fusion peptide, 3) improved stability (e.g., reduced aggregation and/or degradation, and 4) more closely resemble active F1/F2 viral protein. These characteristics provide advantages for the immunogenic compositions and for the manufacture of the

5 immunogenic compositions. For example, as described herein, non-post fusion conformations of RSV F protein (i.e., prefusion conformation, intermediate conformations) can be better immunogens and elicit a better neutralizing antibody response. Reducing or eliminating the exposure of the fusion peptide, e.g., by introducing mutations or deletions into the furin cleavage sites, will reduce the

10 hydrophobicity of the polypeptide and facilitate purifications, and also reduce or eliminate the RSV F protein from associating with cell membranes in a subject to whom the protein is administered. Improved stability of the protein facilitates producing immunogenic compositions in which the protein has a decreased tendency to aggregate or degrade, which provides a more predictable immune response when the

15 composition is administered to a subject. Finally, mutant RSV F polypeptides or proteins that resemble F1/F2 viral protein, for example by deletion of all or part of the p27 linker region, may elicit a better neutralizing antibody response. Other advantages of the invention are described herein.

The invention also relates to methods for preparing compositions that contain

20 RSV F protein, in particular RSV F ecto-domain polypeptides, and to compositions including immunogenic compositions comprising RSV F protein. Preferably, the RSV F ecto-domain polypeptides are in a single form or in a dynamic equilibrium between known forms.

## 25 Definitions

As used herein "population" refers to more than one RSV F polypeptide or protein that is present in a composition. The population can be substantially homogenous, in which substantially all RSV F polypeptides or proteins are substantially the same (e.g., same amino acid sequence, same conformation),

30 heterogenous, or have a desired degree of homogeneity (e.g., at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about



95%, at least about 99% of the RSV F polypeptides or proteins are prefusion conformation, are postfusion conformation, are monomers, are trimers).

The “post fusion conformation” of RSV F protein is a trimer characterized by the presence of a six-helix bundle comprising 3 HRB and 3HRA regions.

5       The “pre-fusion conformation” of RSV F protein is a conformation characterized by a trimer that contains a triple helix comprising 3 HRB regions.

As used herein, “RSV F ecto-domain polypeptide” refers to an RSV F protein polypeptide that contains substantially the extracellular portion of mature RSV F protein, with or without the signal peptide (e.g., about amino acids 1 to about amino acid 524, or about amino acid 22 to about amino acid 524) but lacks the transmembrane domain and cytoplasmic tail of naturally occurring RSV F protein.

As used herein, “cleaved RSV F ecto-domain polypeptide” refers to a RSV F ectodomain polypeptide that has been cleaved at one or more positions from about 101/102 to about 160/161 to produce two subunits, in which one of the subunits  
15       comprises F<sub>1</sub> and the other subunit comprises F<sub>2</sub>.

As used herein, “C-terminal uncleaved RSV F ecto-domain polypeptide” refers to an RSV F ectodomain polypeptide that is cleaved at one or more positions from about 101/102 to about 131/132, and is not cleaved at one or more positions from about 132/133 to about 160/161, to produce two subunits, in which one of the subunits  
20       comprises F<sub>1</sub> and the other subunit comprises F<sub>2</sub>.

As used herein, “uncleaved RSV F ecto-domain polypeptide” refers to an RSV F ectodomain polypeptide that is not cleaved at one or more positions from about 101/102 to about 160/161. An uncleaved RSV F ecto-domain polypeptide can be, for example, a monomer or a trimer.

25       As used herein, “fusion peptide” refers to amino acids 137-154 of RSV F protein.

As used herein, “altered fusion peptide” refers to a fusion peptide in which one or more amino acids are independently replaced or deleted, including replacement or deletion of all amino acids from positions 137-154. Preferably, cleaved RSV F ecto-domain polypeptides that contain an “altered fusion peptide” do not form rosettes.

As used herein, a “purified” protein or polypeptide is a protein or polypeptide which is recombinantly or synthetically produced, or produced by its natural host, and

has been isolated from other components of the recombinant or synthetic production system or natural host such that the amount of the protein relative to other macromolecular components present in a composition is substantially higher than that present in a crude preparation. In general, a purified protein will be at least about 50%  
5 homogeneous and more preferably at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or substantially homogeneous.

As used herein, “substantially free of lipids and lipoproteins” refers to compositions, proteins and polypeptides that are at least about 95% free of lipids and lipoproteins on a mass basis when protein and/or polypeptide (e.g., RSV F polypeptide)  
10 purity is observed on an SDS PAGE gel and total protein content is measured using either UV280 absorption or BCA analysis, and lipid and lipoprotein content is determined using the Phospholipase C assay (Wako, code no. 433-36201).

As used herein, “altered furin cleavage site” refers the amino acid sequence at about positions 106-109 and at about positions 133-136 in naturally occurring RSV F  
15 protein that are recognized and cleaved by furin or furin-like proteases, but in an uncleaved RSV F protein ecto-domain polypeptide contains one or more amino acid replacements, one or more amino acid deletions, or a combination of one or more amino acid replacement and one or more amino acid deletion, so that an RSV F ecto-domain polypeptide that contains an altered furin cleavage site is secreted from a cell  
20 that produces it uncleaved at the altered furin cleavage site.

Features of RSV F protein ecto-domains suitable for use in this invention are described herein with reference to particular amino acids that are identified by the position of the amino acid in the sequence of RSV F protein from the A2 strain (SEQ ID NO:1). RSV F protein ecto-domains can have the amino acid sequence of the F  
25 protein from the A2 strain or any other desired strain. When the F protein ecto-domain from a strain other than the A2 strain is used, the amino acids of the F protein are to be numbered with reference to the numbering of the F protein from the A2 strain, with the insertion of gaps as needed. This can be achieved by aligning the sequence of any desired RSV F protein with the F protein of the strain A2, as shown herein for F  
30 proteins from the A2 strain, CP52 strain, B strain, long strain, and the 18537 strain. *See, FIG. 5.* Sequence alignments are preferably produced using the algorithm disclosed by Corpet, Nucleic Acids Research, 1998, 16(22):10881-10890, using default

parameters (Blossum 62 symbol comparison table, gap open penalty: 12, gap extension penalty: 2).

The invention provides soluble RSV F polypeptides and proteins, and immunogenic compositions comprising the soluble RSV F polypeptides and proteins, as well as compositions comprising nucleic acids (e.g., self-replicating RNA molecules) that encode the soluble RSV F polypeptides and proteins.

The RSV F polypeptides (e.g., ecto-domain polypeptides) can be in any desired form, such as in a single form, such as uncleaved monomers, uncleaved trimers, cleaved trimers, or rosettes of cleaved trimers. The RSV F ectodomain polypeptides can also be in two or more forms, for example two or more forms that exist in equilibrium, such as equilibrium between uncleaved monomers and uncleaved trimers. The invention provides several advantages. For example, the presence of a single desired form of RSV, or a dynamic equilibrium between known forms, in an immunogenic composition, provides for more predictable formulation, solubility and stability, and for a more predictable immune response when the composition is administered to a subject.

Preferably, the RSV F ecto-domain polypeptides are in a single form, such as uncleaved monomers, uncleaved trimers, cleaved trimers, rosettes of cleaved trimers, or in a dynamic equilibrium between a subset of such forms (e.g., equilibrium between uncleaved monomers and uncleaved trimers).

In one aspect of the invention, the RSV F polypeptides and proteins are in pre-fusion conformation. The epitopes of the pre-fusion conformation may be better able to elicit antibodies that can recognize and neutralize natural virions.

In one embodiment of the invention an immunogenic composition comprises a population of respiratory syncytial virus F glycoproteins in pre-fusion conformation. In another aspect of the invention, an immunogenic composition comprises a population of respiratory syncytial virus F glycoproteins which disfavor the post-fusion conformation as compared to a population of isolated RSV F glycoproteins.

The invention also provides an immunogenic composition comprising a polypeptide that displays an epitope present in a pre-fusion or an intermediate fusion conformation of respiratory syncytial virus F glycoprotein but absent the glycoprotein's post-fusion conformation.

### The F glycoprotein

The F glycoprotein of RSV directs viral penetration by fusion between the virion envelope and the host cell plasma membrane. It is a type I single-pass integral membrane protein having four general domains: N-terminal ER-translocating signal sequence (SS), ectodomain (ED), transmembrane domain (TM), and a cytoplasmic tail (CT). CT contains a single palmitoylated cysteine residue. The sequence of F protein is highly conserved among RSV isolates, but is constantly evolving (7). Unlike most paramyxoviruses, the F protein in RSV can mediate entry and syncytium formation independent of the other viral proteins (HN is usually necessary in addition to F in other paramyxoviruses).

The hRSV F mRNA is translated into a 574 amino acid precursor protein designated F<sub>0</sub>, which contains a signal peptide sequence at the N-terminus that is removed by a signal peptidase in the endoplasmic reticulum. F<sub>0</sub> is cleaved at two sites (a.a. 109/110 and 136/137) by cellular proteases (in particular furin) in the trans-Golgi, removing a short glycosylated intervening sequence and generating two subunits designated F<sub>1</sub> (~50 kDa; C-terminus; residues 137-574) and F<sub>2</sub> (~20 kDa; N-terminus; residues 1-109) (See, *e.g.*, FIG. 1). F<sub>1</sub> contains a hydrophobic fusion peptide at its N-terminus and also two hydrophobic heptad-repeat regions (HRA and HRB). HRA is near the fusion peptide and HRB is near to the transmembrane domain (See, *e.g.*, FIG. 1). The F<sub>1</sub>-F<sub>2</sub> heterodimers are assembled as homotrimers in the virion.

RSV exists as a single serotype but has two antigenic subgroups: A and B. The F glycoproteins of the two groups are about 90% identical. The A subgroup, the B subgroup, or a combination or hybrid of both can be used in the invention. An example sequence for the A subgroup is SEQ ID NO: 1 (A2 strain; GenBank GI: 138251; Swiss Prot P03420), and for the B subgroup is SEQ ID NO: 2 (18537 strain; GI: 138250; Swiss Prot P13843). SEQ ID NO:1 and SEQ ID NO:2 are both 574 amino acid sequences. The signal peptide in A2 strain is a.a. 1-21, but in 18537 strain it is 1-22. In both sequences the TM domain is from about a.a. 530-550, but has alternatively been reported as 525-548.

SEQ ID NO: 1

1 MELLILKANAIITILTAVTF CFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE 60

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	61	LSNIKENKCNCGTDAKVKLKQELDKYKNAVTELQLLMQSTPPTNNRARRELPRFMNYTLN	120
	121	NAKKTNTVLSKKRKRRLGFLGVSASIAVGAVSKVLHLEGEVNKIKSALLSTNKAVVS	180
	181	LSNGVSVLTSKVLDLKNIYIDKQLLPVNVKQSCSISNIETVIEFQQKNNRLLLEITREFSVN	240
	241	AGVTPPVSTYMLTNSSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV	300
5	301	VQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKV	360
	361	QSNRVFCDTMNSLTLPSEINLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT	420
	421	KCTASNKNRGIKTFSSNGCDYVSNKGMDTVSVGNTRYVNVKQEGKSLYVKGEPIINFYDP	480
	481	LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTTIIVIVILLS	540
	541	LIAVGLLLYCKARSTPVTLSKDQLSGINNIASFSN	574
10			
		SEQ ID NO: 2	
	1	MELLIHRSSAIFLTLAVNALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE	60
	61	LSNIKETKCNCGTDTKVKLKQELDKYKNAVTELQLLMQNTPAANNRARREAPQYMNYTIN	120
	121	TTKNLNVSISKRRKRRLGFLGVSASIAVGAVSKVLHLEGEVNKIKNALLSTNKAVVS	180
15	181	LSNGVSVLTSKVLDLKNIYINNRLLPVNVKQSCRISNIETVIEFQQMNSRLLLEITREFSVN	240
	241	AGVTPPLSTYMLTNSSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYV	300
	301	VQLPIYGVIDTPCWKLHTSPLCTTNIKEGSNICLTRDRGWYCDNAGSVSFFPQADTCKV	360
	361	QSNRVFCDTMNSLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKT	420
	421	KCTASNKNRGIKTFSSNGCDYVSNKGVDTVSVGNTRYVNVKLEGKNLYVKGEPIINYYDP	480
20	481	LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNTGKSTTNIMITTTIIVIVVLLS	540
	541	LIAIGLLLYCKAKNTPVTLSKDQLSGINNIASFSK	574

The invention may use any desired RSV F amino acid sequence, such as the amino acid sequence of SEQ ID NO: 1 or 2, or a sequence having identity to SEQ ID NO: 1 or 2. Typically it will have at least 75% identity to SEQ ID NO: 1 or 2 *e.g.*, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, identity to SEQ ID NO: 1 or 2. The sequence may be found naturally in RSV.

Where the invention uses an ectodomain of F protein, in whole or in part, it may comprise:

- (i) a polypeptide comprising about amino acid 22-525 of SEQ ID NO: 1.
- (ii) a polypeptide comprising about amino acids 23-525 of SEQ ID NO: 2.
- (iii) a polypeptide comprising an amino acid sequence having at least 75% identity (*e.g.*, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99% identity) to (i) or (ii).
- (iv) a polypeptide comprising a fragment of (i), (ii) or (iii), wherein the fragment comprises at least one F protein epitope. The fragment will usually be at least about

100 amino acids long, *e.g.*, at least about 150, at least about 200, at least about 250, at least about 300, at least about 350, at least about 400, at least about 450 amino acids long.

The ectodomain can be an F<sub>0</sub> form with or without the signal peptide, or can  
5 comprises two separate peptide chains (*e.g.*, an F<sub>1</sub> subunit and a F<sub>2</sub> subunit) that are associated with each other, for example, the subunits may be linked by a disulfide bridge. Accordingly, all or a portion of about amino acid 101 to about 161, such as amino acids 110-136, may be absent from the ectodomain. Thus the ectodomain, in whole or in part, can comprise:

10 (v) a first peptide chain and a second peptide chain that is associated with the first polypeptide chain, where the first peptide chain comprises an amino acid sequence having at least 75% identity (*e.g.*, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or even 100% identity) to about amino acid 22 to about amino acid 101 of SEQ ID NO: 1 or to about amino acid 23 to about amino  
15 acid 101 of SEQ ID NO: 2, and the second peptide chain comprises an amino acid sequence having at least 75% identity (*e.g.*, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or even 100% identity) to about amino acid 162 to about 525 of SEQ ID NO: 1 or to about amino acid 162 to 525 of SEQ ID NO: 2.

20 (vi) a first peptide chain and a second peptide chain that is associated with the first polypeptide chain, where the first peptide chain comprises an amino acid sequence comprising a fragment of about amino acid 22 to about amino acid 101 of SEQ ID NO: 1 or of about amino acid 23 to about amino acid 109 of SEQ ID NO: 2, and the second peptide chain comprises a fragment of about amino acid 162 to about amino acid 525 of  
25 SEQ ID NO: 1 or of about amino acid 161 to about amino acid 525 of SEQ ID NO: 2. One or both of the fragments will comprises at least one F protein epitope. The fragment in the first peptide chain will usually be at least 20 amino acids long, *e.g.*, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80 amino acids long. The fragment in the second peptide chain will usually be at least 100 amino acids long, *e.g.*,  
30 at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450 amino acids long.

(vii) a molecule obtainable by furin digestion of (i), (ii), (iii) or (iv).

Thus an amino acid sequence used with the invention may be found naturally within RSV F protein (e.g., a soluble RSV F protein lacking TM and CT, about amino acids 522-574 of SEQ ID NOS: 1 or 2), and/or it may have one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30) single amino acid mutations (insertions, deletions or substitutions) relative to a natural RSV sequence. For instance, it is known to mutate F proteins to eliminate their furin cleavage sequences, thereby preventing intracellular processing. In certain embodiments, the RSV F protein lacks TM and CT (about amino acids 522-574 of SEQ ID NOS: 1 or 2) and contains one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30) single amino acid mutations (insertions, deletions or substitutions) relative to a natural RSV sequence.

#### **Furin-cleavage, Trypsin-cleavage and Fusion Peptide Mutations**

RSV F polypeptides or proteins may contain one or more mutations that prevent cleavage at one or both of the furin cleavage sites (*i.e.*, amino acids 109 and 136 of SEQ ID NOS: 1 and 2). These mutations can prevent aggregation of the soluble polypeptides or proteins and thereby facilitate purifications, can prevent cell-cell fusion if the RSV F protein is expressed on the surface of a cell, such as by expression from a viral replicon (e.g., alphavirus replicon particles), or if the RSV F protein is a component of a virus-like particle. These mutations, alone or in combination with other mutations described herein, may also stabilize the protein in the pre-fusion conformation.

Examples of suitable furin cleavage mutations include replacement of amino acid residues 106 – 109 of SEQ ID NO: 1 or 2 with RARK (SEQ ID NO:77), RARQ (SEQ ID NO:78), QAQN (SEQ ID NO:79), or IEGR (SEQ ID NO:80). Alternatively, or in addition, amino acid residues 133 – 136 of SEQ ID NO: 1 or 2 can be replaced with RKKK (SEQ ID NO:81),  $\Delta\Delta\Delta$ R, QNQN (SEQ ID NO:82), QQQR (SEQ ID NO:83) or IEGR (SEQ ID NO:80). ( $\Delta$  indicates that the amino acid residue has been deleted.) These mutations can be combined, if desired, with other mutations described herein, such as mutations in the p27 region (amino acids 110-136 of SEQ ID NOS: 1 or 2), including deletion of the p27 region in whole or in part.

These furin cleavage mutations can be combined, if desired, with other mutations described herein, such as trypsin cleavage mutations and fusion peptide mutations. Examples of suitable trypsin cleavage mutations include deletion of any lysine or arginine residue between about position 101 and position 161 of SEQ ID NO:1 or 2, or replacement of any such lysine or arginine residue with an amino acid other than lysine or arginine. For example, lysine and/or arginine residues in the p27 region (about amino acids 110-136 of SEQ ID NOS: 1 or 2) can be substituted or deleted, including deletion of the p27 region in whole or in part.

Alternatively or in addition to the furin-cleavage mutations, RSV F polypeptides or proteins may contain one or more mutations in the fusion peptide region (amino acids 137 and 153 of SEQ ID NOS: 1 or 2). For example, this region can be deleted in whole or in part.

In particular embodiments, the sequence of amino acid residue 100 – 150 of the RSV F polypeptide or protein, such as SEQ ID NO:1, SEQ ID NO:2, or the soluble ectodomains thereof, is

(Furmt) TPATNNRARKELPRFMNYTLNNAKKTNVTLSKKRKKKFLGFLLGVGSAIAS (SEQ ID NO:3)

(Furdel) TPATNNRARQELPRFMNYTLNNAKKTNVTLSKK---RFLGFLLGVGSAIAS (SEQ ID NO:4)

(Furx) TPATNNQAQNELPRFMNYTLNNAKKTNVTLSQNQNQNFLGFLLGVGSAIAS (SEQ ID NO:6)

(Furx R113Q, K123N, K124N)  
TPATNNQAQNELPQFMNYTLNNAANTNVTLSQNQNQNFLGFLLGVGSAIAS (SEQ ID NO:5)

(Furx R113Q, K123Q, K124Q)  
TPATNNQAQNELPQFMNYTLNNAQQTNVTLSQNQNQNFLGFLLGVGSAIAS (SEQ ID NO:92)

(Delp21Furx) TPATNNQAQN-----QNQNQNFLGFLLGVGSAIAS (SEQ ID NO:7)

(Delp23Furx) TPATNNQAQN-----QNQNQNFLGFLLGVGSAIAS (SEQ ID NO:8)



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(Delp21 furdel) TPATNNRARQ-----QNQQQRFLGFLLGVGSAIAS  
(SEQ ID NO:109)

(Delp23furdel) TPATNNRARQ-----QQQRFLGFLLGVGSAIAS (SEQ  
ID NO:9)

5 (Nterm Furin) TPATNNRARREL PQFMNYTLNNAQQTNVTLSQNQNQNFLGFLLGVGSAIAS (SEQ  
ID NO:10)

(Cterm Furin) TPATNNQAQNELPQFMNYTLNNAQQTNVTLSKKRKRRLGFLLGVGSAIAS (SEQ  
ID NO:11)

10 (Fusion peptide deletion 1) TPATNNRARRELPRFMNYTLNNAKKTNVTLSKKRKR-----  
-----SAIAS (SEQ ID NO:12),

(Fusion peptide deletion 2) TPATNNRARRELPRFMNYTLNNAKKTNVTLSKKRKR-----  
-GVGSAIAS (SEQ ID NO:91), or

(Factor Xa) TPATNNIEGRELPRFMNYTLNNAKKTNVTLSKKIEGRFLGFLLGVGSAIAS (SEQ  
ID NO:13); wherein the symbol “-” indicates that the amino acid at that position is  
15 deleted.

In addition to furin-cleavage and fusion peptide mutations, or alternatively,  
soluble RSV F polypeptides or proteins, such as those that lack the transmembrane  
region and cytoplasmic tail, may contain one or more oligomerization sequences.  
When an oligomerization sequence is present, it is preferably a trimerization sequence.  
20 Suitable oligomerization sequences are well known in the art and include, for example,  
the coiled coil of the yeast GCN4 leucine zipper protein, trimerizing sequence from  
bacteriophage T4 fibrin (“foldon”), and the trimer domain of influenza HA. These  
and other suitable oligomerization sequences are described in greater detail herein.

In particular embodiments, the sequence of the carboxy terminus of the RSV F  
25 polypeptide or protein, starting from position 480, is

(GCN) PLVFPSEFDASISQVNEKINQSLAFIRKSDELLHNVNDKIEEILSKIYHIENEIARIKKLIGE  
(SEQ ID NO:14)

30 (HA) PLVFPSEFDASISQVNEKINQSLAFIRKSDELLHNVNEKFHQIEKEFSEVEGRIQDLEK (SEQ  
ID NO:15)

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(Idealized helix) PLVFPSDEFDASISQINEKINQILAFIRKIDELLHNIN (SEQ ID NO:16)

(foldon short)

PLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNGSGYIPEAPRDGQAYVRKDGWVLLSTFL

5 (SEQ ID NO:17); or

(foldon long)

PLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNNKNDDKSGYIPEAPRDGQAYVRKDGWVLLS  
TFL (SEQ ID NO:18)

In addition to any combination of furin-cleavage mutations, fusion peptide  
10 mutations and added oligomerization sequences, or alternatively, RSV F polypeptides  
or proteins that contain a transmembrane region may contain an added amino acid  
sequence that provides a protease cleavage site. This type of RSV F polypeptide or  
protein can be produced by expression on the surface of a cell, and recovered in soluble  
form after cleavage from the cell surface using an appropriate protease. Generally, the  
15 amino acid sequence that provides a protease cleavage site will be located within about  
60 amino acids, about 50 amino acids, about 40 amino acids, about 30 amino acids,  
about 20 amino acids, about 10 amino acids, or substantially adjacent to the amino  
terminus of the transmembrane domain (amino acid 525 of SEQ ID NO:1 or 2). Many  
suitable amino acid sequences that are cleaved by commercially available proteases are  
20 well-known in the art. For example, thrombin cleaves the sequence LVPR (SEQ ID  
NO:75), factor Xa cleaves the sequence IEGR and enterokinase cleaves the sequence  
DDDDK (SEQ ID NO:76). These amino acid sequences can be introduced into an  
RSV F polypeptide. In particular embodiments, the sequence of the RSV F polypeptide  
or protein, starting from position 488 to the TM region is a sequence shown in FIG. 2.

25 Immunogenic polypeptides used according to the invention will usually be  
isolated or purified. Thus, they will not be associated with molecules with which they  
are normally, if applicable, found in nature. For example, an F protein used with the  
invention will not be in the form of a RSV virion (although it may be in the form of an  
artificial virion, such as a virosome or VLP).

30 Polypeptides will usually be prepared by expression in a recombinant host  
system. Generally, they (e.g., RSV ecto-domains) are produced by expression of  
recombinant constructs that encode the ecto-domains in suitable recombinant host cells,

although any suitable methods can be used. Suitable recombinant host cells include, for example, insect cells (e.g., *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*), mammalian cells (e.g., human, non-human primate, horse, cow, sheep, dog, cat, and rodent (e.g., hamster), avian cells (e.g., chicken, duck, and geese), bacteria (e.g., *E. coli*, *Bacillus subtilis*, and *Streptococcus* spp.), yeast cells (e.g., *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillermondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*), Tetrahymena cells (e.g., *Tetrahymena thermophila*) or combinations thereof. Many suitable insect cells and mammalian cells are well-known in the art. Suitable insect cells include, for example, Sf9 cells, Sf21 cells, Tn5 cells, Schneider S2 cells, and High Five cells (a clonal isolate derived from the parental *Trichoplusia ni* BTI-TN-5B1-4 cell line (Invitrogen)). Suitable mammalian cells include, for example, Chinese hamster ovary (CHO) cells, human embryonic kidney cells (HEK293 cells, typically transformed by sheared adenovirus type 5 DNA), NIH-3T3 cells, 293-T cells, Vero cells, HeLa cells, PERC.6 cells (ECACC deposit number 96022940), Hep G2 cells, MRC-5 (ATCC CCL-171), WI-38 (ATCC CCL-75), fetal rhesus lung cells (ATCC CL-160), Madin-Darby bovine kidney ("MDBK") cells, Madin-Darby canine kidney ("MDCK") cells (e.g., MDCK (NBL2), ATCC CCL34; or MDCK 33016, DSM ACC 2219), baby hamster kidney (BHK) cells, such as BHK21-F, HKCC cells, and the like. Suitable avian cells include, for example, chicken embryonic stem cells (e.g., EBx® cells), chicken embryonic fibroblasts, chicken embryonic germ cells, duck cells (e.g., AGE1.CR and AGE1.CR.pIX cell lines (ProBioGen) which are described, for example, in *Vaccine* 27:4975-4982 (2009) and WO2005/042728), EB66 cells, and the like.

Suitable insect cell expression systems, such as baculovirus systems, are known to those of skill in the art and described in, e.g., Summers and Smith, *Texas Agricultural Experiment Station Bulletin* No. 1555 (1987). Materials and methods for baculovirus/insert cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA. Avian cell expression systems are also known to those of skill in the art and described in, e.g., U.S. Patent Nos. 5,340,740; 5,656,479; 5,830,510; 6,114,168; and 6,500,668; European Patent No. EP 0787180B; European

Patent Application No. EP03291813.8 ;WO 03/043415; and WO 03/076601. Similarly, bacterial and mammalian cell expression systems are also known in the art and described in, *e.g.*, *Yeast Genetic Engineering* (Barr *et al.*, eds., 1989) Butterworths, London.

5           Recombinant constructs encoding RSV F protein ecto-domains can be prepared in suitable vectors using conventional methods. A number of suitable vectors for expression of recombinant proteins in insect or mammalian cells are well-known and conventional in the art. Suitable vectors can contain a number of components, including, but not limited to one or more of the following: an origin of replication; a  
10   selectable marker gene; one or more expression control elements, such as a transcriptional control element (*e.g.*, a promoter, an enhancer, a terminator), and/or one or more translation signals; and a signal sequence or leader sequence for targeting to the secretory pathway in a selected host cell (*e.g.*, of mammalian origin or from a heterologous mammalian or non-mammalian species). For example, for expression in  
15   insect cells a suitable baculovirus expression vector, such as pFastBac (Invitrogen), is used to produce recombinant baculovirus particles. The baculovirus particles are amplified and used to infect insect cells to express recombinant protein. For expression in mammalian cells, a vector that will drive expression of the construct in the desired mammalian host cell (*e.g.*, Chinese hamster ovary cells) is used.

20           RSV F protein ecto-domain polypeptides can be purified using any suitable methods. For example, methods for purifying RSV F ecto-domain polypeptides by immunoaffinity chromatography are known in the art. *Ruiz-Arguello et al.*, *J. Gen. Virol.*, 85:3677-3687 (2004). Suitable methods for purifying desired proteins including precipitation and various types of chromatography, such as hydrophobic interaction, ion  
25   exchange, affinity, chelating and size exclusion are well-known in the art. Suitable purification schemes can be created using two or more of these or other suitable methods. If desired, the RSV F protein ecto-domain polypeptides can include a “tag” that facilitates purification, such as an epitope tag or a HIS tag. Such tagged polypeptides can conveniently be purified, for example from conditioned media, by  
30   chelating chromatography or affinity chromatography.

The RSV F polypeptides may also be produced *in situ* by expression of nucleic acids that encode them in the cells of a subject. For example, by expression of a self-replicating RNA described herein.

Polypeptides may include additional sequences in addition to the RSV sequences. For example, a polypeptide may include a sequence to facilitate purification (e.g., a poly-His sequence). Similarly, for expression purposes, the natural leader peptide of F protein may be substituted for a different one. For example, reference 6 used a honeybee melittin leader peptide in place of the natural one.

#### **Form and Conformation of Polypeptides**

10 The invention includes immunogenic compositions that include any of the forms and conformations of RSV F polypeptides and proteins disclosed herein, including any desired combination of the forms and conformations of RSV F polypeptides and proteins disclosed herein. The RSV F polypeptide can be a monomer, or the RSV F protein can be a trimer comprising three monomer polypeptides. Trimers can be  
15 monodispersed or can be in the form of a rosette, for example, due to interactions between the fusion peptides of individual trimers. Immunogenic compositions may comprise polypeptides that are monomers, trimers, a combination of monomers and trimers (e.g., in dynamic equilibrium), rosettes of trimers, and any combination of the foregoing. In addition, as described further herein, the RSV F protein can be in a post-  
20 fusion conformation, a pre-fusion conformation, or intermediate conformation.

The RSV F protein can be in a pre-fusion conformation, a post-fusion conformation or an intermediate conformation. The “post fusion conformation” of RSV F protein is believed to be the low energy conformation of native RSV F, and is a trimer characterized by the presence of a six-helix bundle comprising 3 HRB and 3HRA  
25 regions. The post-fusion conformation has a characteristic “crutch” or “golf tee” shape by electron microscopy. The “pre-fusion conformation” of RSV F protein is a conformation characterized by a trimer that contains a coiled coil comprising 3 HRB regions. The fusion peptide is not exposed in the pre-fusion conformation and, therefore, prefusion conformations generally do not form rosettes, and have a a  
30 “lollipop” or “ball and stem” shape by electron microscopy.

In some aspects, the RSV F protein is in the post-fusion conformation. For example, the RSV F protein can be in the form of a monodisperse trimer in the post-fusion conformation, or in the form of a rosette comprised of post-fusion trimers.

In some embodiments, the RSV F polypeptide is a monomer. In some  
5       embodiments, the RSV F polypeptide is a trimer.

In other aspects, the RSV F protein is in the pre-fusion conformation. Without wishing to be bound by any particular theory, it is believed that the pre-fusion conformation or intermediate forms 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  
10       provide advantages for eliciting neutralizing antibodies.

Some aspects of the invention use a polypeptide that disfavors the post-fusion conformation of the F protein. Preferably, the polypeptides (in whole or in part) will display an epitope of the pre-fusion F protein or an epitope of an intermediate conformation in the conversion from the pre-fusion conformation to the post-fusion  
15       conformation. These polypeptides may be native or mutated F proteins in a pre-fusion state, may be native or mutated F proteins in an intermediate conformation, or may be a population of native or mutated proteins where the post-fusion conformation has been disfavored or preferentially excluded. In certain instances the native or mutated protein may be combined with one or more additional molecules that assist in maintaining the  
20       polypeptides in one of the foregoing states such as a monoclonal antibody that preferentially binds the pre-fusion conformation or an intermediate conformation. In addition, the polypeptides may be derivatives of native F proteins. Such derivatives include polypeptides comprising one or more fragments of a native F protein, fusion polypeptides comprising a native F protein (or fragment thereof) and a heterologous  
25       sequence, and polypeptides comprising a native F protein sequence having one or more mutations. These (or other) modifications may disfavor the post-fusion conformation. Exemplary approaches to disfavor the post-fusion conformation include stabilizing the pre-fusion conformation, stabilizing an intermediate conformation, destabilizing the post-fusion conformation or increasing the activation barrier of one or more steps  
30       leading to the post fusion conformation.

In another embodiment, the invention is a polypeptide that displays at least one epitope that is specific to the pre-fusion conformation F protein or an intermediate

conformation F protein. An epitope that is specific to the pre-fusion conformation F protein or an intermediate conformation F protein is an epitope that is not presented in the post-fusion conformation. It is preferred that the at least one epitope is stably presented, *e.g.*, the epitope is stably presented in solution for at least twelve hours, at least one day, at least two days, at least four days, at least six days, at least one week, at least two weeks, at least four weeks, or at least six weeks.

Such polypeptides may be native or mutated F proteins in the pre-fusion state, an intermediate state or a population of states where the post-fusion state is underrepresented or at a lower percentage than for isolated native F proteins, or it may be a derivative of a native F protein. Such derivatives include polypeptides comprising one or more fragments of a native F protein, fusion polypeptides comprising a native F protein (or fragment thereof) and a heterologous sequence, and polypeptides comprising a native F protein sequence having one or more mutations. These (or other) modifications may stabilize an F protein amino acid sequence in its pre-fusion conformation, stabilize an F protein amino acid sequence in an intermediate conformation, destabilize the post fusion conformation of an F protein amino acid sequence, increase the energy barrier in a transition leading to the post-fusion conformation of an F protein amino acid sequence, or a combination of two or more of the foregoing.

The TM and/or CT domains of F proteins are important for the stability of the pre-fusion conformation (8). Thus these domains may usefully be retained in immunogens of the invention. As it may be desirable not to include transmembrane domains in soluble immunogens, though, the functional effect of TM may be achieved by other means. For instance, the pre- and post-fusion behavior of F protein of parainfluenza virus 5 has been studied in some detail (6), and the authors stabilized the ED's pre-fusion structure by fusing a heterologous trimerization domain to the C-terminus of the ED.

#### **Oligomerization domain**

In another embodiment of the invention, the composition may comprise a polypeptide (*e.g.*, recombinant polypeptide) that comprises a first domain and a second domain, wherein (i) the first domain comprises the RSV F protein (*e.g.* RSV ectodomain, in whole or part), and (ii) the second domain comprises a heterologous

oligomerization domain. The second domain permits oligomerization of the polypeptide, thereby facilitating the first domain to adopt a pre-fusion state or an intermediate state. The polypeptide preferably will be present as an oligomer, and in particular as a trimer.

5           Various oligomerization domains are available to the skilled person. These are sequences of amino acids that can form a structure that can interact with oligomerization domains (whether the same or different) in other polypeptides (whether the same or different) such that the multiple polypeptides can associate (usually non-covalently) to form oligomers, *e.g.*, trimers. For instance, trimerization of the F protein of HIV (*i.e.*, gp160) has been achieved by fusing it to the catalytic subunit of *E. coli* aspartate transcarbamoylase (ATCase), which is naturally a stable trimer (9). Thus this subunit of ATCase may be used with the present invention. Similarly, trimerization of the F proteins of HIV (10) and PIV5 (6) has been achieved by fusing their ectodomains to GCNt. Thus the oligomerization domain used with the present invention may  
10           comprise the coiled coil of the yeast GCN4 leucine zipper protein (11). Trimerization of the ectodomain of HA protein from influenza A virus has been achieved by using a trimerizing sequence ('foldon') from the bacteriophage T4 fibrin (GSGYIPEAPRDGQ AYVRKDGEWVLLSTFL – SEQ ID NO:19) (12). Thus the oligomerization domain used with the present invention may comprise such a foldon.

20           Naturally-occurring protein oligomers (both hetero-oligomers and homo-oligomers) associate in a variety of different ways, *e.g.*, by association of  $\beta$ -sheets in different monomers, by association of  $\alpha$ -helices in different monomers, by association of hydrophobic surface patches, etc. One common structural motif involved in protein oligomerization is the coiled-coil domain. The coiled  $\alpha$ -helix structural motif can itself  
25           form coils, and two, three, four or five  $\alpha$ -helices can wrap around each other to form a left-handed super-helix known as the "coiled coil" though artificial right-handed super helices have been designed (13-19). The simplicity of the coiled-coil domain has made it a popular choice for designing chimeric proteins with defined oligomerization states (16).

30           In a coiled-coil structure the  $\alpha$ -helices interact through hydrophobic residues that form an apolar stripe along one side of each helix, and there may also be stabilizing electrostatic interactions between side chains on either side of this stripe. Within the



abcdefg heptad repeat of an  $\alpha$ -helix, the apolar stripe is defined by hydrophobic side chains at residues a and d, with any electrostatic interactions being primarily at residues e and g. Position a is most frequently Leu, Ile or Ala and position d is usually Leu or Ala. Residues e and g are often Glu or Gln, with Arg and Lys also prominent at position g. Charged residues are common at positions b, c and f as these residues are in contact with solvent. There are exceptions to this general heptad pattern, however, and Pro residues are sometimes found within the heptad. Such exceptions usually have functional significance including, by way of example, destabilization of the oligomerization domain to allow refolding and rearrangement such as occurs in the F protein.

Hundreds of coiled-coil domain sequences are known in the art, and any suitable sequence can be used as an oligomerization domain with the invention, provided that it retains the ability to oligomerize with other coiled-coil domains and that it does not destroy the function of the other domains within the polypeptide. It is preferred to use a coiled-coil domain which is found extracellularly (20) and which naturally acts as an oligomerization domain. As an alternative to using a natural coiled-coil domain, artificial coiled-coil domains can be used (21, 22). Owing to the highly repetitive structure of a coiled-coil domain, the domain is particularly amenable to computer modeling as the backbone portions of each amino acid residue may be parameterized rather than treating each backbone portion of a residue as a unique unit with its own variables. Domain (b) may include a leucine zipper sequence or an alanine zipper sequence (23).

The coiled-coil domain used in the polypeptide of the invention is preferably one which forms a trimer, such that the polypeptide of the invention can also assemble into a trimer. Preferred coiled-coil domains are those taken from bacterial transmembrane proteins. A preferred subset of transmembrane proteins is the adhesins (*i.e.*, cell-surface proteins that mediate adhesion to other cells or to surfaces), and particularly non-fimbrial adhesins (*e.g.*, in the oligomerization coiled-coil adhesins, or 'Oca', family). Specific sequences for use with the invention include those disclosed in reference 24 from *Yersinia enterocolitica* adhesin YadA, *Neisseria meningitidis* adhesin NadA, *Moraxella catarrhalis* surface protein UspA2, and other adhesins, such as the HadA adhesin from *Haemophilus influenzae* biogroup *aegyptius* etc (SEQ ID NOs 28-

31 & 42-58 of ref. 24). In addition, the eukaryotic heat-shock transcription factor has a coiled-coil trimerization domain that can be separately expressed and therefore used with the invention.

Within the amino acid sequence of a polypeptide having a coiled-coil region, the  
5 heptad-repeat nature of the  $\alpha$ -helices means that the boundary of the coiled-coil domain can be determined with some precision, but the precise residue where a coiled-coil arrangement can be said to end may not be known with absolute accuracy. This lack of absolute precision is not a problem for practicing the invention, however, as routine testing can reveal whether the coiled-coil requires any particular amino acid residue for  
10 which there might be doubt. Even so, the invention does not require the boundaries to be known with absolute precision, as the only basic requirement for the invention is that the coiled-coil domain should function in a way that allows the polypeptide to oligomerize with other coiled-coil domains without destroying the function of the other domains within the polypeptide.

15 Another class of oligomerization domain that can be used with the invention is found in the left-handed triple helix known as the collagen helix (25). These triple helix-forming sequences involve a basic tripeptide repeat sequence of  $^1\text{Gly}-^2\text{Xaa}-^3\text{Xaa}$ , where  $^2\text{Xaa}$  is often Pro, and  $^3\text{Xaa}$  is often 4-hydroxyproline. Although this motif is known as the "collagen" helix, it is found in many proteins beyond just collagen. The  
20 oligomerization domain may thus be a sequence comprising multiple repeats of the sequence motif  $^1\text{Gly}-^2\text{Xaa}-^3\text{Xaa}$ , which motif folds to form a helical structure that can oligomerize with corresponding helical structures in other polypeptide chains.

Collagen also provides another class of oligomerization domain. Reference 26 describes a motif found in the non-collagenous domain 1 (NC1) of type X collagen, and  
25 this motif can be used for trimer and higher order multimer formation without a triple helix. This trimeric association is highly thermostable without intermolecular disulfide bonds. The oligomerization domain may thus comprise an NC1 sequence.

Other oligomerization domains may be derived from the transmembrane domains of oligomeric TM proteins. As these are usually lipophilic, hydrophobic  
30 residues positioned on the outside of their TM regions may be substituted with charged residues, to provide a soluble domain. Such methods of solubilizing transmembrane domains by protein engineering are known in the art, for example from reference 27.

This method has also been used for GCN4, where the “a” and “d” heptad repeat positions were replaced with isoleucine (11):

KQIEDKIEEILSKIYHIENEIARIKKLIGEA (SEQ ID NO: 20). Suitable coiled coil sequences for use within the oligomerization domain will usually be between 20 and 35 amino acids long, *e.g.*, 23 to 30 amino acid residues long.

Oligomerization domains used with the invention can generally maintain an oligomeric structure without the need for the formation of inter-monomer disulfide bridges, but oligomers containing disulfide-linked monomers are not excluded from the invention.

As an alternative, or in addition, to using an oligomerization domain to stabilize an F protein in its pre-fusion conformation, mutation can be used. For instance, reference 28 reports that mutation in a conserved region of the F2 subunit of the F proteins in simian virus 5 or hendra virus can influence the stability of the pre-fusion conformation.

In some circumstances a low pH may also be used to favor the pre-fusion conformation.

#### **Stabilization of the HRB domain trimer**

In another preferred aspect of the present invention, the post-fusion conformation of the F protein may be disfavored by stabilization of the HRB domain trimer. The HRB domain forms a triple stranded coiled coil in the pre-fusion and likely the intermediate forms. As discussed in the preceding section, due to their simplicity, coiled-coils have been extensively studied as model systems for intermolecular interactions between proteins and as model systems for longer range intra-molecular interactions (*i.e.*, tertiary folding interactions). These studies are useful in teaching methods that may be used to stabilize the HRB domain in the trimeric coiled-coil form. By way of example, one or more residues at the *a* and/or *d* positions of the heptad repeat may be replaced with residues that favor formation of stable trimeric coiled-coils such as Ile residues. In addition, though less preferred, disfavorable ionic interactions at the *e* and *g* positions may be deleted or favorable ionic interactions at the *e* and *g* positions may be added.

The preferred region of the HRB domain for manipulation is the heptad repeat between P484-N517. Preferred examples of *a* and *d* residues to target for mutations are

F488, I492, V495, I499, S502, I506, S509, L512, and V516. The serine residues are especially preferred as replacement of the hydrophilic residues with hydrophobic residues would stabilize the hydrophobic core of the coiled-coil. Another preferred target would be the phenylalanine with a smaller hydrophobic residue that would pack  
5 better in the core such as an isoleucine.

#### **Destabilization of the HRA domain trimer**

In another preferred aspect of the present invention, the post-fusion conformation of the F protein may be disfavored by destabilization of the HRA domain trimer. The HRA domain forms a triple stranded coiled coil in the post-fusion and  
10 possibly one or more intermediate forms. By way of example, one or more residues at the *a* and/or *d* positions of the heptad repeat may be replaced with residues that disfavor formation of stable trimeric coiled-coils. In addition, though less preferred, favorable ionic interactions at the *e* and *g* positions may be deleted or disfavorable ionic interactions at the *e* and *g* positions may be added. Preferably such mutations will be  
15 selected that have minimal impact on the stability of the HRA domain in the pre-fusion conformation as may be modeled based upon the available crystal structures of the PIV5 F protein in the pre-fusion and post fusion forms.

#### **Other modifications**

In addition to the foregoing modifications, modifications can further be  
20 designed based upon molecular modeling of the hRSV F proteins based upon the available crystal structures of the PIV5 F protein in the pre-fusion and post fusion forms. Mutations may be made that destabilize the post-fusion conformation such as the 6HB fold of the HRA and HRB domains or that stabilize the pre-fusion conformation such as the HRA fold in the pre-fusion conformation. In addition, the  
25 energy barrier of the transitions leading to the post-fusion conformation may be increased. While one of skill in the art will appreciate that stabilizing the starting conformation or destabilizing the end conformation can have the effect of increasing the energy barrier, other modifications that affect the transition state itself may be introduced.

30 As an additional example, the amino acids N-terminal to the HRB domain (approximately a.a. 449-482, preferably V459-F483) act as a "tether" that allows the HRB domain to shift from one side of the F protein trimer to the other side so that the

HRB domain can participate in the 6HB of the post-fusion conformation. Deletion of one or more of these amino acids will impair or outright prevent the HRB domain from participating in the 6HB fold of the post-fusion conformation of the F protein (see Figure 3). In addition, the interaction between the tether and the F protein in the pre-fusion conformation can be stabilized to prevent the tether from pulling away to allow the HRB domain to participate in the 6HB fold. Examples of stabilizing mutations that could be made are cysteine bridges between the tether and the portion of the F protein which the tether contacts in the pre-fusion conformation.

Yet another example is stabilization of the HRA in the pre-fusion conformation (residues T50-Y306). Again, based upon the crystal structures of homologous F proteins, the hydrophobic core may be stabilized by replacing buried hydrophilic or ionic residues with similarly sized hydrophobic residues. Also, cysteine bridges may be introduced at the surface or within the core. In addition, as was demonstrated with extensive crystal structure analysis of lysozyme mutants, the hydrophobic cores or proteins are relatively rigid and therefore introducing holes predictably destabilized the lysozyme mutants. Similarly, repacking the core of the F protein in the pre-fusion conformation to eliminate any natural holes can stabilize the F protein in the pre-fusion or intermediate forms, thus disfavoring the post-fusion conformation.

## 20 **Methods for Preparing Compositions**

The invention relates to methods for preparing compositions and to compositions that contain RSV F protein, in particular soluble RSV F ecto-domain polypeptides, including immunogenic compositions. Preferably, the RSV F ecto-domain polypeptides are in a single form, such as uncleaved monomers, uncleaved trimers, cleaved trimers, rosettes of cleaved trimers, or in a dynamic equilibrium between a subset of such forms (e.g., equilibrium between uncleaved monomers and uncleaved trimers). The invention provides several advantages. For example, as described herein, the invention provides methods for producing compositions that contain a predominate desired form of RSV F protein, or a single desired form of RSV F protein, such as uncleaved monomers, uncleaved trimers, cleaved trimers, rosettes of cleaved trimers, a dynamic equilibrium between a subset of such forms (e.g., equilibrium between uncleaved monomers and uncleaved trimers), or a mixture of

desired form of RSV F protein. These types of compositions can be used for a variety of purposes, such as, in the production of immunogenic compositions that can be used to produce vaccines. The presence of a single desired form of RSV F, or a dynamic equilibrium between known forms, in an immunogenic composition, provides for more predictable formulation, solubility and stability, and for a more predictable immune response when the composition is administered to a subject.

When RSV F protein ecto-domain polypeptides are produced by conventional recombinant expression in host cells, the polypeptides are cleaved at the furin cleavage sites at about position 109/110 and at about position 136/137 during production in the host cell before they are secreted into the culture media. Cleavage of the polypeptides by the host cells is permissive for RSV F protein ecto-domain polypeptide refolding, which results in exposure of the hydrophobic fusion peptide. Accordingly, the cleaved RSV F protein ecto-domain polypeptides, due to the presence of an exposed fusion peptide, form rosettes and associate with lipids and lipoproteins that are derived from the host cells and culture media. In fact, electron microscopy of cleaved RSV F ectodomain that are produced in insect cells and purified by virtue of a HIS<sub>6</sub>-tag showed that the polypeptides had a crutch shape consistent with a post-fusion form and were bound to what appeared to residual cell debris. Accordingly, high purity preparations of rosettes and other forms and confirmations of RSV F protein ecto-domain polypeptides cannot be readily obtained by conventional recombinant expression in host cells.

#### **Methods for producing cleaved RSV F protein ecto-domain polypeptides**

In one aspect, the invention is a method for preparing a composition that contains cleaved RSV F protein ecto-domain polypeptides. In general, the method involves providing uncleaved RSV F protein ecto-domain polypeptides and then cleaving them to produce a F<sub>1</sub> subunit and a F<sub>2</sub> subunit. As described herein, uncleaved RSV F protein ecto-domain polypeptides can be readily purified and separated from contaminating lipids and lipoproteins using suitable methods, such as size exclusion chromatography. Without wishing to be bound by any particular theory, it is believed that the hydrophobic fusion peptide is not exposed in the uncleaved RSV F protein ecto-domain polypeptides and, therefore, the uncleaved polypeptides do not associate

with lipid and lipoprotein contaminants. As further described herein, uncleaved RSV F protein ecto-domains can be cleaved to produce F<sub>1</sub> and F<sub>2</sub> subunits, which can be purified as trimers, rosettes of trimers, or a mixture of trimers and rosettes of trimers.

Uncleaved RSV F protein ecto-domain polypeptides can be produced using any suitable method. For example, by recombinant production in host cells that do not contain active furin or furin-like proteases at the time the RSV F protein ecto-domain polypeptides are being produced. A variety of methods can be used to achieve this method of production, such as, production in recombinant host cells that are mutated to prevent expression of furin or furin-like protease (conditionally or complete “knock-out”), and various methods that reduce or prevent expression of furin or furin-like proteases in the host cells, for example, using RNA interference or other similar methods, or inhibiting furin or furin-like protease activity in host cells using inhibitors of the proteases.

Uncleaved RSV F protein ecto-domain polypeptides are preferably produced by recombinant expression of constructs that encode a RSV F protein ecto-domain in which the amino acid sequence of the furin cleavage sites are altered, so that the RSV F protein ecto-domain polypeptides are secreted by a host cell that produces the polypeptides uncleaved. The uncleaved RSV F protein ecto-domain polypeptides can be produced using any suitable host cell, such as insect cells (e.g., *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*), mammalian cells (e.g., human, non-human primate, horse, cow, sheep, dog, cat, and rodent (e.g., hamster), avian cells (e.g., chicken, duck, and geese, bacteria (e.g., *E. coli*, *Bacillus subtilis*, and *Streptococcus* spp.), yeast cells (e.g., *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guilliermondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*), Tetrahymena cells (e.g., *Tetrahymena thermophila*) or combinations thereof. Many suitable insect cells and mammalian cells are well-known in the art. Suitable insect cells include, for example, Sf9 cells, Sf21 cells, Tn5 cells, Schneider S2 cells, and High Five cells (a clonal isolate derived from the parental *Trichoplusia ni* BTI-TN-5B1-4 cell line (Invitrogen)). Suitable mammalian cells include, for example, Chinese hamster ovary (CHO) cells, human embryonic kidney cells (HEK293 cells, typically transformed by

sheared adenovirus type 5 DNA), NIH-3T3 cells, 293-T cells, Vero cells, HeLa cells, PERC.6 cells (ECACC deposit number 96022940), Hep G2 cells, MRC-5 (ATCC CCL-171), WI-38 (ATCC CCL-75), fetal rhesus lung cells (ATCC CL-160), Madin-Darby bovine kidney ("MDBK") cells, Madin-Darby canine kidney ("MDCK") cells  
 5 (e.g., MDCK (NBL2), ATCC CCL34; or MDCK 33016, DSM ACC 2219), baby hamster kidney (BHK) cells, such as BHK21-F, HKCC cells, and the like. Suitable avian cells include, for example, chicken embryonic stem cells (e.g., EBx® cells), chicken embryonic fibroblasts, chicken embryonic germ cells, duck cells (e.g., AGE1.CR and AGE1.CR.pIX cell lines (ProBioGen) which are described, for example,  
 10 in *Vaccine* 27:4975-4982 (2009) and WO2005/042728), EB66 cells, and the like.

Suitable insect cell expression systems, such as baculovirus systems, are known to those of skill in the art and described in, e.g., Summers and Smith, *Texas Agricultural Experiment Station Bulletin* No. 1555 (1987). Materials and methods for baculovirus/insert cell expression systems are commercially available in kit form from,  
 15 *inter alia*, Invitrogen, San Diego CA. Avian cell expression systems are also known to those of skill in the art and described in, e.g., U.S. Patent Nos. 5,340,740; 5,656,479; 5,830,510; 6,114,168; and 6,500,668; European Patent No. EP 0787180B; European Patent Application No. EP03291813.8 ;WO 03/043415; and WO 03/076601. Similarly, bacterial and mammalian cell expression systems are also known in the art and  
 20 described in, e.g., *Yeast Genetic Engineering* (Barr *et al.*, eds., 1989) Butterworths, London.

Generally, the amino acid sequence of an uncleaved RSV F protein ecto-domain is altered to prevent cleavage at the furin cleavage sites at about position 109/110 and about position 136/137, but contains a naturally occurring or introduced protease  
 25 cleavage site, that when cleaved produce a F<sub>1</sub> subunit and a F<sub>2</sub> subunit. For example, the uncleaved RSV F protein ecto-domain polypeptide can have an amino acid sequence that is altered to prevent cleavage at the furin cleavage sites at about position 109/110 and about position 136/137, but contain one or more naturally occurring or introduced protease cleavage sites from about position 101 to about position 161.

30 A variety of particular amino acid sequences that will allow uncleaved RSV F protein ecto-domain polypeptides to be produced and expressed by host cells, including amino acid sequences that are not cleaved at the furin cleavage sites at about position



109/110 and about position 136/137 can be readily designed and envisioned by a person of ordinary skill in the art. In general, one or more amino acids that are part of, or are located near by, the furin cleavage sites at about position 109/110 and about position 136/137 are independently replaced or deleted. Some amino acid substitutions and deletions that are suitable to prevent cleavage of RSV F protein ecto-domain polypeptides are known. For example, the substitutions R108N, R109N, R108N/R109N, which inhibit cleavage at 109/110, and the substitution K131Q or the deletion of the amino acids at positions 131-134, which inhibit cleavage at 136/137, have been described Gonzalez-Reyes *et al.*, *Proc. Natl. Acad. Sci. USA*, 98:9859-9864 (2001). An uncleaved RSV F ecto-domain polypeptide that contains the amino acid substitutions R108N/R109N/ K131Q/R133Q/ R135Q/R136Q has been described. Ruiz-Arguello *et al.*, *J. Gen. Virol.* 85:3677687 (2004). As described in detail herein, additional RSV F protein amino acid sequences that result in the RSV F ecto-domain polypeptide being secreted from a host cell uncleaved contain altered furin cleavage sites, e.g., alter amino acid sequences at about positions 106-109 and at about positions 133-136. The altered furin cleavage sites contain at least one amino acid substitution or deletion at about positions 106-109, and at least one amino acid substitution or deletion at about positions 133-136.

Similarly, a variety of particular amino acid sequences of uncleaved RSV F protein ecto-domain polypeptides that contain a protease cleavage site (e.g., naturally occurring or introduced) that when cleaved produce a first subunit that comprises an F<sub>1</sub> and a second subunit that comprises F<sub>2</sub>, are possible and can be readily designed and envisioned. For example, the amino acid sequence of RSV F protein from about position 101 to about position 161 contains trypsin cleavage sites, and one or more of the trypsin cleavage sites can be cleaved by trypsin to generate F<sub>1</sub> and F<sub>2</sub> subunits. If desired, one or more suitable protease recognition sites can be introduced into the uncleaved RSV F protein ecto-domain polypeptide, for example, between about positions 101 to about position 161. The introduced protease recognition sites can be cleaved using the appropriate protease to generate F<sub>1</sub> and F<sub>2</sub> subunits. When a protease recognition site is introduced into the amino acid sequence of an uncleaved RSV F protein ecto-domain polypeptide, it is preferred that the site is recognized by a protease that does not cleave the ecto-domain of naturally occurring RSV F protein.

The method of this aspect of the invention includes: a) providing uncleaved RSV F protein ecto-domain polypeptides containing a protease cleavage site that, when cleaved, produces F<sub>1</sub> and F<sub>2</sub> subunits, and b) cleaving the uncleaved RSV F protein ecto-domain polypeptides with a protease that recognizes the protease cleavage site. In  
5 general, the amino acid sequence of the uncleaved RSV F protein ecto-domain polypeptides contains altered furin cleavage sites, and the RSV F protein ecto-domain polypeptides are secreted from a host cell that produces them uncleaved at the furin cleavage sites at about positions 106-109 and about positions 131-136.

The provided uncleaved RSV F protein ecto-domain polypeptides can be  
10 purified to the desired degree. For example, the provided uncleaved RSV F protein ecto-domain polypeptides can be provided as a cell lysate, cell homogenate or cell culture conditioned media that is substantially unprocessed (*e.g.*, unprocessed, or clarified only), or in partially or substantially purified form. In particular examples, the provided uncleaved RSV F protein ecto-domain polypeptides are provided in cell  
15 culture conditioned media selected from the group consisting of insect cell conditioned media, mammalian cell conditioned media, avian cell conditioned media, yeast cell conditioned media, Tetrahymena cell conditioned media, and combinations thereof.

It is generally preferred that the provided uncleaved RSV F protein ecto-domain polypeptides are purified, for example, purified to be at least about 80%, at least about  
20 85%, at least about 90%, at least about 95% or substantially homogenous. As described herein, uncleaved RSV F protein ecto-domain polypeptides can be readily purified from lipids and lipoproteins, while conventionally produced cleaved forms of RSV F protein co-purify with lipid and lipoprotein contaminants. Accordingly, when purified  
uncleaved RSV F protein ecto-domain polypeptides are provided, the method can be  
25 used to readily produce a composition containing cleaved RSV F protein ecto-domains that are substantially free of lipids or lipoproteins.

Suitable methods for cleaving polypeptides using a protease are well-known and conventional in the art. Generally, the polypeptides to be cleaved are combined with a sufficient amount of protease under conditions (*e.g.*, pH, polypeptide and protease  
30 concentration, temperature) suitable for cleavage of the polypeptide. Many suitable proteases are commercially available, and suitable conditions for performing

polypeptide cleavage are well-known for many proteases. If desired, the cleaved RSV F protein ecto-domain polypeptides can be purified following cleavage with protease.

In one example of the method, uncleaved RSV F protein ecto-domain polypeptides are provided that contain an intact fusion peptide, such as an uncleaved  
5 RSV F protein ecto-domain polypeptide in which none of the amino acids from positions 137-154 are substituted or deleted. In some embodiments, the provided uncleaved RSV F protein ecto-domain polypeptides are purified. The provided  
10 uncleaved RSV F protein ecto-domain polypeptides that contain an intact fusion peptide are cleaved, and cleavage results in the formation of rosettes of cleaved RSV F protein ecto-domain polypeptide trimers. If desired, the rosettes can be purified further using any suitable methods, such as size exclusion chromatography.

In another example of the method, uncleaved RSV F protein ecto-domain polypeptides are provided that contain an altered fusion peptide, such as an uncleaved RSV F protein ecto-domain polypeptide in which about amino 137-152, about amino  
15 acids 137-153, about amino acids 137-145 or about amino acids 137-142 are deleted. Other suitable fusion peptide deletions have also been described, such as the deletion of the amino acids at positions 137-146. *Ruiz-Arguello et al., J. Gen. Virol.*, 85:3677-3687 (2004).

In some embodiments, the provided uncleaved RSV F protein ecto-domain polypeptides are purified. The provided uncleaved RSV F protein ecto-domain polypeptides are cleaved, and cleavage results in the formation of trimers of cleaved  
20 RSV F protein ecto-domain polypeptides. If desired, the trimers can be purified further using any suitable methods, such as size exclusion chromatography.

In particular examples of the method, the provided uncleaved RSV F protein ecto-domain polypeptides contain at least one polypeptide selected from the group  
25 consisting of furdel and delp23 furdel (*e.g.*, homogenous trypsin-cleavable furdel, homogenous trypsin-cleavable delp23 furdel, or a mixture of trypsin-cleavable furdel and trypsin-cleavable delp23 furdel). The provided uncleaved RSV F protein ecto-domain polypeptides are cleaved, for example with trypsin, and cleavage results in the  
30 formation of cleaved trimers, rosettes of cleaved trimers, or a combination of cleaved trimers and rosettes of cleaved trimers of RSV F protein ecto-domain polypeptides. If

desired, the cleaved trimers and/or rosettes of cleaved trimers can be purified further using any suitable methods, such as size exclusion chromatography.

#### **Methods for producing uncleaved RSV F protein ecto-domain polypeptides**

- 5           In another aspect, the invention is a method for preparing a composition that contains uncleaved RSV F protein ecto-domain polypeptides. In general, the method involves providing a biological material that contains uncleaved RSV F protein ecto-domain polypeptides, such as a cell lysate, cell homogenate or cell culture conditioned medium, and then purifying the uncleaved RSV F protein ecto-domain polypeptides.
- 10       As described herein, it has been discovered that purified uncleaved RSV F protein ecto-domain polypeptide monomers can self associate to form uncleaved trimers, and that there is a mixture of uncleaved monomers and uncleaved trimers or an equilibrium between the uncleaved monomers and uncleaved trimers. Without wishing to be bound by any particular theory, it is believed that the equilibrium favors the monomer, but that
- 15       the equilibrium will shift toward the trimer in concentrated solutions.

- The method of this aspect of the invention includes: a) providing a biological material that contains uncleaved RSV F protein ecto-domain polypeptides, such as a cell lysate, cell homogenate or cell culture conditioned medium; and b) purifying
- 20       uncleaved RSV F protein ecto-domain polypeptide monomers, trimers or a combination of monomers and trimers from the biological material. In some embodiments, uncleaved RSV F protein ecto-domain polypeptide monomers are purified, or uncleaved RSV F protein ecto-domain polypeptide trimers are purified, or monomers and trimers are purified.

- In general, the amino acid sequence of the uncleaved RSV F protein ecto-
- 25       domain polypeptides contains altered furin cleavage sites, and the RSV F protein ecto-domain polypeptides are secreted from a host cell that produces them uncleaved between about position 101 to about position 161 (including at the furin cleavage sites at positions 106-109 and 131-136). In more particular examples, the biological material that contains uncleaved RSV F protein ecto-domain polypeptides; includes at least one
- 30       polypeptide selected from the group consisting of furmt, furdel, delp21 furx, delp23 furx, delp21 furdel, delp23 furdel, and the factor Xa construct, which can be cleaved using factor Xa.

In some embodiments, the amino acid sequence of the RSV F protein ecto-domain polypeptide contains altered furin cleavage sites, and other protease cleavage sites (e.g., trypsin cleavage sites) between about position 101 and about position 161 are altered or deleted to prevent protease (e.g., trypsin) cleavage. For example, trypsin is well-known to cleave after lysine and arginine residues. In certain preferred embodiments, the amino acid sequence of the uncleaved RSV F protein ecto-domain polypeptide contains altered furin cleavage sites, one or more lysine and/or arginine residues (e.g., all lysine and arginine residues) present between about position 101 and about position 161 are deleted or replaced with an amino acid that is not lysine or arginine, the RSV F protein ecto-domain polypeptides are secreted from a host cell that produces them uncleaved between about position 101 and about position 161, and the RSV F protein ecto-domain polypeptides are not cleaved by trypsin between about position 101 and about position 161. Preferably, the RSV F protein ecto-domain polypeptides are not cleaved by trypsin when a 1 mg/ml solution of RSV F protein ecto-domain polypeptide (diluted in 25 mM Tris pH 7.5, 300 mM NaCl) is treated with one-one thousandth volume of trypsin solution (trypsin from bovine plasma diluted to a 1 mg/ml concentration in 25 mM Tris pH 7.5, 300 mM NaCl; final mass ratio in digestion reaction is 0.001:1 trypsin:RSV F ecto-domain; trypsin used at 10-15 BAEE units per mg protein) for 1 hour at 37°C.

If desired, the uncleaved RSV F protein ecto-domain polypeptides (e.g., the polypeptides that contain alter furin cleavage sites, and polypeptide that contain altered furin cleavage sites and altered trypsin cleavage sites) can further contain an altered fusion peptide, such as an uncleaved RSV F protein ecto-domain polypeptide in which, for example, about amino acids 137-152 are deleted, about amino acids 137-154 are deleted, about amino acids 137-145 are deleted or about amino acids 137-142 are deleted. Other suitable fusion peptide deletions have also been described, such as the deletion of the amino acids at positions 137-146. *Ruiz-Arguello et al., J. Gen. Virol.*, 85:3677-3687 (2004).

In particular embodiments, the method includes: a) providing a biological material that contains uncleaved RSV F protein ecto-domain polypeptides, such as a cell lysate, cell homogenate or cell culture conditioned medium, wherein the amino acid sequence of the uncleaved RSV F protein ecto-domain polypeptide contains altered

furin cleavage sites, the lysine and arginine residues present between about position 101 and about position 161 are deleted or replaced with an amino acid that is not lysine or arginine, the RSV F protein ecto-domain polypeptides are secreted from a host cell that produces them uncleaved between about position 101 and about position 161, and the  
5 RSV F protein ecto-domain polypeptides are not cleaved by trypsin between about position 101 and about position 161; and b) purifying uncleaved RSV F protein ecto-domain polypeptide monomers, trimers or a combination of monomers and trimers from the biological material.

In more particular examples, the biological material that contains uncleaved  
10 RSV F protein ecto-domain polypeptides; includes at least one polypeptide selected from the group consisting of Furx, Furx R113Q K123N K124N, delp21 furx and delp23 furx.

In other particular embodiments, the method includes: a) providing a biological material that contains uncleaved RSV F protein ecto-domain polypeptides in which the  
15 fusion peptide is mutated (*e.g.*, at least a portion of the fusion peptide is deleted), such as a cell lysate, cell homogenate or cell culture conditioned medium; and b) purifying uncleaved RSV F protein ecto-domain polypeptides from the biological material. The uncleaved RSV F protein ecto-domain polypeptide can contain altered furin cleavage sites, and the RSV F protein ecto-domain polypeptides are secreted from a host cell that  
20 produces them uncleaved between about position 101 to about position 161 (including at the furin cleavage sites at positions 106-109 and 131-136). If desired, the uncleaved RSV F protein ecto-domain polypeptide with altered furin cleavage sites further contains altered or deleted sites for other proteases (*e.g.*, trypsin cleavage sites) between about position 101 and about position 161 to prevent protease (*e.g.*, trypsin) cleavage.  
25 For example, one or more lysine and/or arginine residues (*e.g.*, all lysine and arginine residues) present between about position 101 and about position 161 are deleted or replaced with an amino acid that is not lysine or arginine, and the RSV F protein ecto-domain polypeptides are not cleaved by trypsin between about position 101 and about position 161.

30 The uncleaved RSV F protein ecto-domain polypeptide monomers, trimers and combinations of monomers and trimers can be purified to the desired degree. It is generally preferred that the uncleaved RSV F protein ecto-domain polypeptide

monomers or trimers are purified, for example, to be at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or substantially homogenous. As described herein, uncleaved RSV F protein ecto-domain polypeptides can be readily purified from lipids and lipoproteins, for example, by size exclusion chromatography. Accordingly, the method can be used to readily produce a composition containing cleaved RSV F protein ecto-domain polypeptide monomers, trimers, or a combination of monomers and trimers that are substantially free of lipids and lipoproteins.

In one example, the method includes providing insect cell culture conditioned medium, mammalian cell culture conditioned medium, avian cell conditioned medium, yeast cell conditioned medium, Tetrahymena cell conditioned medium, or a combination thereof. In some embodiments, uncleaved RSV F protein ecto-domain polypeptide trimers are purified. In other embodiments, uncleaved RSV F protein ecto-domain polypeptide monomers are purified. In other embodiments, uncleaved RSV F protein ecto-domain polypeptide monomers and trimers are purified.

#### **Methods for producing cleaved RSV F protein ecto-domain polypeptides with altered fusion peptides**

In one aspect, the invention is a method for preparing a composition that contains cleaved RSV F protein ecto-domain polypeptides that contain an altered fusion peptide. When RSV F protein ecto-domain polypeptides that do not contain altered furin cleavage sites are expressed in host cells, the host cells process the polypeptides, in part by cleaving the polypeptide at the furin sites at about positions 109/110 and about positions 136/137 to produce F<sub>1</sub> and F<sub>2</sub> subunits. The processed polypeptides are secreted into the culture and can be recovered as associated F<sub>1</sub>-F<sub>2</sub> subunits (e.g., disulphide bonding F<sub>1</sub> and F<sub>2</sub> subunits), which can form rosettes of trimers through aggregation of exposed fusion peptides. RSV F protein ecto-domain polypeptides that contain altered fusion peptides can be produced in and secreted from host cells as associated F<sub>1</sub>-F<sub>2</sub> subunits, and preferably do not aggregate into rosettes or with lipids or lipoprotein contaminants. Without wishing to be bound by any particular theory, it is believed that the polypeptides do not form rosettes or associate with lipid and

lipoprotein contaminants because the altered fusion peptide does not mediate aggregation.

The method of this aspect of the invention includes: a) providing a biological material that contains cleaved RSV F protein ecto-domain polypeptides that contain an altered fusion peptide (*e.g.*, at least a portion of the fusion peptide is deleted), such as a cell lysate, cell homogenate or cell culture conditioned medium; and b) purifying cleaved RSV F protein ecto-domain polypeptides from the biological material. The purified cleaved RSV F protein ecto-domain polypeptides can be purified as cleaved trimers, rosettes of cleaved trimers, or a mixture of cleaved trimers and rosettes of cleaved trimers. Suitable RSV F protein ecto-domain polypeptides that contain altered fusion peptides contain cleavable furin cleavage sites at about 109/110 and about 136/137 and further contain an altered fusion peptide as described herein. For example, an RSV F protein ecto-domain polypeptide in which about amino acids 137-152 are deleted, about amino acids 137-153 are deleted, about amino acids 137-145 are deleted, about amino acids 137-146 are deleted or about amino acids 137-142 are deleted, can be used in the method. In particular examples, the biological material that contains uncleaved RSV F protein ecto-domain polypeptides; includes at least the fusion peptide deletion 1.

The cleaved RSV F protein ecto-domain polypeptides (*e.g.*, cleaved trimers or a mixture of cleaved trimers and rosettes of cleaved trimers ) can be purified to the desired degree. It is generally preferred that the cleaved RSV F protein ecto-domain polypeptides are purified, for example, to be at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or substantially homogenous. As described herein, cleaved RSV F protein ecto-domain polypeptides that contain an altered fusion peptide can be readily purified from lipids and lipoproteins, for example, by size exclusion chromatography. Accordingly, the method can be used to readily produce a composition containing cleaved RSV F protein ecto-domain polypeptide trimers, rosettes of cleaved trimers, or a combination of cleaved trimers and rosettes of cleaved trimers that are substantially free of lipids and lipoproteins.



**Methods for producing RSV F protein ecto-domain polypeptides with C-terminal furin mutations**

In another aspect, the invention is a method for preparing a composition that  
5 contains C-terminal uncleaved RSV ecto-domain polypeptides and a method for  
preparing cleaved RSV F protein ecto-domain polypeptides. Without wishing to be  
bound by any particular theory, it is believed that C-terminal uncleaved RSV F protein  
ecto-domain polypeptides are cleaved by cells that produce the proteins at the furin  
cleavage site at about position 109/110, but not at the furin cleavage site at about  
10 position 136/137, and are secreted into the media as an F<sub>1</sub> subunit that is associated  
with an F<sub>2</sub> subunit. It is further believed that the hydrophobic fusion peptide is not  
exposed in the C-terminal uncleaved RSV F protein ecto-domain polypeptides and,  
therefore, the C-terminal uncleaved polypeptides do not associate with lipid and  
lipoprotein contaminants. As further described herein, C-terminal uncleaved RSV F  
15 protein ecto-domains can be cleaved further to produce a F<sub>1</sub> subunit, in which the amino  
terminus is from position 110 to about position 161, that is associated with a F<sub>2</sub> subunit.  
Such F<sub>1</sub> and F<sub>2</sub> subunits, which can be purified as trimers, rosettes of trimers, or a  
mixture of trimers and rosettes of trimers.

Generally, the amino acid sequence of a C-terminal uncleaved RSV F protein  
20 ecto-domain is altered to prevent cleavage at the furin cleavage site at about position  
136/137, but contains a naturally occurring or introduced protease cleavage site, that  
when cleaved produces a F<sub>1</sub> subunit, in which the amino terminus is from position 110  
to about position 161, and a F<sub>2</sub> subunit. For example, the C-terminal uncleaved RSV F  
protein ecto-domain polypeptide can have an amino acid sequence that is altered to  
25 prevent cleavage at the furin cleavage sites at about position 136/137, but contain one  
or more naturally occurring or introduced protease cleavage sites from about position  
101 to about position 161. In a particular example, the amino acid sequence of a C-  
terminal uncleaved RSV F protein ecto-domain is altered to prevent cleavage at the  
furin cleavage site at about position 136/137, but contains a naturally occurring furin  
30 cleavage site at about position 109/110.

A variety of particular amino acid sequences that will allow C-terminal  
uncleaved RSV F protein ecto-domain polypeptides to be produced and expressed by

host cells, including amino acid sequences that are not cleaved at the furin cleavage sites at about position 136/137, can be readily designed and envisioned by a person of ordinary skill in the art. In general, one or more amino acids that are part of, or are located near by, the furin cleavage sites at about position 136/137 are independently  
5 replaced or deleted. Suitable amino acid substitutions and deletions that prevent cleavage at about position 136/137 are described herein. For example, the substitution K131Q, the deletion of the amino acids at positions 131-134, or the substitutions K131Q/R133Q/ R135Q/R136Q, each of which inhibit cleavage at 136/137, can be used. In certain embodiments, C-terminal uncleaved RSV F protein ecto-domain  
10 polypeptides comprise at least one amino acid substitution or deletion at about positions 133-136.

Similarly, a variety of particular amino acid sequences of C-terminal uncleaved RSV F protein ecto-domain polypeptides that contain a protease cleavage site (e.g., naturally occurring or introduced) that when cleaved produce a first subunit that  
15 comprises an F<sub>1</sub> and a second subunit that comprises F<sub>2</sub>, are possible and can be readily designed and envisioned. For example, the amino acid sequence of RSV F protein from about position 101 to about position 161 contains trypsin cleavage sites, and one or more of the trypsin cleavage sites can be cleaved by trypsin to generate F<sub>1</sub> and F<sub>2</sub> subunits. If desired, one or more suitable protease recognition sites can be introduced  
20 into the C-terminal uncleaved RSV F protein ecto-domain polypeptide, for example, between about positions 101 to about position 161. The introduced protease recognition sites can be cleaved using the appropriate protease to generate F<sub>1</sub> and F<sub>2</sub> subunits. When a protease recognition site is introduced into the amino acid sequence of a C-terminal uncleaved RSV F protein ecto-domain polypeptide, it is preferred that  
25 the site is recognized by a protease that does not cleave the ecto-domain of naturally occurring RSV F protein.

C-terminal uncleaved RSV F protein ecto-domain polypeptides can be produced using any suitable method. A preferred method is by recombinant expression of constructs that encode a RSV F protein ecto-domain in which that amino acid sequence  
30 of the furin cleavage site at about positions 136/137 is altered, so that the C-terminal uncleaved RSV F protein ecto-domain polypeptides are secreted by a host cell that produces the polypeptides uncleaved at the furin cleavage site at about position

136/137. Preferably, the C-terminal uncleaved RSV F protein ecto-domain polypeptide is secreted by a host cell that produces it as an F<sub>1</sub> subunit that is associated with an F<sub>2</sub> subunit, wherein the amino terminus of the F<sub>1</sub> subunit is from position 132 to about position 161, but not position 137. The C-terminal uncleaved RSV F protein ecto-domain polypeptides can be produced using any suitable host cell, as described herein.

One method of this aspect of the invention includes: a) providing C-terminal uncleaved RSV F protein ecto-domain polypeptides that comprise an altered furin cleavage site at position 136/137, and said C-terminal uncleaved RSV F protein ecto-domain polypeptides are secreted from a cell that produces them in the form of an F<sub>2</sub> fragment that is associated with a subunit that comprises F<sub>1</sub> but is uncleaved at position 136/137, and b) cleaving the provided C-terminal uncleaved RSV F protein ecto-domain polypeptides with a protease that cleaves RSV F protein ecto-domain at a site between positions 101 and 161, thereby producing said composition. In particular embodiments, step b) comprises cleaving the provided C-terminal uncleaved RSV F protein ecto-domain polypeptides with a protease that cleaves RSV F protein ecto-domain at a site between about positions 101 and 132, or about positions 132 and 161, or about positions 110 and 132. Alternatively or in addition, in some embodiments, the C-terminal uncleaved RSV F protein ecto-domain polypeptides comprise an altered furin cleavage site at position 136/137, with the proviso that the altered furin cleavage site is not deletion of amino acids 131-134. In particular examples, the biological material that contains C-terminal uncleaved RSV F protein ecto-domain polypeptides; includes at least the N-term Furin polypeptide.

The provided C-terminal uncleaved RSV F protein ecto-domain polypeptides can be purified to the desired degree. For example, the provided C-terminal uncleaved RSV F protein ecto-domain polypeptides can be provided in a cell lysate, cell homogenate, or cell culture conditioned media that is substantially unprocessed (*e.g.*, unprocessed, or clarified only), or in partially or substantially purified form. In particular examples, the provided C-terminal uncleaved RSV F protein ecto-domain polypeptides are provided in cell culture conditioned media selected from the group consisting of insect cell conditioned media, mammalian cell conditioned media, avian cell conditioned media, yeast cell conditioned media, Tetrahymena cell conditioned media, and combinations thereof.

It is generally preferred that the provided C-terminal uncleaved RSV F protein ecto-domain polypeptides are purified, for example, purified to be at least about 80%, at least about 85%, at least about 90%, at least about 95% or substantially homogenous. As described herein, C-terminal uncleaved RSV F protein ecto-domain polypeptides  
5 can be readily purified from lipids and lipoproteins, while conventionally produced cleaved forms of RSV F protein co-purify with lipid and lipoprotein contaminants. Accordingly, when purified C-terminal uncleaved RSV F protein ecto-domain polypeptides are provided, the method can be used to readily produce a composition containing cleaved RSV F protein ecto-domains that are substantially free of lipids or  
10 phospholipids.

Suitable methods for cleaving polypeptides using a protease are well-known and conventional in the art. Generally, the polypeptides to be cleaved are combined with a sufficient amount of protease under conditions (*e.g.*, pH, polypeptide and protease concentration, temperature) suitable for cleavage of the polypeptide. Many suitable  
15 proteases are commercially available, and suitable conditions for performing polypeptide cleavage are well-known for many proteases. If desired, the RSV F protein ecto-domain polypeptides can be purified following cleavage with protease.

In one example of the method, C-terminal uncleaved RSV F protein ecto-domain polypeptides are provided that contain an intact fusion peptide, such as a C-terminal uncleaved RSV F protein ecto-domain polypeptide in which none of the amino  
20 acids from positions 137-154 are substituted or deleted. In another example of the method, C-terminal uncleaved RSV F protein ecto-domain polypeptides are provided that contain an altered fusion peptide, such as a C-terminal uncleaved RSV F protein ecto-domain polypeptide in which about amino 137-152, about amino acids 137-153,  
25 about amino acids 137-145 or about amino acids 137-142 are deleted. Other suitable fusion peptide deletions have also been described, such as the deletion of the amino acids at positions 137-146. *Ruiz-Arguello et al., J. Gen. Virol.*, 85:3677-3687 (2004).

In some embodiments, the provided C-terminal uncleaved RSV F protein ecto-domain polypeptides are purified. The provided uncleaved RSV F protein ecto-domain  
30 polypeptides are cleaved, and cleavage results in the formation of trimers of cleaved RSV F protein ecto-domain polypeptides. If desired, the trimers can be purified further using any suitable methods, such as size exclusion chromatography.

In particular examples of the method, the provided C-terminal uncleaved RSV F protein ecto-domain polypeptides contain at least the N-terminal Furin polypeptide (FIG. 1). The provided C-terminal uncleaved RSV F protein ecto-domain polypeptides are cleaved, for example with trypsin, and cleavage results in the formation of cleaved trimers, rosettes of cleaved trimers, or a combination of cleaved trimers and rosettes of cleaved trimers of RSV F protein ecto-domain polypeptides. If desired, the cleaved trimers and/or rosettes of cleaved trimers can be purified further using any suitable methods, such as size exclusion chromatography.

Another method of this aspect of the invention includes: a) providing a biological material, such as a cell lysate, cell homogenate or cell culture conditioned medium, that contains a C-terminal uncleaved RSV F protein ecto-domain polypeptides that comprise an altered furin cleavage site at position 136/137, and said soluble RSV F protein ecto-domain polypeptides are secreted from a cell that produces them in the form of an F<sub>2</sub> fragment that is associated with a subunit that comprises F<sub>1</sub> but is uncleaved at position 136/137, with the proviso that the altered furin cleavage site is not deletion of amino acids 131-134; and b) purifying the C-terminal uncleaved RSV F protein ecto-domain polypeptides from the biological material, thereby producing the composition. Preferably, the amino terminus of the F<sub>1</sub> subunit is from about position 110 to about position 132. More preferably, the amino terminus of the F<sub>1</sub> subunit is about position 110. It is particularly preferred that the amino terminus of the F<sub>1</sub> subunit is not position 137. In particular examples, the biological material that contains C-terminal uncleaved RSV F protein ecto-domain polypeptides; includes at least the N-term Furin polypeptide.

If desired, the C-terminal uncleaved RSV F protein ecto-domain polypeptide further contains altered or deleted sites for other proteases (e.g., trypsin cleavage sites) between about position 101 and about position 161 to prevent protease (e.g., trypsin) cleavage. For example, one or more lysine and/or arginine residues (e.g., all lysine and arginine residues) present between about position 101 and about position 161 are deleted or replaced with an amino acid that is not lysine or arginine, and the C-terminal uncleaved RSV F protein ecto-domain polypeptides are not cleaved by trypsin between about position 101 and about position 161. The C-terminal uncleaved RSV F protein

ecto-domain polypeptides can contain an intact fusion peptide or an altered fusion peptide, as described herein.

The C-terminal uncleaved RSV F protein ecto-domain polypeptides, e.g., monomers, trimers and combinations of monomers and trimers can be purified to the  
5 desired degree. It is generally preferred that the C-terminal uncleaved RSV F protein ecto-domain polypeptide monomers or trimers are purified, for example, to be at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or substantially homogenous. As described herein, C-terminal uncleaved RSV F protein ecto-domain polypeptides can be readily purified from lipids and lipoproteins,  
10 for example, by size exclusion chromatography. Accordingly, the method can be used to readily produce a composition containing C-terminal uncleaved RSV F protein ecto-domain polypeptides, e.g., monomers, trimers, or a combination of monomers and trimers, that are substantially free of lipids and lipoproteins. In particular examples of the method, the C-terminal uncleaved RSV F protein ecto-domain polypeptides contain  
15 at least the N-terminal Furin polypeptide (FIG. 1).

In one example, the method includes providing insect cell culture conditioned medium, mammalian cell culture conditioned medium, avian cell conditioned media, yeast cell conditioned media, Tetrahymena cell conditioned media or a combination thereof. In some embodiments, C-terminal uncleaved RSV F protean ecto-domain  
20 polypeptide trimers are purified. In other embodiments, C-terminal uncleaved RSV F protein ecto-domain polypeptide monomers are purified. In other embodiments, C-terminal uncleaved RSV F protein ecto-domain polypeptide monomers and trimers are purified.

## 25           **Self-Replicating RNA**

The RSV-F polypeptides described herein can be produced by expression of recombinant nucleic acids that encode the polypeptides in the cells of a subject. Preferred nucleic acids that can be administered to a subject to cause the production of RSV-F polypeptides are self-replicating RNA molecules. The self-replicating RNA  
30 molecules of the invention are based on the genomic RNA of RNA viruses, but lack the genes encoding one or more structural proteins. The self-replicating RNA molecules

are capable of being translated to produce non-structural proteins of the RNA virus and heterologous proteins encoded by the self-replicating RNA.

The self-replicating RNA generally contains at least one or more genes selected from the group consisting of viral replicase, viral proteases, viral helicases and other nonstructural viral proteins, and also comprise 5'- and 3'-end cis-active replication sequences, and if desired, a heterologous sequences that encode a desired amino acid sequences (e.g., a protein, an antigen). A subgenomic promoter that directs expression of the heterologous sequence can be included in the self-replicating RNA. If desired, the heterologous sequence may be fused in frame to other coding regions in the self-replicating RNA and/or may be under the control of an internal ribosome entry site (IRES).

Self-replicating RNA molecules of the invention can be designed so that the self-replicating RNA molecule cannot induce production of infectious viral particles. This can be achieved, for example, by omitting one or more viral genes encoding structural proteins that are necessary for the production of viral particles in the self-replicating RNA. For example, when the self-replicating RNA molecule is based on an alpha virus, such as Sinebis virus (SIN), Semliki forest virus and Venezuelan equine encephalitis virus (VEE), one or more genes encoding viral structural proteins, such as capsid and/or envelope glycoproteins, can be omitted. If desired, self-replicating RNA molecules of the invention can be designed to induce production of infectious viral particles that are attenuated or virulent, or to produce viral particles that are capable of a single round of subsequent infection.

A self-replicating RNA molecule can, when delivered to a vertebrate cell even without any proteins, lead to the production of multiple daughter RNAs by transcription from itself (or from an antisense copy of itself). The self-replicating RNA can be directly translated after delivery to a cell, and this translation provides a RNA-dependent RNA polymerase which then produces transcripts from the delivered RNA. Thus the delivered RNA leads to the production of multiple daughter RNAs. These transcripts are antisense relative to the delivered RNA and may be translated themselves to provide in situ expression of a gene product, or may be transcribed to provide further transcripts with the same sense as the delivered RNA which are translated to provide in situ expression of the encoded RSV-F polypeptide.

One suitable system for achieving self-replication is to use an alphavirus-based RNA replicon. These + stranded replicons are translated after delivery to a cell to give of a replicase (or replicase-transcriptase). The replicase is translated as a polyprotein which auto cleaves to provide a replication complex which creates genomic – strand  
5 copies of the + strand delivered RNA. These – strand transcripts can themselves be transcribed to give further copies of the + stranded parent RNA and also to give a subgenomic transcript which encodes the RSV-F polypeptide. Translation of the subgenomic transcript thus leads to in situ expression of the RSV-F polypeptide by the infected cell. Suitable alphavirus replicons can use a replicase from a sindbis virus, a  
10 semliki forest virus, an eastern equine encephalitis virus, a venezuelan equine encephalitis virus, etc.

A preferred self-replicating RNA molecule thus encodes (i) a RNA-dependent RNA polymerase which can transcribe RNA from the self-replicating RNA molecule and (ii) an RSV-F polypeptide. The polymerase can be an alphavirus replicase e.g.  
15 comprising alphavirus protein nsP4.

Whereas natural alphavirus genomes encode structural virion proteins in addition to the non structural replicase polyprotein, it is preferred that an alphavirus based self-replicating RNA molecule of the invention does not encode alphavirus structural proteins. Thus the self replicating RNA can lead to the production of  
20 genomic RNA copies of itself in a cell, but not to the production of RNA-containing alphavirus virions. The inability to produce these virions means that, unlike a wild-type alphavirus, the self-replicating RNA molecule cannot perpetuate itself in infectious form. The alphavirus structural proteins which are necessary for perpetuation in wild-type viruses are absent from self replicating RNAs of the invention and their place is  
25 taken by gene(s) encoding the desired gene product, such that the subgenomic transcript encodes the desired gene product rather than the structural alphavirus virion proteins.

Thus a self-replicating RNA molecule useful with the invention may have two open reading frames. The first (5') open reading frame encodes a replicase; the second (3') open reading frame encodes an RSV-F polypeptide. In some embodiments the  
30 RNA may have additional (downstream) open reading frames e.g. that encode further desired gene products. A self-replicating RNA molecule can have a 5' sequence which is compatible with the encoded replicase.



In one aspect, the self-replicating RNA molecule is derived from or based on an alphavirus. In other aspects, the self-replicating RNA molecule is derived from or based on a virus other than an alphavirus, preferably, a positive-stranded RNA viruses, and more preferably a picornavirus, flavivirus, rubivirus, pestivirus, hepacivirus, calicivirus, or coronavirus. Suitable wild-type alphavirus sequences are well-known and are available from sequence depositories, such as the American Type Culture Collection, Rockville, Md. Representative examples of suitable alphaviruses include Aura (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mayaro virus (ATCC VR-1277), Middleburg (ATCC VR-370), Mucambo virus (ATCC VR-580, ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest (ATCC VR-67, ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248), Tonate (ATCC VR-925), Trinita (ATCC VR-469), Una (ATCC VR-374), Venezuelan equine encephalomyelitis (ATCC VR-69, ATCC VR-923, ATCC VR-1250 ATCC VR-1249, ATCC VR-532), Western equine encephalomyelitis (ATCC VR-70, ATCC VR-1251, ATCC VR-622, ATCC VR-1252), Whataroa (ATCC VR-926), and Y-62-33 (ATCC VR-375).

The self-replicating RNA may be associated with a delivery system. The self-replicating RNA may be administered with or without an adjuvant.

#### **RNA DELIVERY SYSTEMS**

The self-replicating RNA of the invention are suitable for delivery in a variety of modalities, such as naked RNA delivery or in combination with lipids, polymers or other compounds that facilitate entry into the cells. Self-replicating RNA molecules of the present invention can be introduced into target cells or subjects using any suitable technique, *e.g.*, by direct injection, microinjection, electroporation, lipofection, biolistics, and the like. The self-replicating RNA molecule may also be introduced into cells by way of receptor-mediated endocytosis. See *e.g.*, U.S. Pat. No. 6,090,619; Wu and Wu, J. Biol. Chem., 263:14621 (1988); and Curiel *et al.*, Proc. Natl. Acad. Sci.

USA, 88:8850 (1991). For example, U.S. Pat. No. 6,083,741 discloses introducing an exogenous nucleic acid into mammalian cells by associating the nucleic acid to a polycation moiety (*e.g.*, poly-L-lysine having 3-100 lysine residues), which is itself coupled to an integrin receptor-binding moiety (*e.g.*, a cyclic peptide having the  
5 sequence Arg-Gly-Asp).

The self-replicating RNA molecule of the present invention can be delivered into cells via amphiphiles. See *e.g.*, U.S. Pat. No. 6,071,890. Typically, a nucleic acid molecule may form a complex with the cationic amphiphile. Mammalian cells contacted with the complex can readily take it up.

10 The self-replicating RNA can be delivered as naked RNA (*e.g.* merely as an aqueous solution of RNA) but, to enhance entry into cells and also subsequent intercellular effects, the self-replicating RNA is preferably administered in combination with a delivery system, such as a particulate or emulsion delivery system. A large number of delivery systems are well known to those of skill in the art. Such delivery  
15 systems include, for example liposome-based delivery (Debs and Zhu (1993) WO 93/24640; Mannino and Gould-Fogerite (1988) BioTechniques 6(7): 682-691; Rose U.S. Pat. No. 5,279,833; Brigham (1991) WO 91/06309; and Felgner *et al.* (1987) Proc. Natl. Acad. Sci. USA 84: 7413-7414), as well as use of viral vectors (*e.g.*, adenoviral (see, *e.g.*, Berns *et al.* (1995) Ann. NY Acad. Sci. 772: 95-104; Ali *et al.* (1994) Gene  
20 Ther. 1: 367-384; and Haddada *et al.* (1995) Curr. Top. Microbiol. Immunol. 199 (Pt 3): 297-306 for review), papillomaviral, retroviral (see, *e.g.*, Buchscher *et al.* (1992) J. Virol. 66(5) 2731-2739; Johann *et al.* (1992) J. Virol. 66 (5): 1635-1640 (1992); Sommerfelt *et al.*, (1990) Virol. 176:58-59; Wilson *et al.* (1989) J. Virol. 63:2374-2378; Miller *et al.*, J. Virol. 65:2220-2224 (1991); Wong-Staal *et al.*, PCT/US94/05700,  
25 and Rosenberg and Fauci (1993) in Fundamental Immunology, Third Edition Paul (ed) Raven Press, Ltd., New York and the references therein, and Yu *et al.*, Gene Therapy (1994) *supra.*), and adeno-associated viral vectors (see, West *et al.* (1987) Virology 160:38-47; Carter *et al.* (1989) U.S. Pat. No. 4,797,368; Carter *et al.* WO 93/24641 (1993); Kotin (1994) Human Gene Therapy 5:793-801; Muzyczka (1994) J. Clin. Invst.  
30 94:1351 and Samulski (*supra*) for an overview of AAV vectors; see also, Lebkowski, U.S. Pat. No. 5,173,414; Tratschin *et al.* (1985) Mol. Cell. Biol. 5(11):3251-3260; Tratschin, *et al.* (1984) Mol. Cell. Biol., 4:2072-2081; Hermonat and Muzyczka (1984)

Proc. Natl. Acad. Sci. USA, 81:6466-6470; McLaughlin *et al.* (1988) and Samulski *et al.* (1989) J. Virol., 63:03822-3828), and the like.

Three particularly useful delivery systems are (i) liposomes (ii) non-toxic and biodegradable polymer microparticles (iii) cationic submicron oil-in-water emulsions.

5

### Liposomes

Various amphiphilic lipids can form bilayers in an aqueous environment to encapsulate a RNA-containing aqueous core as a liposome. These lipids can have an anionic, cationic or zwitterionic hydrophilic head group. Formation of liposomes from anionic phospholipids dates back to the 1960s, and cationic liposome-forming lipids have been studied since the 1990s. Some phospholipids are anionic whereas other are zwitterionic. Suitable classes of phospholipid include, but are not limited to, phosphatidylethanolamines, phosphatidylcholines, phosphatidylserines, and phosphatidylglycerols, and some useful phospholipids are listed in Table 20. Useful cationic lipids include, but are not limited to, dioleoyl trimethylammonium propane (DOTAP), 1,2-distearoyloxy-N,N-dimethyl-3-aminopropane (DSDMA), 1,2-dioleoyloxy-N,N-dimethyl-3-aminopropane (DODMA), 1,2-dilinoleoyloxy-N,N-dimethyl-3-aminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethyl-3-aminopropane (DLenDMA). Zwitterionic lipids include, but are not limited to, acyl zwitterionic lipids and ether zwitterionic lipids. Examples of useful zwitterionic lipids are DPPC, DOPC and dodecylphosphocholine. The lipids can be saturated or unsaturated.

Liposomes can be formed from a single lipid or from a mixture of lipids. A mixture may comprise (i) a mixture of anionic lipids (ii) a mixture of cationic lipids (iii) a mixture of zwitterionic lipids (iv) a mixture of anionic lipids and cationic lipids (v) a mixture of anionic lipids and zwitterionic lipids (vi) a mixture of zwitterionic lipids and cationic lipids or (vii) a mixture of anionic lipids, cationic lipids and zwitterionic lipids. Similarly, a mixture may comprise both saturated and unsaturated lipids. For example, a mixture may comprise DSPC (zwitterionic, saturated), DlinDMA (cationic, unsaturated), and/or DMPG (anionic, saturated). Where a mixture of lipids is used, not all of the component lipids in the mixture need to be amphiphilic *e.g.* one or more amphiphilic lipids can be mixed with cholesterol.

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The hydrophilic portion of a lipid can be PEGylated (*i.e.* modified by covalent attachment of a polyethylene glycol). This modification can increase stability and prevent non-specific adsorption of the liposomes. For instance, lipids can be conjugated to PEG using techniques such as those disclosed in Heyes *et al.* (2005) *J Controlled Release* 107:276-287.

A mixture of DSPC, DlinDMA, PEG-DMPG and cholesterol is used in the examples. A separate aspect of the invention is a liposome comprising DSPC, DlinDMA, PEG-DMG and cholesterol. This liposome preferably encapsulates RNA, such as a self-replicating RNA *e.g.* encoding an immunogen.

Liposomes are usually divided into three groups: multilamellar vesicles (MLV); small unilamellar vesicles (SUV); and large unilamellar vesicles (LUV). MLVs have multiple bilayers in each vesicle, forming several separate aqueous compartments. SUVs and LUVs have a single bilayer encapsulating an aqueous core; SUVs typically have a diameter  $\leq 50\text{nm}$ , and LUVs have a diameter  $> 50\text{nm}$ . Liposomes useful with of the invention are ideally LUVs with a diameter in the range of 50-220nm. For a composition comprising a population of LUVs with different diameters: (i) at least 80% by number should have diameters in the range of 20-220nm, (ii) the average diameter ( $Z_{av}$ , by intensity) of the population is ideally in the range of 40-200nm, and/or (iii) the diameters should have a polydispersity index  $< 0.2$ .

Techniques for preparing suitable liposomes are well known in the art *e.g.* see Liposomes: Methods and Protocols, Volume 1: Pharmaceutical Nanocarriers: Methods and Protocols. (ed. Weissig). Humana Press, 2009. ISBN 160327359X; Liposome Technology, volumes I, II & III. (ed. Gregoriadis). Informa Healthcare, 2006; and Functional Polymer Colloids and Microparticles volume 4 (Microspheres, microcapsules & liposomes). (eds. Arshady & Guyot). Citus Books, 2002. One useful method involves mixing (i) an ethanolic solution of the lipids (ii) an aqueous solution of the nucleic acid and (iii) buffer, followed by mixing, equilibration, dilution and purification (Heyes *et al.* (2005) *J Controlled Release* 107:276-87.).

RNA is preferably encapsulated within the liposomes, and so the liposome forms a outer layer around an aqueous RNA-containing core. This encapsulation has been found to protect RNA from RNase digestion.. The liposomes can include some

external RNA (*e.g.* on the surface of the liposomes), but at least half of the RNA (and ideally all of it) is encapsulated.

### **Polymeric microparticles**

5           Various polymers can form microparticles to encapsulate or adsorb RNA. The use of a substantially non-toxic polymer means that a recipient can safely receive the particles, and the use of a biodegradable polymer means that the particles can be metabolised after delivery to avoid long-term persistence. Useful polymers are also sterilisable, to assist in preparing pharmaceutical grade formulations.

10           Suitable non-toxic and biodegradable polymers include, but are not limited to, poly( $\alpha$ -hydroxy acids), polyhydroxy butyric acids, polylactones (including polycaprolactones), polydioxanones, polyvalerolactone, polyorthoesters, polyanhydrides, polycyanoacrylates, tyrosine-derived polycarbonates, polyvinylpyrrolidinones or polyester-amides, and combinations thereof.

15           In some embodiments, the microparticles are formed from poly( $\alpha$ -hydroxy acids), such as a poly(lactides) ("PLA"), copolymers of lactide and glycolide such as a poly(D,L-lactide-co-glycolide) ("PLG"), and copolymers of D,L-lactide and caprolactone. Useful PLG polymers include those having a lactide/glycolide molar ratio ranging, for example, from 20:80 to 80:20 *e.g.* 25:75, 40:60, 45:55, 55:45, 60:40,  
20   75:25. Useful PLG polymers include those having a molecular weight between, for example, 5,000-200,000 Da *e.g.* between 10,000-100,000, 20,000-70,000, 40,000-50,000 Da.

          The microparticles ideally have a diameter in the range of 0.02  $\mu\text{m}$  to 8  $\mu\text{m}$ . For a composition comprising a population of microparticles with different diameters at  
25   least 80% by number should have diameters in the range of 0.03-7  $\mu\text{m}$ .

          Techniques for preparing suitable microparticles are well known in the art *e.g.* see Functional Polymer Colloids and Microparticles volume 4 (Microspheres, microcapsules & liposomes). (eds. Arshady & Guyot). Citus Books, 2002; *Polymers in Drug Delivery*. (eds. Uchegbu & Schatzlein). CRC Press, 2006. (in particular chapter 7)  
30   and *Microparticulate Systems for the Delivery of Proteins and Vaccines*. (eds. Cohen & Bernstein). CRC Press, 1996. To facilitate adsorption of RNA, a microparticle may include a cationic surfactant and/or lipid *e.g.* as disclosed in O'Hagan *et al.* (2001) *J*

*Virology* 75:9037-9043; and Singh *et al.* (2003) *Pharmaceutical Research* 20: 247-251.

An alternative way of making polymeric microparticles is by molding and curing *e.g.* as disclosed in WO2009/132206.

Microparticles of the invention can have a zeta potential of between 40-100 mV.

- 5 RNA can be adsorbed to the microparticles, and adsorption is facilitated by including cationic materials (*e.g.* cationic lipids) in the microparticle.

### **Oil-in-water cationic emulsions**

- Oil-in-water emulsions are known for adjuvanting influenza vaccines *e.g.* the MF59<sup>TM</sup> adjuvant in the FLUAD<sup>TM</sup> product, and the AS03 adjuvant in the PREPANDRIX<sup>TM</sup> product. RNA delivery according to the present invention can utilise an oil-in-water emulsion, provided that the emulsion includes one or more cationic molecules. For instance, a cationic lipid can be included in the emulsion to provide a positive droplet surface to which negatively-charged RNA can attach.

- 15 The emulsion comprises one or more oils. Suitable oil(s) include those from, for example, an animal (such as fish) or a vegetable source. The oil is ideally biodegradable (metabolisable) and biocompatible. Sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Jojoba oil can be used *e.g.* obtained from the jojoba bean. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like may also be used. 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, may be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. Fats and oils from mammalian milk are metabolizable and so may be used. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art.

- Most fish contain metabolizable oils which may be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which may be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as

terpenoids. Squalane, the saturated analog to squalene, can also be used. Fish oils, including squalene and squalane, are readily available from commercial sources or may be obtained by methods known in the art.

Other useful oils are the tocopherols, particularly in combination with squalene.

- 5 Where the oil phase of an emulsion includes a tocopherol, any of the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  or  $\xi$  tocopherols can be used, but  $\alpha$ -tocopherols are preferred. D- $\alpha$ -tocopherol and DL- $\alpha$ -tocopherol can both be used. A preferred  $\alpha$ -tocopherol is DL- $\alpha$ -tocopherol. An oil combination comprising squalene and a tocopherol (*e.g.* DL- $\alpha$ -tocopherol) can be used.

- Preferred emulsions comprise squalene, a shark liver oil which is a branched, 10 unsaturated terpenoid ( $C_{30}H_{50}$ ;  $[(CH_3)_2C(=CHCH_2CH_2C(CH_3)_2=CHCH_2-)]_2$ ; 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene; CAS RN 7683-64-9).

The oil in the emulsion may comprise a combination of oils *e.g.* squalene and at least one further oil.

- The aqueous component of the emulsion can be plain water (*e.g.* w.f.i.) or can 15 include further components *e.g.* solutes. For instance, it may include salts to form a buffer *e.g.* citrate or phosphate salts, such as sodium salts. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer; or a citrate buffer. A buffered aqueous phase is preferred, and buffers will typically be included in the 5-20mM range.

- 20 The emulsion also includes a cationic lipid. Preferably this lipid is a surfactant so that it can facilitate formation and stabilisation of the emulsion. Useful cationic lipids generally contains a nitrogen atom that is positively charged under physiological conditions *e.g.* as a tertiary or quaternary amine. This nitrogen can be in the hydrophilic head group of an amphiphilic surfactant. Useful cationic lipids include, but are not 25 limited to: 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 3'-[N-(N',N'-Dimethylaminoethane)-carbamoyl]Cholesterol (DC Cholesterol), dimethyldioctadecylammonium (DDA *e.g.* the bromide), 1,2-Dimyristoyl-3-Trimethyl-AmmoniumPropane (DMTAP), dipalmitoyl(C16:0)trimethyl ammonium propane (DPTAP), distearoyltrimethylammonium propane (DSTAP). Other useful cationic lipids are: 30 benzalkonium chloride (BAK), benzethonium chloride, cetramide (which contains tetradecyltrimethylammonium bromide and possibly small amounts of dedecyltrimethylammonium bromide and hexadecyltrimethyl ammonium bromide),

cetylpyridinium chloride (CPC), cetyl trimethylammonium chloride (CTAC), N,N',N'-polyoxyethylene (10)-N-tallow-1,3 -diaminopropane, dodecyltrimethylammonium bromide, hexadecyltrimethyl-ammonium bromide, mixed alkyl-trimethyl-ammonium bromide, benzyldimethyldodecylammonium chloride, benzyldimethylhexadecyl-ammonium chloride, benzyltrimethylammonium methoxide, cetyl dimethylethylammonium bromide, dimethyldioctadecyl ammonium bromide (DDAB), methylbenzethonium chloride, decamethonium chloride, methyl mixed trialkyl ammonium chloride, methyl trioctylammonium chloride), N,N-dimethyl-N-[2 (2-methyl-4-(1,1,3,3-tetramethylbutyl)- phenoxy]-ethoxy)ethyl]-benzenemetha-naminium chloride (DEBDA), dialkyldimethylammonium salts, [1-(2,3-dioleoyloxy)-propyl]-N,N,N,trimethylammonium chloride, 1,2-diacyl-3-(trimethylammonio) propane (acyl group=dimyristoyl, dipalmitoyl, distearoyl, dioleoyl), 1,2-diacyl-3 (dimethylammonio)propane (acyl group=dimyristoyl, dipalmitoyl, distearoyl, dioleoyl), 1,2-dioleoyl-3-(4'-trimethyl- ammonio)butanoyl-sn-glycerol, 1,2-dioleoyl 3-succinyl-sn-glycerol choline ester, cholesteryl (4'-trimethylammonio) butanoate), N-alkyl pyridinium salts (e.g. cetylpyridinium bromide and cetylpyridinium chloride), N-alkylpiperidinium salts, dicationic bolaform electrolytes (C12Me6; C12BU6), dialkylglycetylphosphorylcholine, lysolecithin, L- $\alpha$  dioleoylphosphatidylethanolamine, cholesterol hemisuccinate choline ester, lipopolyamines, including but not limited to dioctadecylamidoglycylspermine (DOGS), dipalmitoyl phosphatidylethanol-amidospermine (DPPES), lipopoly-L (or D)- lysine (LPLL, LPDL), poly (L (or D)-lysine conjugated to N- glutarylphosphatidylethanolamine, didodecyl glutamate ester with pendant amino group (C<sup>+</sup>GluPhCnN<sup>+</sup>), ditetradecyl glutamate ester with pendant amino group (Cl4GIuCnN<sup>+</sup>), cationic derivatives of cholesterol, including but not limited to cholesteryl-3  $\beta$ -oxysuccinamidoethylenetrimethylammonium salt, cholesteryl-3  $\beta$ -oxysuccinamidoethylene-dimethylamine, cholesteryl-3  $\beta$ -carboxyamidoethylenetrimethylammonium salt, and cholesteryl-3  $\beta$ -carboxyamidoethylenedimethylamine. Other useful cationic lipids are described in US 2008/0085870 and US 2008/0057080, which are incorporated herein by reference.

The cationic lipid is preferably biodegradable (metabolisable) and biocompatible.



In addition to the oil and cationic lipid, an emulsion can include a non-ionic surfactant and/or a zwitterionic surfactant. Such surfactants include, but are not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide  
5 (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxy polyethoxyethanol) being of particular interest; (octylphenoxy) polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as  
10 phosphatidylcholine (lecithin); polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); polyoxyethylene-9-lauryl ether; and sorbitan esters (commonly known as the Spans), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Preferred surfactants for including in the emulsion are polysorbate 80  
15 (Tween 80; polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

Mixtures of these surfactants can be included in the emulsion *e.g.* Tween 80/Span 85 mixtures, or Tween 80/Triton-X100 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (Tween  
20 80) and an octoxynol such as t-octylphenoxy-polyethoxyethanol (Triton X-100) is also suitable. Another useful combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol. Useful mixtures can comprise a surfactant with a HLB value in the range of 10-20 (*e.g.* polysorbate 80, with a HLB of 15.0) and a surfactant with a HLB value in the range of 1-10 (*e.g.* sorbitan trioleate, with a HLB of  
25 1.8).

Preferred amounts of oil (% by volume) in the final emulsion are between 2-20% *e.g.* 5-15%, 6-14%, 7-13%, 8-12%. A squalene content of about 4-6% or about 9-11% is particularly useful.

Preferred amounts of surfactants (% by weight) in the final emulsion are  
30 between 0.001% and 8%. For example: polyoxyethylene sorbitan esters (such as polysorbate 80) 0.2 to 4%, in particular between 0.4-0.6%, between 0.45-0.55%, about 0.5% or between 1.5-2%, between 1.8-2.2%, between 1.9-2.1%, about 2%, or 0.85-

0.95%, or about 1%; sorbitan esters (such as sorbitan trioleate) 0.02 to 2%, in particular about 0.5% or about 1%; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100) 0.001 to 0.1%, in particular 0.005 to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1 to 8%, preferably 0.1 to 10% and in particular 0.1 to 1% or about 0.5%.

5           The absolute amounts of oil and surfactant, and their ratio, can be varied within wide limits while still forming an emulsion. A skilled person can easily vary the relative proportions of the components to obtain a desired emulsion, but a weight ratio of between 4:1 and 5:1 for oil and surfactant is typical (excess oil).

          An important parameter for ensuring immunostimulatory activity of an  
10   emulsion, particularly in large animals, is the oil droplet size (diameter). The most effective emulsions have a droplet size in the submicron range. Suitably the droplet sizes will be in the range 50-750nm. Most usefully the average droplet size is less than 250nm *e.g.* less than 200nm, less than 150nm. The average droplet size is usefully in the range of 80-180nm. Ideally, at least 80% (by number) of the emulsion's oil droplets  
15   are less than 250 nm in diameter, and preferably at least 90%. Apparatuses for determining the average droplet size in an emulsion, and the size distribution, are commercially available. These these typically use the techniques of dynamic light scattering and/or single-particle optical sensing *e.g.* the Accusizer™ and Nicomp™ series of instruments available from Particle Sizing Systems (Santa Barbara, USA), or  
20   the Zetasizer™ instruments from Malvern Instruments (UK), or the Particle Size Distribution Analyzer instruments from Horiba (Kyoto, Japan).

          Ideally, the distribution of droplet sizes (by number) has only one maximum *i.e.* there is a single population of droplets distributed around an average (mode), rather than having two maxima. Preferred emulsions have a polydispersity of <0.4 *e.g.* 0.3,  
25   0.2, or less.

          Suitable emulsions with submicron droplets and a narrow size distribution can be obtained by the use of microfluidisation. This technique reduces average oil droplet size by propelling streams of input components through geometrically fixed channels at high pressure and high velocity. These streams contact channel walls, chamber walls  
30   and each other. The results shear, impact and cavitation forces cause a reduction in droplet size. Repeated steps of microfluidisation can be performed until an emulsion with a desired droplet size average and distribution are achieved.

As an alternative to microfluidisation, thermal methods can be used to cause phase inversion. These methods can also provide a submicron emulsion with a tight particle size distribution.

Preferred emulsions can be filter sterilised *i.e.* their droplets can pass through a  
5 220nm filter. As well as providing a sterilisation, this procedure also removes any large droplets in the emulsion.

In certain embodiments, the cationic lipid in the emulsion is DOTAP. The cationic oil-in-water emulsion may comprise from about 0.5 mg/ml to about 25 mg/ml DOTAP. For example, the cationic oil-in-water emulsion may comprise DOTAP at  
10 from about 0.5 mg/ml to about 25 mg/ml, from about 0.6 mg/ml to about 25 mg/ml, from about 0.7 mg/ml to about 25 mg/ml, from about 0.8 mg/ml to about 25 mg/ml, from about 0.9 mg/ml to about 25 mg/ml, from about 1.0 mg/ml to about 25 mg/ml, from about 1.1 mg/ml to about 25 mg/ml, from about 1.2 mg/ml to about 25 mg/ml, from about 1.3 mg/ml to about 25 mg/ml, from about 1.4 mg/ml to about 25 mg/ml,  
15 from about 1.5 mg/ml to about 25 mg/ml, from about 1.6 mg/ml to about 25 mg/ml, from about 1.7 mg/ml to about 25 mg/ml, from about 0.5 mg/ml to about 24 mg/ml, from about 0.5 mg/ml to about 22 mg/ml, from about 0.5 mg/ml to about 20 mg/ml, from about 0.5 mg/ml to about 18 mg/ml, from about 0.5 mg/ml to about 15 mg/ml, from about 0.5 mg/ml to about 12 mg/ml, from about 0.5 mg/ml to about 10 mg/ml,  
20 from about 0.5 mg/ml to about 5 mg/ml, from about 0.5 mg/ml to about 2 mg/ml, from about 0.5 mg/ml to about 1.9 mg/ml, from about 0.5 mg/ml to about 1.8 mg/ml, from about 0.5 mg/ml to about 1.7 mg/ml, from about 0.5 mg/ml to about 1.6 mg/ml, from about 0.6 mg/ml to about 1.6 mg/ml, from about 0.7 mg/ml to about 1.6 mg/ml, from about 0.8 mg/ml to about 1.6 mg/ml, about 0.5 mg/ml, about 0.6 mg/ml, about 0.7  
25 mg/ml, about 0.8 mg/ml, about 0.9 mg/ml, about 1.0 mg/ml, about 1.1 mg/ml, about 1.2 mg/ml, about 1.3 mg/ml, about 1.4 mg/ml, about 1.5 mg/ml, about 1.6 mg/ml, about 12 mg/ml, about 18 mg/ml, about 20 mg/ml, about 21.8 mg/ml, about 24 mg/ml, *etc.* In an exemplary embodiment, the cationic oil-in-water emulsion comprises from about 0.8 mg/ml to about 1.6 mg/ml DOTAP, such as 0.8 mg/ml, 1.2 mg/ml, 1.4 mg/ml or 1.6  
30 mg/ml.

In certain embodiments, the cationic lipid is DC Cholesterol. The cationic oil-in-water emulsion may comprise DC Cholesterol at from about 0.1 mg/ml to about 5

mg/ml DC Cholesterol. For example, the cationic oil-in-water emulsion may comprise DC Cholesterol from about 0.1 mg/ml to about 5 mg/ml, from about 0.2 mg/ml to about 5 mg/ml, from about 0.3 mg/ml to about 5 mg/ml, from about 0.4 mg/ml to about 5 mg/ml, from about 0.5 mg/ml to about 5 mg/ml, from about 0.62 mg/ml to about 5 mg/ml, from about 1 mg/ml to about 5 mg/ml, from about 1.5 mg/ml to about 5 mg/ml, from about 2 mg/ml to about 5 mg/ml, from about 2.46 mg/ml to about 5 mg/ml, from about 3 mg/ml to about 5 mg/ml, from about 3.5 mg/ml to about 5 mg/ml, from about 4 mg/ml to about 5 mg/ml, from about 4.5 mg/ml to about 5 mg/ml, from about 0.1 mg/ml to about 4.92 mg/ml, from about 0.1 mg/ml to about 4.5 mg/ml, from about 0.1 mg/ml to about 4 mg/ml, from about 0.1 mg/ml to about 3.5 mg/ml, from about 0.1 mg/ml to about 3 mg/ml, from about 0.1 mg/ml to about 2.46 mg/ml, from about 0.1 mg/ml to about 2 mg/ml, from about 0.1 mg/ml to about 1.5 mg/ml, from about 0.1 mg/ml to about 1 mg/ml, from about 0.1 mg/ml to about 0.62 mg/ml, about 0.15 mg/ml, about 0.3 mg/ml, about 0.6 mg/ml, about 0.62 mg/ml, about 0.9 mg/ml, about 1.2 mg/ml, about 2.46 mg/ml, about 4.92 mg/ml, *etc.* In an exemplary embodiment, the cationic oil-in-water emulsion comprises from about 0.62 mg/ml to about 4.92 mg/ml DC Cholesterol, such as 2.46 mg/ml.

In certain embodiments, the cationic lipid is DDA. The cationic oil-in-water emulsion may comprise from about 0.1 mg/ml to about 5 mg/ml DDA. For example, the cationic oil-in-water emulsion may comprise DDA at from about 0.1 mg/ml to about 5 mg/ml, from about 0.1 mg/ml to about 4.5 mg/ml, from about 0.1 mg/ml to about 4 mg/ml, from about 0.1 mg/ml to about 3.5 mg/ml, from about 0.1 mg/ml to about 3 mg/ml, from about 0.1 mg/ml to about 2.5 mg/ml, from about 0.1 mg/ml to about 2 mg/ml, from about 0.1 mg/ml to about 1.5 mg/ml, from about 0.1 mg/ml to about 1.45 mg/ml, from about 0.2 mg/ml to about 5 mg/ml, from about 0.3 mg/ml to about 5 mg/ml, from about 0.4 mg/ml to about 5 mg/ml, from about 0.5 mg/ml to about 5 mg/ml, from about 0.6 mg/ml to about 5 mg/ml, from about 0.73 mg/ml to about 5 mg/ml, from about 0.8 mg/ml to about 5 mg/ml, from about 0.9 mg/ml to about 5 mg/ml, from about 1.0 mg/ml to about 5 mg/ml, from about 1.2 mg/ml to about 5 mg/ml, from about 1.45 mg/ml to about 5 mg/ml, from about 2 mg/ml to about 5 mg/ml, from about 2.5 mg/ml to about 5 mg/ml, from about 3 mg/ml to about 5 mg/ml, from about 3.5 mg/ml to about 5 mg/ml, from about 4 mg/ml to about 5 mg/ml, from

about 4.5 mg/ml to about 5 mg/ml, about 1.2 mg/ml, about 1.45 mg/ml, etc.

Alternatively, the cationic oil-in-water emulsion may comprise DDA at about 20 mg/ml, about 21 mg/ml, about 21.5 mg/ml, about 21.6 mg/ml, about 25 mg/ml. In an exemplary embodiment, the cationic oil-in-water emulsion comprises from about 0.73  
5 mg/ml to about 1.45 mg/ml DDA, such as 1.45 mg/ml.

Catheters or like devices may be used to deliver the self-replicating RNA molecules of the invention, as naked RNA or in combination with a delivery system, into a target organ or tissue. Suitable catheters are disclosed in, *e.g.*, U.S. Pat. Nos. 4,186,745; 5,397,307; 5,547,472; 5,674,192; and 6,129,705, all of which are  
10 incorporated herein by reference.

The present invention includes the use of suitable delivery systems, such as liposomes, polymer microparticles or submicron emulsion microparticles with encapsulated or adsorbed self-replicating RNA, to deliver a self-replicating RNA molecule that encodes an RSV-F polypeptide, for example, to elicit an immune  
15 response alone, or in combination with another macromolecule. The invention includes liposomes, microparticles and submicron emulsions with adsorbed and/or encapsulated self-replicating RNA molecules, and combinations thereof.

As demonstrated further in the Examples, the self-replicating RNA molecules associated with liposomes and submicron emulsion microparticles can be effectively  
20 delivered to the host cell, and can induce an immune response to the protein encoded by the self-replicating RNA.

### **The immunogenic composition**

The invention provides immunogenic compositions. The immunogenic  
25 compositions may include a single active immunogenic agent, or several immunogenic agents. For example, the immunogenic composition can comprise RSV F polypeptides that are in a single form (*e.g.*, monomer, trimer, or rosettes) or in two or more forms (*e.g.*, a mixture of monomer and trimer or a dynamic equilibrium between monomer and trimer). The immunogenic composition can comprise a self-replicating RNA  
30 encoding an RSV-F polypeptide, and preferably also comprises a suitable delivery system, such as liposomes, polymeric microparticles, an oil-in-water emulsion and combinations thereof.

Immunogenic compositions of the invention may also comprise one or more immunoregulatory agents. Preferably, one or more of the immunoregulatory agents include one or more adjuvants, for example two, three, four or more adjuvants. The adjuvants may include a TH1 adjuvant and/or a TH2 adjuvant, further discussed below.

5 In another embodiment, an immunogenic composition of the invention comprises a polypeptide that displays an epitope present in a pre-fusion or an intermediate conformation of RSV-F glycoprotein, but does not display the glycoprotein's post-fusion conformation.

In another embodiment, an immunogenic composition of the invention  
10 comprises a first polypeptide and a second polypeptide, wherein the first polypeptide comprises an RSV F protein, in whole or in part, and the second polypeptide comprises a heterologous oligomerization domain. The first polypeptide can comprise an RSV F protein ectodomain. The second polypeptide can be a trimerization domain from influenza hemagglutinin, a trimerization domain from SARS spike, a trimerization  
15 domain from HIV gp41, NadA, modified GCN4, or ATCase.

In one aspect, the invention is a composition comprising cleaved RSV F protein ecto-domain polypeptides produced by providing uncleaved RSV F protein ecto-domain polypeptides, or C-terminal uncleaved RSV F protein ecto-domain polypeptides, and cleaving them to produce F<sub>1</sub> and F<sub>2</sub> subunits, as described herein.

20 In another aspect, the invention is a composition comprising uncleaved RSV F protein ecto-domain polypeptide trimers and/or monomers produced by providing a biological material that contains uncleaved RSV F protein ecto-domain polypeptides, and purifying uncleaved RSV F protein ecto-domain polypeptides monomers, uncleaved trimers, or a combination of uncleaved monomers and uncleaved trimers  
25 (e.g., a mixture or a dynamic equilibrium) from the biological material, as described herein. In some embodiments, the RSV F protein ecto-domain polypeptide contains altered furin cleavage sites at about positions 106-109 and at about positions 133-136, and if desired can further contain an altered fusion peptide. In other embodiments, the RSV F protein ecto-domain contains altered furin cleavage sites about positions 106-  
30 109 and at about positions 133-136, and altered trypsin cleavage sites between about position 101 and about position 161, and if desired can further contain an altered fusion peptide.

In another aspect, the invention is a composition comprising C-terminal uncleaved RSV F protein ecto-domain polypeptide trimers and/or monomers produced by providing a biological material that contains C-terminal uncleaved RSV F protein ecto-domain polypeptides, and purifying uncleaved RSV F protein ecto-domain polypeptides monomers, uncleaved trimers, or a combination of uncleaved monomers and uncleaved trimers (e.g., a mixture or a dynamic equilibrium) from the biological material, as described herein.

In another aspect, the invention is a composition comprising cleaved RSV F protein ecto-domain polypeptides produced by providing a biological material that contains cleaved RSV F protein ecto-domain polypeptides that contain an altered fusion peptide (e.g., at least a portion of the fusion peptide is deleted) and purifying cleaved RSV F protein ecto-domain polypeptide trimers from the biological material, as described herein.

In another aspect, the invention is a composition comprising uncleaved RSV F protein ecto-domain polypeptides produced by providing a biological material that contains uncleaved RSV F protein ecto-domain polypeptides that contain an altered fusion peptide (e.g., at least a portion of the fusion peptide is deleted) and purifying uncleaved RSV F protein ecto-domain polypeptide monomers from the biological material, as described herein.

The compositions of the invention are preferably suitable for administration to a mammalian subject, such as a human, and include one or more pharmaceutically acceptable carrier(s) and/or excipient(s), including adjuvants. A thorough discussion of such components is available in reference 29. Compositions will generally be in aqueous form. When the composition is an immunogenic composition, it will elicit an immune response when administered to a mammal, such as a human. The immunogenic composition can be used to prepare a vaccine formulation for immunizing a mammal.

The immunogenic compositions may include a single active immunogenic agent, or several immunogenic agents. For example, the RSV F protein ecto-domain polypeptide can be in a single form (e.g., uncleaved monomer, cleaved monomer, uncleaved trimer, cleaved trimer, or rosettes of cleaved trimers) or in two or more forms (e.g., a mixture of uncleaved monomer and uncleaved trimer or a dynamic equilibrium

between uncleaved monomer and uncleaved trimer). In addition, the compositions can contain an RSV F protein ecto-domain polypeptide and one or more other RSV proteins (*e.g.*, a G protein and/or an M protein) and/or it may be combined with immunogens from other pathogens.

5           The composition may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the vaccine should be substantially free from (*i.e.*, less than 5µg/ml) mercurial material, *e.g.*, thiomersal-free. Immunogenic compositions containing no mercury are more preferred. Preservative-free immunogenic compositions are particularly preferred.

10           To control tonicity, it is preferred to include a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 20 mg/ml. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride, calcium chloride, and the like.

15           Compositions will generally have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, preferably between 240-360 mOsm/kg, and will more preferably fall within the range of 290-310 mOsm/kg.

            Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer  
20 (particularly with an aluminum hydroxide adjuvant); or a citrate buffer. Buffers will typically be included in the 5-20mM range. The pH of a composition will generally be between 5.0 and 8.1, and more typically between 6.0 and 8.0, *e.g.*, between 6.5 and 7.5, or between 7.0 and 7.8. A process of the invention may therefore include a step of adjusting the pH of the bulk vaccine prior to packaging.

25           The composition is preferably sterile. The composition is preferably non-pyrogenic, *e.g.*, containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. The composition is preferably gluten free. Human vaccines are typically administered in a dosage volume of about 0.5ml, although a half dose (*i.e.*, about 0.25ml) may be administered to children.

30



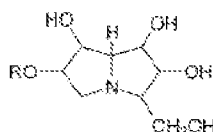
### Adjuvants

Compositions of the invention, that contain RSV-F polypeptids, or nucleic acids that encode RSV-F polypeptids, may also include one or more adjuvants, for example two, three, four or more adjuvants, which can function to enhance the immune  
5 responses (humoral and/or cellular) elicited in a patient who receives the composition. The adjuvants may include a TH1 adjuvant and/or a TH2 adjuvant. Adjuvants which may be used in compositions of the invention include, but are not limited to:

- 10 • Mineral-containing compositions. Mineral-containing compositions suitable for use as adjuvants in the invention include mineral salts, such as calcium salts and aluminum salts (or mixtures thereof). The invention includes mineral salts such as hydroxides (*e.g.* oxyhydroxides), phosphates (*e.g.* hydroxyphosphates, orthophosphates), sulphates, *etc.*, or mixtures of different mineral compounds, with the compounds taking any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*), and with adsorption being preferred. Calcium salts include calcium  
15 phosphate (*e.g.*, the “CAP” particles disclosed in ref. 38). Aluminum salts include hydroxides, phosphates, sulfates, and the like. The mineral containing compositions may also be formulated as a particle of metal salt (39). Aluminum salt adjuvants are described in more detail below.
- 20 • Oil emulsion compositions (see in more detail below). Oil emulsion compositions suitable for use as adjuvants in the invention include squalene-water emulsions, such as MF59 (5% Squalene, 0.5% Tween 80 and 0.5% Span, formulated into submicron particles using a microfluidizer).
- 25 • Cytokine-inducing agents (see in more detail below). Cytokine-inducing agents suitable for use in the invention include toll-like receptor 7 (TLR7) agonists (*e.g.* benzonaphthyridine compounds disclosed in WO 2009/111337).
- 30 • Saponins (chapter 22 of ref. 74), which are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponin from the bark of the *Quillaja saponaria* Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from *Smilax ornata* (sarsapilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as

- well as lipid formulations, such as ISCOMs. QS21 is marketed as STIMULON (TM). Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 40. Saponin formulations may also comprise a sterol, such as cholesterol (41). Combinations of saponins and cholesterol can be used to form unique particles called immunostimulating complexes (ISCOMs) (chapter 23 of ref. 74). ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of QuilA, QHA & QHC. ISCOMs are further described in refs. 41-43. Optionally, the ISCOMS may be devoid of additional detergent (44). A review of the development of saponin based adjuvants can be found in refs. 45 & 46.
- Fatty adjuvants (see in more detail below), including oil-in-water emulsions, modified natural lipid As derived from enterobacterial lipopolysaccharides, phospholipid compounds (such as the synthetic phospholipid dimer, E6020) and the like.
  - Bacterial ADP-ribosylating toxins (*e.g.*, the *E. coli* heat labile enterotoxin “LT”, cholera toxin “CT”, or pertussis toxin “PT”) and detoxified derivatives thereof, such as the mutant toxins known as LT-K63 and LT-R72 (47). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 48 and as parenteral adjuvants in ref. 49.
  - Bioadhesives and mucoadhesives, such as esterified hyaluronic acid microspheres (50) or chitosan and its derivatives (51).
  - Microparticles (*i.e.*, a particle of ~100 nm to ~150 µm in diameter, more preferably ~200 nm to ~30 µm in diameter, or ~500 nm to ~10 µm in diameter) formed from materials that are biodegradable and non-toxic (*e.g.*, a poly( $\alpha$ -hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, and the like), with poly(lactide-co-glycolide) being preferred, optionally treated to have a negatively-charged surface (*e.g.*, with SDS) or a positively-charged surface (*e.g.*, with a cationic detergent, such as CTAB).

- Liposomes (Chapters 13 & 14 of ref. 74). Examples of liposome formulations suitable for use as adjuvants are described in refs. 52-54.
- Polyoxyethylene ethers and polyoxyethylene esters (55). Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol (56) as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol (57). Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-stearyl ether, polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.
- Muramyl peptides, such as N-acetylmuramyl-L-threonyl-D-isoglutamine (“thr-MDP”), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylglucosaminyl-N-acetylmuramyl-L-Al-D-isoglu-L-Ala-dipalmitoyl propylamide (“DTP-DPP”, or “Theramide™”), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (“MTP-PE”).
- An outer membrane protein proteosome preparation prepared from a first Gram-negative bacterium in combination with a liposaccharide preparation derived from a second Gram-negative bacterium, wherein the outer membrane protein proteosome and liposaccharide preparations form a stable non-covalent adjuvant complex. Such complexes include “IVX-908”, a complex comprised of *Neisseria meningitidis* outer membrane and lipopolysaccharides.
- A polyoxidonium polymer (58, 59) or other N-oxidized polyethylene-piperazine derivative.
- Methyl inosine 5'-monophosphate (“MIMP”) (60).
- A polyhydroxylated pyrrolizidine compound (61), such as one having formula:



where R is selected from the group comprising hydrogen, straight or branched, unsubstituted or substituted, saturated or unsaturated acyl, alkyl (e.g., cycloalkyl), alkenyl, alkynyl and aryl groups, or a pharmaceutically acceptable

salt or derivative thereof. Examples include, but are not limited to: casuarine, casuarine-6- $\alpha$ -D-glucopyranose, 3-*epi*-casuarine, 7-*epi*-casuarine, 3,7-*diepi*-casuarine, and the like

- A CD1d ligand, such as an  $\alpha$ -glycosylceramide (62-69) (*e.g.*,  $\alpha$ -galactosylceramide), phytosphingosine-containing  $\alpha$ -glycosylceramides, OCH, KRN7000 [(2S,3S,4R)-1-O-( $\alpha$ -D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol], CRONY-101, 3"-O-sulfo-galactosylceramide, *etc.*
- A gamma inulin (70) or derivative thereof, such as alammulin.
- Virosomes and virus-like particles (VLPs). These structures generally contain one or more proteins from a virus optionally combined or formulated with a phospholipid. They are generally non-pathogenic, non-replicating and generally do not contain any of the native viral genome. The viral proteins may be recombinantly produced or isolated from whole viruses. These viral proteins suitable for use in virosomes or VLPs include proteins derived from influenza virus (such as HA or NA), Hepatitis B virus (such as core or capsid proteins), Hepatitis E virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth Disease virus, Retrovirus, Norwalk virus, human Papilloma virus, HIV, RNA-phages, Q $\beta$ -phage (such as coat proteins), GA-phage, fr-phage, AP205 phage, and Ty (such as retrotransposon Ty protein p1).

These and other adjuvant-active substances are discussed in more detail in references 74 & 75.

Compositions may include two, three, four or more adjuvants. For example, compositions of the invention may advantageously include both an oil-in-water emulsion and a cytokine-inducing agent, or both a mineral-containing composition and a cytokine-inducing agent, or two oil-in-water emulsion adjuvants, or two benzonaphthyridine compounds, *etc.*

Antigens and adjuvants in a composition will typically be in admixture.

#### **Oil emulsion adjuvants**

Oil emulsion compositions suitable for use as adjuvants in the invention include squalene-water emulsions, such as MF59 (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) may also be used.

Various oil-in-water emulsions are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolizable) and biocompatible. The oil droplets in the emulsion are generally less than 5  $\mu\text{m}$  in diameter, and may even have a sub-micron diameter, with these small sizes being achieved with a microfluidizer to provide stable emulsions. Droplets with a size less than 220 nm are preferred as they can be subjected to filter sterilization.

The invention can be used with oils such as those from an animal (such as fish) or vegetable source. Sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Jojoba oil can be used, *e.g.*, obtained from the jojoba bean. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like may also be used. 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, may be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. Fats and oils from mammalian milk are metabolizable and may therefore be used in the practice of this invention. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art. Most fish contain metabolizable oils which may be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which may be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Shark liver oil contains a branched, unsaturated terpenoid known as squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, which is particularly preferred herein. Squalane, the saturated analog to squalene, is also preferred oil. Fish oils, including squalene and squalane, are readily available from commercial sources or may be obtained by methods known in the art. Other preferred oils are the tocopherols (see below). Mixtures of oils can be used.

Surfactants can be classified by their 'HLB' (hydrophile/lipophile balance). Preferred surfactants of the invention have a HLB of at least 10, preferably at least 15, and more preferably at least 16. The invention can be used with surfactants including,

but not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX (TM) tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the TERGITOL (TM) NP series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Non-ionic surfactants are preferred. Preferred surfactants for including in the emulsion are TWEEN 80 (TM) (polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

Mixtures of surfactants can be used *e.g.*, TWEEN 80 (TM)/Span 85 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (TWEEN 80 (TM)) and an octoxynol such as t-octylphenoxypolyethoxyethanol (Triton X-100) is also suitable. Another useful combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol.

Preferred amounts of surfactants (% by weight) are: polyoxyethylene sorbitan esters (such as TWEEN 80 (TM)) 0.01 to 1%, in particular about 0.1 %; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100, or other detergents in the Triton series) 0.001 to 0.1 %, in particular 0.005 to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1 to 20 %, preferably 0.1 to 10 % and in particular 0.1 to 1 % or about 0.5%.

Specific oil-in-water emulsion adjuvants useful with the invention include, but are not limited to:

- A submicron emulsion of squalene, TWEEN 80 (TM), and Span 85. The composition of the emulsion by volume can be about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85. In weight terms, these ratios become

4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85. This adjuvant is known as 'MF59' (71-73), as described in more detail in Chapter 10 of ref. 74 and chapter 12 of ref. 75. The MF59 emulsion advantageously includes citrate ions, *e.g.*, 10mM sodium citrate buffer.

- 5      • An emulsion of squalene, a tocopherol, and TWEEN 80 (TM). The emulsion may include phosphate buffered saline. It may also include Span 85 (*e.g.*, at 1%) and/or lecithin. These emulsions may have from 2 to 10% squalene, from 2 to 10% tocopherol and from 0.3 to 3% TWEEN 80 (TM), and the weight ratio of squalene:tocopherol is preferably <1 as this provides a more stable emulsion.  
10      Squalene and TWEEN 80 (TM) may be present volume ratio of about 5:2. One such emulsion can be made by dissolving TWEEN 80 (TM) in PBS to give a 2% solution, then mixing 90ml of this solution with a mixture of (5g of DL- $\alpha$ -tocopherol and 5ml squalene), then microfluidizing the mixture. The resulting emulsion may have submicron oil droplets, *e.g.*, with an average  
15      diameter of between 100 and 250nm, preferably about 180nm.
- An emulsion of squalene, a tocopherol, and a Triton detergent (*e.g.*, Triton X-100). The emulsion may also include a 3d-MPL (see below). The emulsion may contain a phosphate buffer.
- An emulsion comprising a polysorbate (*e.g.*, polysorbate 80), a Triton detergent  
20      (*e.g.*, Triton X-100) and a tocopherol (*e.g.*, an  $\alpha$ -tocopherol succinate). The emulsion may include these three components at a mass ratio of about 75:11:10 (*e.g.*, 750  $\mu$ g/ml polysorbate 80, 110  $\mu$ g/ml Triton X-100 and 100  $\mu$ g/ml  $\alpha$ -tocopherol succinate), and these concentrations should include any contribution of these components from antigens. The emulsion may also include squalene. The  
25      emulsion may also include a 3d-MPL (see below). The aqueous phase may contain a phosphate buffer.
- An emulsion of squalane, polysorbate 80 and poloxamer 401 ("PLURONIC (TM) L121"). The emulsion can be formulated in phosphate buffered saline, pH 7.4. This emulsion is a useful delivery vehicle for muramyl dipeptides, and has been  
30      used with threonyl-MDP in the "SAF-1" adjuvant (76) (0.05-1% Thr-MDP, 5% squalane, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the "AF" adjuvant (77) (5% squalane, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidization is preferred.

- An emulsion comprising squalene, an aqueous solvent, a polyoxyethylene alkyl ether hydrophilic nonionic surfactant (*e.g.* polyoxyethylene (12) cetostearyl ether) and a hydrophobic nonionic surfactant (*e.g.* a sorbitan ester or mannide ester, such as sorbitan monoleate or 'Span 80'). The emulsion is preferably  
5 thermoreversible and/or has at least 90% of the oil droplets (by volume) with a size less than 200 nm. The emulsion may also include one or more of: alditol; a cryoprotective agent (*e.g.* a sugar, such as dodecylmaltoside and/or sucrose); and/or an alkylpolyglycoside. Such emulsions may be lyophilized.
- An emulsion having from 0.5-50% of an oil, 0.1-10% of a phospholipid, and  
10 0.05-5% of a non-ionic surfactant. As described in reference 78, preferred phospholipid components are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, sphingomyelin and cardiolipin. Submicron droplet sizes are advantageous.
- A submicron oil-in-water emulsion of a non-metabolizable oil (such as light  
15 mineral oil) and at least one surfactant (such as lecithin, TWEEN 80 (TM) or Span 80). Additives may be included, such as QuilA saponin, cholesterol, a saponin-lipophile conjugate (such as GPI-0100, described in reference 79, produced by addition of aliphatic amine to desacylsaponin via the carboxyl group of glucuronic acid), dimethyldioctadecylammonium bromide and/or N,N-  
20 dioctadecyl-N,N-bis (2-hydroxyethyl)propanediamine.
- An emulsion comprising a mineral oil, a non-ionic lipophilic ethoxylated fatty alcohol, and a non-ionic hydrophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer).
- An emulsion comprising a mineral oil, a non-ionic hydrophilic ethoxylated fatty  
25 alcohol, and a non-ionic lipophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer).
- An emulsion in which a saponin (*e.g.*, QuilA or QS21) and a sterol (*e.g.*, a cholesterol) are associated as helical micelles (80).

The emulsions may be mixed with antigen extemporaneously, at the time of  
30 delivery. Thus the adjuvant and antigen may be kept separately in a packaged or distributed vaccine, ready for final formulation at the time of use. The antigen will generally be in an aqueous form, such that the vaccine is finally prepared by mixing



two liquids. The volume ratio of the two liquids for mixing can vary (*e.g.*, between 5:1 and 1:5) but is generally about 1:1.

### Cytokine-inducing agents

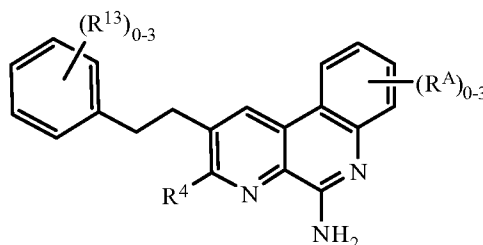
5           Cytokine-inducing agents for inclusion in compositions of the invention are able, when administered to a patient, to elicit the immune system to release cytokines, including interferons and interleukins. Preferred agents can elicit the release of one or more of: interferon- $\gamma$ ; interleukin-1; interleukin-2; interleukin-12; TNF- $\alpha$ ; TNF- $\beta$ ; and GM-CSF. Preferred agents elicit the release of cytokines associated with a Th1-type  
10 immune response, *e.g.*, interferon- $\gamma$ , TNF- $\alpha$ , interleukin-2. Stimulation of both interferon- $\gamma$  and interleukin-2 is preferred.

As a result of receiving a composition of the invention, therefore, a patient will have T cells that, when stimulated with a RSV F protein, will release the desired cytokine(s) in an antigen-specific manner. For example, T cells purified from their  
15 blood will release  $\gamma$ -interferon when exposed *in vitro* to F protein. Methods for measuring such responses in peripheral blood mononuclear cells (PBMC) are known in the art, and include ELISA, ELISPOT, flow-cytometry and real-time PCR. For example, reference 81 reports a study in which antigen-specific T cell-mediated immune responses against tetanus toxoid, specifically  $\gamma$ -interferon responses, were  
20 monitored, and found that ELISPOT was the most sensitive method to discriminate antigen-specific TT-induced responses from spontaneous responses, but that intracytoplasmic cytokine detection by flow cytometry was the most efficient method to detect re-stimulating effects.

Suitable cytokine-inducing agents include, but are not limited to:

- 25           • An immunostimulatory oligonucleotide, such as one containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine linked by a phosphate bond to a guanosine), or a double-stranded RNA, or an oligonucleotide containing a palindromic sequence, or an oligonucleotide containing a poly(dG) sequence.
- 30           • 3-O-deacylated monophosphoryl lipid A ('3dMPL', also known as 'MPL (TM)') (82-85).

- An imidazoquinoline compound, such as IMIQUIMOD (TM) ("R-837") (86, 87), RESIQUIMOD (TM) ("R-848") (88), and their analogs; and salts thereof (e.g., the hydrochloride salts). Further details about immunostimulatory imidazoquinolines can be found in references 89 to 93.
- 5 • A benzonaphthyridine compound, such as: (a) a compound having the formula:



wherein:

- $R^4$  is selected from H, halogen,  $-C(O)OR^7$ ,  $-C(O)R^7$ ,  $-C(O)N(R^{11}R^{12})$ ,  $-N(R^{11}R^{12})$ ,  $-N(R^9)_2$ ,  $-NHN(R^9)_2$ ,  $-SR^7$ ,  $-(CH_2)_nOR^7$ ,  $-(CH_2)_nR^7$ ,  $-LR^8$ ,  $-LR^{10}$ ,  $-OLR^8$ ,  $-OLR^{10}$ ,  $C_1$ - $C_6$ alkyl,  $C_1$ - $C_6$ heteroalkyl,  $C_1$ - $C_6$ haloalkyl,  $C_2$ - $C_8$ alkene,  $C_2$ - $C_8$ alkyne,  $C_1$ - $C_6$ alkoxy,  $C_1$ - $C_6$ haloalkoxy, aryl, heteroaryl,  $C_3$ - $C_8$ cycloalkyl, and  $C_3$ - $C_8$ heterocycloalkyl, wherein the  $C_1$ - $C_6$ alkyl,  $C_1$ - $C_6$ heteroalkyl,  $C_1$ - $C_6$ haloalkyl,  $C_2$ - $C_8$ alkene,  $C_2$ - $C_8$ alkyne,  $C_1$ - $C_6$ alkoxy,  $C_1$ - $C_6$ haloalkoxy, aryl, heteroaryl,  $C_3$ - $C_8$ cycloalkyl, and  $C_3$ - $C_8$ heterocycloalkyl groups of  $R^4$  are each optionally substituted with 1 to 3 substituents independently selected from halogen,  $-CN$ ,  $-NO_2$ ,  $-R^7$ ,  $-OR^8$ ,  $-C(O)R^8$ ,  $-OC(O)R^8$ ,  $-C(O)OR^8$ ,  $-N(R^9)_2$ ,  $-P(O)(OR^8)_2$ ,  $-OP(O)(OR^8)_2$ ,  $-P(O)(OR^{10})_2$ ,  $-OP(O)(OR^{10})_2$ ,  $-C(O)N(R^9)_2$ ,  $-S(O)_2R^8$ ,  $-S(O)R^8$ ,  $-S(O)_2N(R^9)_2$ , and  $-NR^9S(O)_2R^8$ ;
- each L is independently selected from a bond,  $-(O(CH_2)_m)_t$ ,  $C_1$ - $C_6$ alkyl,  $C_2$ - $C_6$ alkenylene and  $C_2$ - $C_6$ alkynylene, wherein the  $C_1$ - $C_6$ alkyl,  $C_2$ - $C_6$ alkenylene and  $C_2$ - $C_6$ alkynylene of L are each optionally substituted with 1 to 4 substituents independently selected from halogen,  $-R^8$ ,  $-OR^8$ ,  $-N(R^9)_2$ ,  $-P(O)(OR^8)_2$ ,  $-OP(O)(OR^8)_2$ ,  $-P(O)(OR^{10})_2$ , and  $-OP(O)(OR^{10})_2$ ;

- $R^7$  is selected from H,  $C_1$ - $C_6$ alkyl, aryl, heteroaryl,  $C_3$ - $C_8$ cycloalkyl,  $C_1$ - $C_6$ heteroalkyl,  $C_1$ - $C_6$ haloalkyl,  $C_2$ - $C_8$ alkene,  $C_2$ - $C_8$ alkyne,  $C_1$ - $C_6$ alkoxy,  $C_1$ - $C_6$ haloalkoxy, and  $C_3$ - $C_8$ heterocycloalkyl, wherein the  $C_1$ - $C_6$ alkyl, aryl, heteroaryl,  $C_3$ - $C_8$ cycloalkyl,  $C_1$ - $C_6$ heteroalkyl,  $C_1$ - $C_6$ haloalkyl,  $C_2$ - $C_8$ alkene,  $C_2$ - $C_8$ alkyne,  $C_1$ - $C_6$ alkoxy,  $C_1$ - $C_6$ haloalkoxy, and  $C_3$ - $C_8$ heterocycloalkyl groups of  $R^7$  are each optionally substituted with 1 to 3  $R^{13}$  groups;
- each  $R^8$  is independently selected from H,  $-CH(R^{10})_2$ ,  $C_1$ - $C_8$ alkyl,  $C_2$ - $C_8$ alkene,  $C_2$ - $C_8$ alkyne,  $C_1$ - $C_6$ haloalkyl,  $C_1$ - $C_6$ alkoxy,  $C_1$ - $C_6$ heteroalkyl,  $C_3$ - $C_8$ cycloalkyl,  $C_2$ - $C_8$ heterocycloalkyl,  $C_1$ - $C_6$ hydroxyalkyl and  $C_1$ - $C_6$ haloalkoxy, wherein the  $C_1$ - $C_8$ alkyl,  $C_2$ - $C_8$ alkene,  $C_2$ - $C_8$ alkyne,  $C_1$ - $C_6$ heteroalkyl,  $C_1$ - $C_6$ haloalkyl,  $C_1$ - $C_6$ alkoxy,  $C_3$ - $C_8$ cycloalkyl,  $C_2$ - $C_8$ heterocycloalkyl,  $C_1$ - $C_6$ hydroxyalkyl and  $C_1$ - $C_6$ haloalkoxy groups of  $R^8$  are each optionally substituted with 1 to 3 substituents independently selected from  $-CN$ ,  $R^{11}$ ,  $-OR^{11}$ ,  $-SR^{11}$ ,  $-C(O)R^{11}$ ,  $-OC(O)R^{11}$ ,  $-C(O)N(R^9)_2$ ,  $-C(O)OR^{11}$ ,  $-NR^9C(O)R^{11}$ ,  $-NR^9R^{10}$ ,  $-NR^{11}R^{12}$ ,  $-N(R^9)_2$ ,  $-OR^9$ ,  $-OR^{10}$ ,  $-C(O)NR^{11}R^{12}$ ,  $-C(O)NR^{11}OH$ ,  $-S(O)_2R^{11}$ ,  $-S(O)R^{11}$ ,  $-S(O)_2NR^{11}R^{12}$ ,  $-NR^{11}S(O)_2R^{11}$ ,  $-P(O)(OR^{11})_2$ , and  $-OP(O)(OR^{11})_2$ ;
- each  $R^9$  is independently selected from H,  $-C(O)R^8$ ,  $-C(O)OR^8$ ,  $-C(O)R^{10}$ ,  $-C(O)OR^{10}$ ,  $-S(O)_2R^{10}$ ,  $-C_1$ - $C_6$ alkyl,  $C_1$ - $C_6$ heteroalkyl and  $C_3$ - $C_6$ cycloalkyl, or each  $R^9$  is independently a  $C_1$ - $C_6$ alkyl that together with N they are attached to form a  $C_3$ - $C_8$ heterocycloalkyl, wherein the  $C_3$ - $C_8$ heterocycloalkyl ring optionally contains an additional heteroatom selected from N, O and S, and wherein the  $C_1$ - $C_6$ alkyl,  $C_1$ - $C_6$ heteroalkyl,  $C_3$ - $C_6$ cycloalkyl, or  $C_3$ - $C_8$ heterocycloalkyl groups of  $R^9$  are each optionally substituted with 1 to 3 substituents independently selected from  $-CN$ ,  $R^{11}$ ,  $-OR^{11}$ ,  $-SR^{11}$ ,  $-C(O)R^{11}$ ,  $-OC(O)R^{11}$ ,  $-C(O)OR^{11}$ ,  $-NR^{11}R^{12}$ ,  $-C(O)NR^{11}R^{12}$ ,  $-C(O)NR^{11}OH$ ,  $-S(O)_2R^{11}$ ,  $-S(O)R^{11}$ ,  $-S(O)_2NR^{11}R^{12}$ ,  $-NR^{11}S(O)_2R^{11}$ ,  $-P(O)(OR^{11})_2$ , and

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-OP(O)(OR<sup>11</sup>)<sub>2</sub>;

each R<sup>10</sup> is independently selected from aryl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl, C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl and heteroaryl, wherein the aryl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl, C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl and heteroaryl groups are optionally substituted with 1 to 3 substituents selected from halogen, -R<sup>8</sup>, -OR<sup>8</sup>, -LR<sup>9</sup>, -LOR<sup>9</sup>, -N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>C(O)R<sup>8</sup>, -NR<sup>9</sup>CO<sub>2</sub>R<sup>8</sup>, -CO<sub>2</sub>R<sup>8</sup>, -C(O)R<sup>8</sup> and -C(O)N(R<sup>9</sup>)<sub>2</sub>;

R<sup>11</sup> and R<sup>12</sup> are independently selected from H, C<sub>1</sub>-C<sub>6</sub>alkyl, C<sub>1</sub>-C<sub>6</sub>heteroalkyl, C<sub>1</sub>-C<sub>6</sub>haloalkyl, aryl, heteroaryl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl, and C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl, wherein the C<sub>1</sub>-C<sub>6</sub>alkyl, C<sub>1</sub>-C<sub>6</sub>heteroalkyl, C<sub>1</sub>-C<sub>6</sub>haloalkyl, aryl, heteroaryl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl, and C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl groups of R<sup>11</sup> and R<sup>12</sup> are each optionally substituted with 1 to 3 substituents independently selected from halogen, -CN, R<sup>8</sup>, -OR<sup>8</sup>, -C(O)R<sup>8</sup>, -OC(O)R<sup>8</sup>, -C(O)OR<sup>8</sup>, -N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>8</sup>C(O)R<sup>8</sup>, -NR<sup>8</sup>C(O)OR<sup>8</sup>, -C(O)N(R<sup>9</sup>)<sub>2</sub>, C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl, -S(O)<sub>2</sub>R<sup>8</sup>, -S(O)<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>S(O)<sub>2</sub>R<sup>8</sup>, C<sub>1</sub>-C<sub>6</sub>haloalkyl and C<sub>1</sub>-C<sub>6</sub>haloalkoxy;

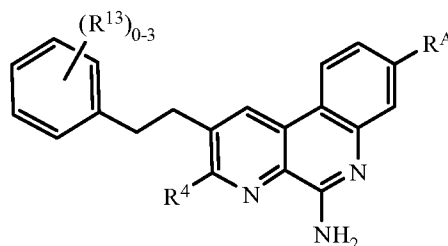
or R<sup>11</sup> and R<sup>12</sup> are each independently C<sub>1</sub>-C<sub>6</sub>alkyl and taken together with the N atom to which they are attached form an optionally substituted C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl ring optionally containing an additional heteroatom selected from N, O and S;

each R<sup>13</sup> is independently selected from halogen, -CN, -LR<sup>9</sup>, -LOR<sup>9</sup>, -OLR<sup>9</sup>, -LR<sup>10</sup>, -LOR<sup>10</sup>, -OLR<sup>10</sup>, -LR<sup>8</sup>, -LOR<sup>8</sup>, -OLR<sup>8</sup>, -LSR<sup>8</sup>, -LSR<sup>10</sup>, -LC(O)R<sup>8</sup>, -OLC(O)R<sup>8</sup>, -LC(O)OR<sup>8</sup>, -LC(O)R<sup>10</sup>, -LOC(O)OR<sup>8</sup>, -LC(O)NR<sup>9</sup>R<sup>11</sup>, -LC(O)NR<sup>9</sup>R<sup>8</sup>, -LN(R<sup>9</sup>)<sub>2</sub>, -LNR<sup>9</sup>R<sup>8</sup>, -LNR<sup>9</sup>R<sup>10</sup>, -LC(O)N(R<sup>9</sup>)<sub>2</sub>, -LS(O)<sub>2</sub>R<sup>8</sup>, -LS(O)R<sup>8</sup>, -LC(O)NR<sup>8</sup>OH, -LNR<sup>9</sup>C(O)R<sup>8</sup>, -LNR<sup>9</sup>C(O)OR<sup>8</sup>, -LS(O)<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -OLS(O)<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -LNR<sup>9</sup>S(O)<sub>2</sub>R<sup>8</sup>, -LC(O)NR<sup>9</sup>LN(R<sup>9</sup>)<sub>2</sub>, -LP(O)(OR<sup>8</sup>)<sub>2</sub>, -LOP(O)(OR<sup>8</sup>)<sub>2</sub>, -LP(O)(OR<sup>10</sup>)<sub>2</sub> and -OLP(O)(OR<sup>10</sup>)<sub>2</sub>;

each R<sup>A</sup> is independently selected from -R<sup>8</sup>, -R<sup>7</sup>, -OR<sup>7</sup>, -OR<sup>8</sup>, -R<sup>10</sup>, -OR<sup>10</sup>, -SR<sup>8</sup>, -NO<sub>2</sub>, -CN, -N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>C(O)R<sup>8</sup>, -NR<sup>9</sup>C(S)R<sup>8</sup>,

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- NR<sup>9</sup>C(O)N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>C(S)N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>CO<sub>2</sub>R<sup>8</sup>,  
 -NR<sup>9</sup>NR<sup>9</sup>C(O)R<sup>8</sup>, -NR<sup>9</sup>NR<sup>9</sup>C(O)N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>NR<sup>9</sup>CO<sub>2</sub>R<sup>8</sup>,  
 -C(O)C(O)R<sup>8</sup>, -C(O)CH<sub>2</sub>C(O)R<sup>8</sup>, -CO<sub>2</sub>R<sup>8</sup>, -(CH<sub>2</sub>)<sub>n</sub>CO<sub>2</sub>R<sup>8</sup>,  
 -C(O)R<sup>8</sup>, -C(S)R<sup>8</sup>, -C(O)N(R<sup>9</sup>)<sub>2</sub>, -C(S)N(R<sup>9</sup>)<sub>2</sub>, -OC(O)N(R<sup>9</sup>)<sub>2</sub>,  
 -OC(O)R<sup>8</sup>, -C(O)N(OR<sup>8</sup>)R<sup>8</sup>, -C(NOR<sup>8</sup>)R<sup>8</sup>, -S(O)<sub>2</sub>R<sup>8</sup>, -S(O)<sub>3</sub>R<sup>8</sup>,  
 -SO<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -S(O)R<sup>8</sup>, -NR<sup>9</sup>SO<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>SO<sub>2</sub>R<sup>8</sup>, -P(O)(OR<sup>8</sup>)<sub>2</sub>, -  
 OP(O)(OR<sup>8</sup>)<sub>2</sub>, -P(O)(OR<sup>10</sup>)<sub>2</sub>, -OP(O)(OR<sup>10</sup>)<sub>2</sub>, -N(OR<sup>8</sup>)R<sup>8</sup>,  
 -CH=CHCO<sub>2</sub>R<sup>8</sup>, -C(=NH)-N(R<sup>9</sup>)<sub>2</sub>, and -(CH<sub>2</sub>)<sub>n</sub>NHC(O)R<sup>8</sup>; or two  
 adjacent R<sup>A</sup> substituents on Ring A form a 5-6 membered ring that  
 contains up to two heteroatoms as ring members;  
 n is, independently at each occurrence, 0, 1, 2, 3, 4, 5, 6, 7 or 8;  
 each m is independently selected from 1, 2, 3, 4, 5 and 6, and  
 t is 1, 2, 3, 4, 5, 6, 7 or 8; (b) a compound having the formula:



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wherein:

- R<sup>4</sup> is selected from H, halogen, -C(O)OR<sup>7</sup>, -C(O)R<sup>7</sup>, -C(O)N(R<sup>11</sup>R<sup>12</sup>),  
 -N(R<sup>11</sup>R<sup>12</sup>), -N(R<sup>9</sup>)<sub>2</sub>, -NHN(R<sup>9</sup>)<sub>2</sub>, -SR<sup>7</sup>, -(CH<sub>2</sub>)<sub>n</sub>OR<sup>7</sup>, -(CH<sub>2</sub>)<sub>n</sub>R<sup>7</sup>,  
 -LR<sup>8</sup>, -LR<sup>10</sup>, -OLR<sup>8</sup>, -OLR<sup>10</sup>, C<sub>1</sub>-C<sub>6</sub>alkyl, C<sub>1</sub>-C<sub>6</sub>heteroalkyl,  
 C<sub>1</sub>-C<sub>6</sub>haloalkyl, C<sub>2</sub>-C<sub>8</sub>alkene, C<sub>2</sub>-C<sub>8</sub>alkyne, C<sub>1</sub>-C<sub>6</sub>alkoxy,  
 C<sub>1</sub>-C<sub>6</sub>haloalkoxy, aryl, heteroaryl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl, and C<sub>3</sub>-  
 C<sub>8</sub>heterocycloalkyl, wherein the C<sub>1</sub>-C<sub>6</sub>alkyl, C<sub>1</sub>-C<sub>6</sub>heteroalkyl, C<sub>1</sub>-  
 C<sub>6</sub>haloalkyl, C<sub>2</sub>-C<sub>8</sub>alkene, C<sub>2</sub>-C<sub>8</sub>alkyne, C<sub>1</sub>-C<sub>6</sub>alkoxy,  
 C<sub>1</sub>-C<sub>6</sub>haloalkoxy, aryl, heteroaryl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl, and  
 C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl groups of R<sup>4</sup> are each optionally substituted  
 with 1 to 3 substituents independently selected from halogen, -CN, -

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NO<sub>2</sub>, -R<sup>7</sup>, -OR<sup>8</sup>, -C(O)R<sup>8</sup>, -OC(O)R<sup>8</sup>, -C(O)OR<sup>8</sup>, -N(R<sup>9</sup>)<sub>2</sub>, -  
P(O)(OR<sup>8</sup>)<sub>2</sub>, -OP(O)(OR<sup>8</sup>)<sub>2</sub>, -P(O)(OR<sup>10</sup>)<sub>2</sub>,  
-OP(O)(OR<sup>10</sup>)<sub>2</sub>, -C(O)N(R<sup>9</sup>)<sub>2</sub>, -S(O)<sub>2</sub>R<sup>8</sup>, -S(O)R<sup>8</sup>,  
-S(O)<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, and -NR<sup>9</sup>S(O)<sub>2</sub>R<sup>8</sup>;

5 each L is independently selected from a bond, -(O(CH<sub>2</sub>)<sub>m</sub>)<sub>t</sub>-, C<sub>1</sub>-C<sub>6</sub>alkyl, C<sub>2</sub>-  
C<sub>6</sub>alkenylene and C<sub>2</sub>-C<sub>6</sub>alkynylene, wherein the C<sub>1</sub>-C<sub>6</sub>alkyl, C<sub>2</sub>-  
C<sub>6</sub>alkenylene and C<sub>2</sub>-C<sub>6</sub>alkynylene of L are each optionally  
substituted with 1 to 4 substituents independently selected from  
halogen, -R<sup>8</sup>, -OR<sup>8</sup>, -N(R<sup>9</sup>)<sub>2</sub>, -P(O)(OR<sup>8</sup>)<sub>2</sub>, -OP(O)(OR<sup>8</sup>)<sub>2</sub>,  
10 -P(O)(OR<sup>10</sup>)<sub>2</sub>, and -OP(O)(OR<sup>10</sup>)<sub>2</sub>;

R<sup>7</sup> is selected from H, C<sub>1</sub>-C<sub>6</sub>alkyl, aryl, heteroaryl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl,  
C<sub>1</sub>-C<sub>6</sub>heteroalkyl, C<sub>1</sub>-C<sub>6</sub>haloalkyl, C<sub>2</sub>-C<sub>8</sub>alkene, C<sub>2</sub>-C<sub>8</sub>alkyne,  
C<sub>1</sub>-C<sub>6</sub>alkoxy, C<sub>1</sub>-C<sub>6</sub>haloalkoxy, and C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl, wherein  
the C<sub>1</sub>-C<sub>6</sub>alkyl, aryl, heteroaryl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl,  
15 C<sub>1</sub>-C<sub>6</sub>heteroalkyl, C<sub>1</sub>-C<sub>6</sub>haloalkyl, C<sub>2</sub>-C<sub>8</sub>alkene, C<sub>2</sub>-C<sub>8</sub>alkyne,  
C<sub>1</sub>-C<sub>6</sub>alkoxy, C<sub>1</sub>-C<sub>6</sub>haloalkoxy, and C<sub>3</sub>-C<sub>8</sub>heterocycloalkyl groups of  
R<sup>7</sup> are each optionally substituted with 1 to 3 R<sup>13</sup> groups;

each R<sup>8</sup> is independently selected from H, -CH(R<sup>10</sup>)<sub>2</sub>, C<sub>1</sub>-C<sub>8</sub>alkyl,  
C<sub>2</sub>-C<sub>8</sub>alkene, C<sub>2</sub>-C<sub>8</sub>alkyne, C<sub>1</sub>-C<sub>6</sub>haloalkyl, C<sub>1</sub>-C<sub>6</sub>alkoxy,  
20 C<sub>1</sub>-C<sub>6</sub>heteroalkyl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl, C<sub>2</sub>-C<sub>8</sub>heterocycloalkyl,  
C<sub>1</sub>-C<sub>6</sub>hydroxyalkyl and C<sub>1</sub>-C<sub>6</sub>haloalkoxy, wherein the C<sub>1</sub>-C<sub>8</sub>alkyl,  
C<sub>2</sub>-C<sub>8</sub>alkene, C<sub>2</sub>-C<sub>8</sub>alkyne, C<sub>1</sub>-C<sub>6</sub>heteroalkyl, C<sub>1</sub>-C<sub>6</sub>haloalkyl,  
C<sub>1</sub>-C<sub>6</sub>alkoxy, C<sub>3</sub>-C<sub>8</sub>cycloalkyl, C<sub>2</sub>-C<sub>8</sub>heterocycloalkyl,  
C<sub>1</sub>-C<sub>6</sub>hydroxyalkyl and C<sub>1</sub>-C<sub>6</sub>haloalkoxy groups of R<sup>8</sup> are each  
25 optionally substituted with 1 to 3 substituents independently selected  
from -CN, R<sup>11</sup>, -OR<sup>11</sup>, -SR<sup>11</sup>, -C(O)R<sup>11</sup>, -OC(O)R<sup>11</sup>,  
-C(O)N(R<sup>9</sup>)<sub>2</sub>, -C(O)OR<sup>11</sup>, -NR<sup>9</sup>C(O)R<sup>11</sup>, -NR<sup>9</sup>R<sup>10</sup>, -NR<sup>11</sup>R<sup>12</sup>,  
-N(R<sup>9</sup>)<sub>2</sub>, -OR<sup>9</sup>, -OR<sup>10</sup>, -C(O)NR<sup>11</sup>R<sup>12</sup>, -C(O)NR<sup>11</sup>OH, -S(O)<sub>2</sub>R<sup>11</sup>, -  
S(O)R<sup>11</sup>, -S(O)<sub>2</sub>NR<sup>11</sup>R<sup>12</sup>, -NR<sup>11</sup>S(O)<sub>2</sub>R<sup>11</sup>, -P(O)(OR<sup>11</sup>)<sub>2</sub>, and  
30 -OP(O)(OR<sup>11</sup>)<sub>2</sub>;

each R<sup>9</sup> is independently selected from H, -C(O)R<sup>8</sup>, -C(O)OR<sup>8</sup>, -C(O)R<sup>10</sup>, -  
C(O)OR<sup>10</sup>, -S(O)<sub>2</sub>R<sup>10</sup>, -C<sub>1</sub>-C<sub>6</sub>alkyl, C<sub>1</sub>-C<sub>6</sub>heteroalkyl and C<sub>3</sub>-C<sub>6</sub>

cycloalkyl, or each  $R^9$  is independently a  $C_1$ - $C_6$ alkyl that together with N they are attached to form a  $C_3$ - $C_8$ heterocycloalkyl, wherein the  $C_3$ - $C_8$ heterocycloalkyl ring optionally contains an additional heteroatom selected from N, O and S, and wherein the  $C_1$ - $C_6$  alkyl,  $C_1$ - $C_6$  heteroalkyl,  $C_3$ - $C_6$  cycloalkyl, or  $C_3$ - $C_8$ heterocycloalkyl groups of  $R^9$  are each optionally substituted with 1 to 3 substituents independently selected from  $-\text{CN}$ ,  $R^{11}$ ,  $-\text{OR}^{11}$ ,  $-\text{SR}^{11}$ ,  $-\text{C}(\text{O})\text{R}^{11}$ ,  $-\text{OC}(\text{O})\text{R}^{11}$ ,  $-\text{C}(\text{O})\text{OR}^{11}$ ,  $-\text{NR}^{11}\text{R}^{12}$ ,  $-\text{C}(\text{O})\text{NR}^{11}\text{R}^{12}$ ,  $-\text{C}(\text{O})\text{NR}^{11}\text{OH}$ ,  $-\text{S}(\text{O})_2\text{R}^{11}$ ,  $-\text{S}(\text{O})\text{R}^{11}$ ,  $-\text{S}(\text{O})_2\text{NR}^{11}\text{R}^{12}$ ,  $-\text{NR}^{11}\text{S}(\text{O})_2\text{R}^{11}$ ,  $-\text{P}(\text{O})(\text{OR}^{11})_2$ , and  $-\text{OP}(\text{O})(\text{OR}^{11})_2$ ;

each  $R^{10}$  is independently selected from aryl,  $C_3$ - $C_8$ cycloalkyl,  $C_3$ - $C_8$ heterocycloalkyl and heteroaryl, wherein the aryl,  $C_3$ - $C_8$ cycloalkyl,  $C_3$ - $C_8$ heterocycloalkyl and heteroaryl groups are optionally substituted with 1 to 3 substituents selected from halogen,  $-\text{R}^8$ ,  $-\text{OR}^8$ ,  $-\text{LR}^9$ ,  $-\text{LOR}^9$ ,  $-\text{N}(\text{R}^9)_2$ ,  $-\text{NR}^9\text{C}(\text{O})\text{R}^8$ ,  $-\text{NR}^9\text{CO}_2\text{R}^8$ ,  $-\text{CO}_2\text{R}^8$ ,  $-\text{C}(\text{O})\text{R}^8$  and  $-\text{C}(\text{O})\text{N}(\text{R}^9)_2$ ;

$R^{11}$  and  $R^{12}$  are independently selected from H,  $C_1$ - $C_6$ alkyl,  $C_1$ - $C_6$ heteroalkyl,  $C_1$ - $C_6$ haloalkyl, aryl, heteroaryl,  $C_3$ - $C_8$ cycloalkyl, and  $C_3$ - $C_8$ heterocycloalkyl, wherein the  $C_1$ - $C_6$ alkyl,  $C_1$ - $C_6$ heteroalkyl,  $C_1$ - $C_6$ haloalkyl, aryl, heteroaryl,  $C_3$ - $C_8$ cycloalkyl, and  $C_3$ - $C_8$ heterocycloalkyl groups of  $R^{11}$  and  $R^{12}$  are each optionally substituted with 1 to 3 substituents independently selected from halogen,  $-\text{CN}$ ,  $\text{R}^8$ ,  $-\text{OR}^8$ ,  $-\text{C}(\text{O})\text{R}^8$ ,  $-\text{OC}(\text{O})\text{R}^8$ ,  $-\text{C}(\text{O})\text{OR}^8$ ,  $-\text{N}(\text{R}^9)_2$ ,  $-\text{NR}^8\text{C}(\text{O})\text{R}^8$ ,  $-\text{NR}^8\text{C}(\text{O})\text{OR}^8$ ,  $-\text{C}(\text{O})\text{N}(\text{R}^9)_2$ ,  $C_3$ - $C_8$ heterocycloalkyl,  $-\text{S}(\text{O})_2\text{R}^8$ ,  $-\text{S}(\text{O})_2\text{N}(\text{R}^9)_2$ ,  $-\text{NR}^9\text{S}(\text{O})_2\text{R}^8$ ,  $C_1$ - $C_6$ haloalkyl and  $C_1$ - $C_6$ haloalkoxy;

or  $R^{11}$  and  $R^{12}$  are each independently  $C_1$ - $C_6$ alkyl and taken together with the N atom to which they are attached form an optionally substituted  $C_3$ - $C_8$ heterocycloalkyl ring optionally containing an additional heteroatom selected from N, O and S;

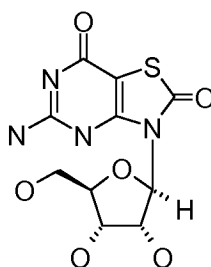
each  $R^{13}$  is independently selected from halogen,  $-\text{CN}$ ,  $-\text{LR}^9$ ,  $-\text{LOR}^9$ ,

- OLR<sup>9</sup>, -LR<sup>10</sup>, -LOR<sup>10</sup>, -OLR<sup>10</sup>, -LR<sup>8</sup>, -LOR<sup>8</sup>, -OLR<sup>8</sup>, -LSR<sup>8</sup>,  
 -LSR<sup>10</sup>, -LC(O)R<sup>8</sup>, -OLC(O)R<sup>8</sup>, -LC(O)OR<sup>8</sup>, -LC(O)R<sup>10</sup>,  
 -LOC(O)OR<sup>8</sup>, -LC(O)NR<sup>9</sup>R<sup>11</sup>, -LC(O)NR<sup>9</sup>R<sup>8</sup>, -LN(R<sup>9</sup>)<sub>2</sub>,  
 -LNR<sup>9</sup>R<sup>8</sup>, -LNR<sup>9</sup>R<sup>10</sup>, -LC(O)N(R<sup>9</sup>)<sub>2</sub>, -LS(O)<sub>2</sub>R<sup>8</sup>, -LS(O)R<sup>8</sup>,  
 5 -LC(O)NR<sup>8</sup>OH, -LNR<sup>9</sup>C(O)R<sup>8</sup>, -LNR<sup>9</sup>C(O)OR<sup>8</sup>,  
 LS(O)<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -OLS(O)<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -LNR<sup>9</sup>S(O)<sub>2</sub>R<sup>8</sup>,  
 -LC(O)NR<sup>9</sup>LN(R<sup>9</sup>)<sub>2</sub>, -LP(O)(OR<sup>8</sup>)<sub>2</sub>, -LOP(O)(OR<sup>8</sup>)<sub>2</sub>,  
 -LP(O)(OR<sup>10</sup>)<sub>2</sub> and -OLP(O)(OR<sup>10</sup>)<sub>2</sub>;  
 each R<sup>A</sup> is independently selected from -R<sup>8</sup>, -R<sup>7</sup>, -OR<sup>7</sup>, -OR<sup>8</sup>, -R<sup>10</sup>,  
 10 -OR<sup>10</sup>, -SR<sup>8</sup>, -NO<sub>2</sub>, -CN, -N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>C(O)R<sup>8</sup>, -NR<sup>9</sup>C(S)R<sup>8</sup>,  
 -NR<sup>9</sup>C(O)N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>C(S)N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>CO<sub>2</sub>R<sup>8</sup>,  
 -NR<sup>9</sup>NR<sup>9</sup>C(O)R<sup>8</sup>, -NR<sup>9</sup>NR<sup>9</sup>C(O)N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>NR<sup>9</sup>CO<sub>2</sub>R<sup>8</sup>,  
 -C(O)C(O)R<sup>8</sup>, -C(O)CH<sub>2</sub>C(O)R<sup>8</sup>, -CO<sub>2</sub>R<sup>8</sup>, -(CH<sub>2</sub>)<sub>n</sub>CO<sub>2</sub>R<sup>8</sup>,  
 -C(O)R<sup>8</sup>, -C(S)R<sup>8</sup>, -C(O)N(R<sup>9</sup>)<sub>2</sub>, -C(S)N(R<sup>9</sup>)<sub>2</sub>, -OC(O)N(R<sup>9</sup>)<sub>2</sub>,  
 15 -OC(O)R<sup>8</sup>, -C(O)N(OR<sup>8</sup>)R<sup>8</sup>, -C(NOR<sup>8</sup>)R<sup>8</sup>, -S(O)<sub>2</sub>R<sup>8</sup>, -S(O)<sub>3</sub>R<sup>8</sup>,  
 -SO<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -S(O)R<sup>8</sup>, -NR<sup>9</sup>SO<sub>2</sub>N(R<sup>9</sup>)<sub>2</sub>, -NR<sup>9</sup>SO<sub>2</sub>R<sup>8</sup>, -P(O)(OR<sup>8</sup>)<sub>2</sub>, -  
 OP(O)(OR<sup>8</sup>)<sub>2</sub>, -P(O)(OR<sup>10</sup>)<sub>2</sub>, -OP(O)(OR<sup>10</sup>)<sub>2</sub>, -N(OR<sup>8</sup>)R<sup>8</sup>,  
 -CH=CHCO<sub>2</sub>R<sup>8</sup>, -C(=NH)-N(R<sup>9</sup>)<sub>2</sub>, and -(CH<sub>2</sub>)<sub>n</sub>NHC(O)R<sup>8</sup>;  
 n is, independently at each occurrence, 0, 1, 2, 3, 4, 5, 6, 7 or 8;  
 20 each m is independently selected from 1, 2, 3, 4, 5 and 6, and  
 t is 1, 2, 3, 4, 5, 6, 7 or 8; or (c) a pharmaceutically acceptable salt of any of (a)  
 or (b). Other benzonaphthyridine compounds, and methods of making  
 benzonaphthyridine compounds, are described in WO 2009/111337. A  
 benzonaphthyridine compound, or a salt thereof, can be used on its own, or in  
 25 combination with one or more further compounds. For example, a  
 benzonaphthyridine compound can be used in combination with an oil-in-water  
 emulsion or a mineral-containing composition. In a particular embodiment, a  
 benzonaphthyridine compound is used in combination with an oil-in-water  
 emulsion (e.g. a squalene-water emulsion, such as MF59) or a mineral-  
 30 containing composition (e.g., a mineral salt such as an aluminum salt or a  
 calcium salt).

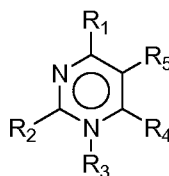


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- A thiosemicarbazone compound, such as those disclosed in reference 94. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 94. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- $\alpha$ .
- A tryptanthrin compound, such as those disclosed in reference 95. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 95. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- $\alpha$ .
- A nucleoside analog, such as: (a) Isatorabine (ANA-245; 7-thia-8-oxoguanosine):



- and prodrugs thereof; (b) ANA975; (c) ANA-025-1; (d) ANA380; (e) the compounds disclosed in references 96 to 98; (f) a compound having the formula:

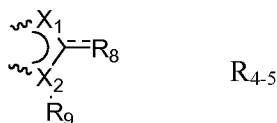


wherein:

- R<sub>1</sub> and R<sub>2</sub> are each independently H, halo, -NR<sub>a</sub>R<sub>b</sub>, -OH, C<sub>1-6</sub> alkoxy, substituted C<sub>1-6</sub> alkoxy, heterocyclyl, substituted heterocyclyl, C<sub>6-10</sub> aryl, substituted C<sub>6-10</sub> aryl, C<sub>1-6</sub> alkyl, or substituted C<sub>1-6</sub> alkyl;
- R<sub>3</sub> is absent, H, C<sub>1-6</sub> alkyl, substituted C<sub>1-6</sub> alkyl, C<sub>6-10</sub> aryl, substituted C<sub>6-10</sub> aryl, heterocyclyl, or substituted heterocyclyl;

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R<sub>4</sub> and R<sub>5</sub> are each independently H, halo, heterocyclyl, substituted heterocyclyl, -C(O)-R<sub>d</sub>, C<sub>1-6</sub> alkyl, substituted C<sub>1-6</sub> alkyl, or bound together to form a 5 membered ring as in R<sub>4-5</sub>:

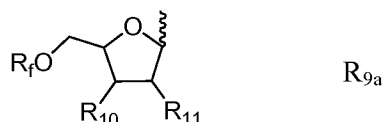


5 the binding being achieved at the bonds indicated by a ~~~

X<sub>1</sub> and X<sub>2</sub> are each independently N, C, O, or S;

R<sub>8</sub> is H, halo, -OH, C<sub>1-6</sub> alkyl, C<sub>2-6</sub> alkenyl, C<sub>2-6</sub> alkynyl, -OH, -NR<sub>a</sub>R<sub>b</sub>, -(CH<sub>2</sub>)<sub>n</sub>-O-R<sub>c</sub>, -O-(C<sub>1-6</sub> alkyl), -S(O)<sub>p</sub>R<sub>e</sub>, or -C(O)-R<sub>d</sub>;

10 R<sub>9</sub> is H, C<sub>1-6</sub> alkyl, substituted C<sub>1-6</sub> alkyl, heterocyclyl, substituted heterocyclyl or R<sub>9a</sub>, wherein R<sub>9a</sub> is:



the binding being achieved at the bond indicated by a ~~~

R<sub>10</sub> and R<sub>11</sub> are each independently H, halo, C<sub>1-6</sub> alkoxy, substituted C<sub>1-6</sub> alkoxy, -NR<sub>a</sub>R<sub>b</sub>, or -OH;

15 each R<sub>a</sub> and R<sub>b</sub> is independently H, C<sub>1-6</sub> alkyl, substituted C<sub>1-6</sub> alkyl, -C(O)R<sub>d</sub>, C<sub>6-10</sub> aryl;

each R<sub>c</sub> is independently H, phosphate, diphosphate, triphosphate, C<sub>1-6</sub> alkyl, or substituted C<sub>1-6</sub> alkyl;

20 each R<sub>d</sub> is independently H, halo, C<sub>1-6</sub> alkyl, substituted C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkoxy, substituted C<sub>1-6</sub> alkoxy, -NH<sub>2</sub>, -NH(C<sub>1-6</sub> alkyl), -NH(substituted C<sub>1-6</sub> alkyl), -N(C<sub>1-6</sub> alkyl)<sub>2</sub>, -N(substituted C<sub>1-6</sub> alkyl)<sub>2</sub>, C<sub>6-10</sub> aryl, or heterocyclyl;

each R<sub>e</sub> is independently H, C<sub>1-6</sub> alkyl, substituted C<sub>1-6</sub> alkyl, C<sub>6-10</sub> aryl, substituted C<sub>6-10</sub> aryl, heterocyclyl, or substituted heterocyclyl;

25 each R<sub>f</sub> is independently H, C<sub>1-6</sub> alkyl, substituted C<sub>1-6</sub> alkyl, -C(O)R<sub>d</sub>, phosphate, diphosphate, or triphosphate;

each n is independently 0, 1, 2, or 3;

each p is independently 0, 1, or 2; or

or (g) a pharmaceutically acceptable salt of any of (a) to (f), a tautomer of any of (a) to (f), or a pharmaceutically acceptable salt of the tautomer.

- Loxoribine (7-allyl-8-oxoguanosine) (99).
- Compounds disclosed in reference 100, including: Acylpiperazine compounds, Indoleione compounds, Tetrahydroisoquinoline (THIQ) compounds, Benzocycloione compounds, Aminoazavinyl compounds, Aminobenzimidazole quinolinone (ABIQ) compounds (101, 102), Hydrapthalamide compounds, Benzophenone compounds, Isoxazole compounds, Sterol compounds, Quinazilinone compounds, Pyrrole compounds (103), Anthraquinone compounds, Quinoxaline compounds, Triazine compounds, Pyrazalopyrimidine compounds, and Benzazole compounds (104).
- Compounds disclosed in reference 105.
- An aminoalkyl glucosaminide phosphate derivative, such as RC-529 (106, 107).
- A phosphazene, such as poly[di(carboxylatophenoxy)phosphazene] ("PCPP") as described, for example, in references 108 and 109.
- Small molecule immunopotentiators (SMIPs) such as:
  - N2-methyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
  - N2,N2-dimethyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
  - N2-ethyl-N2-methyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
  - N2-methyl-1-(2-methylpropyl)-N2-propyl-1H-imidazo[4,5-c]quinoline-2,4-diamine
  - 1-(2-methylpropyl)-N2-propyl-1H-imidazo[4,5-c]quinoline-2,4-diamine
  - N2-butyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
  - N2-butyl-N2-methyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
  - N2-methyl-1-(2-methylpropyl)-N2-pentyl-1H-imidazo[4,5-c]quinoline-2,4-diamine
  - N2-methyl-1-(2-methylpropyl)-N2-prop-2-enyl-1H-imidazo[4,5-c]quinoline-2,4-diamine

- 1-(2-methylpropyl)-2-[(phenylmethyl)thio]-1H-imidazo[4,5-c]quinolin-4-amine
- 1-(2-methylpropyl)-2-(propylthio)-1H-imidazo[4,5-c]quinolin-4-amine
- 2-[[4-amino-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-2-yl](methyl)amino]ethanol
- 2-[[4-amino-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-2-yl](methyl)amino]ethyl acetate
- 4-amino-1-(2-methylpropyl)-1,3-dihydro-2H-imidazo[4,5-c]quinolin-2-one
- N2-butyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
- N2-butyl-N2-methyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
- N2-methyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
- N2,N2-dimethyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine
- 1-[4-amino-2-[methyl(propyl)amino]-1H-imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol
- 1-[4-amino-2-(propylamino)-1H-imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol
- N4,N4-dibenzyl-1-(2-methoxy-2-methylpropyl)-N2-propyl-1H-imidazo[4,5-c]quinoline-2,4-diamine.

The cytokine-inducing agents for use in the present invention may be modulators and/or agonists of Toll-Like Receptors (TLR). For example, they may be agonists of one or more of the human TLR1, TLR2, TLR3, TLR4, TLR7, TLR8, and/or TLR9 proteins. Preferred agents are agonists of TLR4 (*e.g.*, modified natural lipid As derived from enterobacterial lipopolysaccharides, phospholipid compounds, such as the synthetic phospholipid dimer, E6020), TLR7 (*e.g.*, benzonaphthyridines, imidazoquinolines) and/or TLR9 (*e.g.*, CpG oligonucleotides). These agents are useful for activating innate immunity pathways.

The cytokine-inducing agent can be added to the composition at various stages during its production. For example, it may be within an antigen composition, and this mixture can then be added to an oil-in-water emulsion. As an alternative, it may be within an oil-in-water emulsion, in which case the agent can either be added to the emulsion components before emulsification, or it can be added to the emulsion after emulsification. Similarly, the agent may be coacervated within the emulsion droplets. The location and distribution of the cytokine-inducing agent within the final composition will depend on its hydrophilic/lipophilic properties, *e.g.*, the agent can be located in the aqueous phase, in the oil phase, and/or at the oil-water interface.

10       The cytokine-inducing agent can be conjugated to a separate agent, such as an antigen (*e.g.*, CRM197). A general review of conjugation techniques for small molecules is provided in ref. 110. As an alternative, the adjuvants may be non-covalently associated with additional agents, such as by way of hydrophobic or ionic interactions.

15       Preferred cytokine-inducing agents are (a) benzonaphthridine compounds; (b) immunostimulatory oligonucleotides and (c) 3dMPL.

Immunostimulatory oligonucleotides can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or (except for RNA) single-stranded. References 111, 112, and 113 disclose possible analog substitutions, *e.g.*, replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in refs. 114 to 119. A CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT (120). The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN (oligodeoxynucleotide), or it may be more specific for inducing a B cell response, such as a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 121-123. Preferably, the CpG is a CpG-A ODN. Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, references 120 & 124-126. A useful CpG adjuvant is CpG7909, also known as PROMUNE (TM) (Coley Pharmaceutical Group, Inc.).

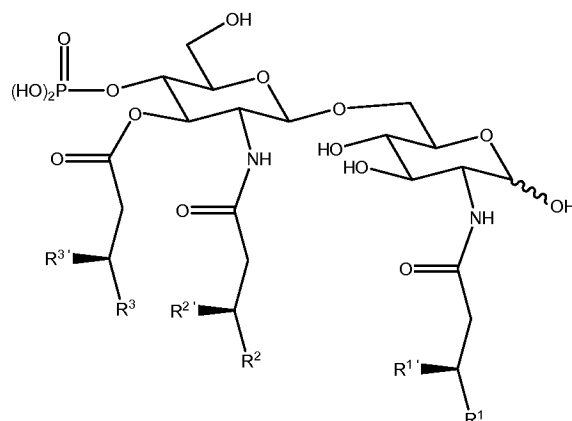
As an alternative, or in addition, to using CpG sequences, TpG sequences can be used (127). These oligonucleotides may be free from unmethylated CpG motifs.

The immunostimulatory oligonucleotide may be pyrimidine-rich. For example, it may comprise more than one consecutive thymidine nucleotide (*e.g.*, TTTT, as disclosed in ref. 127), and/or it may have a nucleotide composition with >25% thymidine (*e.g.*, >35%, >40%, >50%, >60%, >80%, *etc.*). For example, it may comprise more than one consecutive cytosine nucleotide (*e.g.*, CCCC, as disclosed in ref. 127), and/or it may have a nucleotide composition with >25% cytosine (*e.g.*, >35%, >40%, >50%, >60%, >80%, *etc.*). These oligonucleotides may be free from unmethylated CpG motifs.

Immunostimulatory oligonucleotides will typically comprise at least 20 nucleotides. They may comprise fewer than 100 nucleotides.

3dMPL (also known as 3 de-O-acylated monophosphoryl lipid A or 3-O-desacyl-4'-monophosphoryl lipid A) is an adjuvant in which position 3 of the reducing end glucosamine in monophosphoryl lipid A has been de-acylated. 3dMPL has been prepared from a heptoseless mutant of *Salmonella minnesota*, and is chemically similar to lipid A but lacks an acid-labile phosphoryl group and a base-labile acyl group. It activates cells of the monocyte/macrophage lineage and stimulates release of several cytokines, including IL-1, IL-12, TNF- $\alpha$  and GM-CSF (see also ref. 128). Preparation of 3dMPL was originally described in reference 129.

3dMPL can take the form of a mixture of related molecules, varying by their acylation (*e.g.*, having 3, 4, 5 or 6 acyl chains, which may be of different lengths). The two glucosamine (also known as 2-deoxy-2-amino-glucose) monosaccharides are N-acylated at their 2-position carbons (*i.e.*, at positions 2 and 2'), and there is also O-acylation at the 3' position. The group attached to carbon 2 has formula -NH-CO-CH<sub>2</sub>-CR<sup>1</sup>R<sup>1'</sup>. The group attached to carbon 2' has formula -NH-CO-CH<sub>2</sub>-CR<sup>2</sup>R<sup>2'</sup>. The group attached to carbon 3' has formula -O-CO-CH<sub>2</sub>-CR<sup>3</sup>R<sup>3'</sup>. A representative structure is:



Groups  $R^1$ ,  $R^2$  and  $R^3$  are each independently  $-(CH_2)_n-CH_3$ . The value of  $n$  is preferably between 8 and 16, more preferably between 9 and 12, and is most preferably 10.

- 5 Groups  $R^{1'}$ ,  $R^{2'}$  and  $R^{3'}$  can each independently be: (a)  $-H$ ; (b)  $-OH$ ; or (c)  $-O-CO-R^4$ , where  $R^4$  is either  $-H$  or  $-(CH_2)_m-CH_3$ , wherein the value of  $m$  is preferably between 8 and 16, and is more preferably 10, 12 or 14. At the 2 position,  $m$  is preferably 14. At the 2' position,  $m$  is preferably 10. At the 3' position,  $m$  is preferably 12. Groups  $R^{1'}$ ,  $R^{2'}$  and  $R^{3'}$  are thus preferably  $-O$ -acyl groups from dodecanoic acid, 10 tetradecanoic acid or hexadecanoic acid.

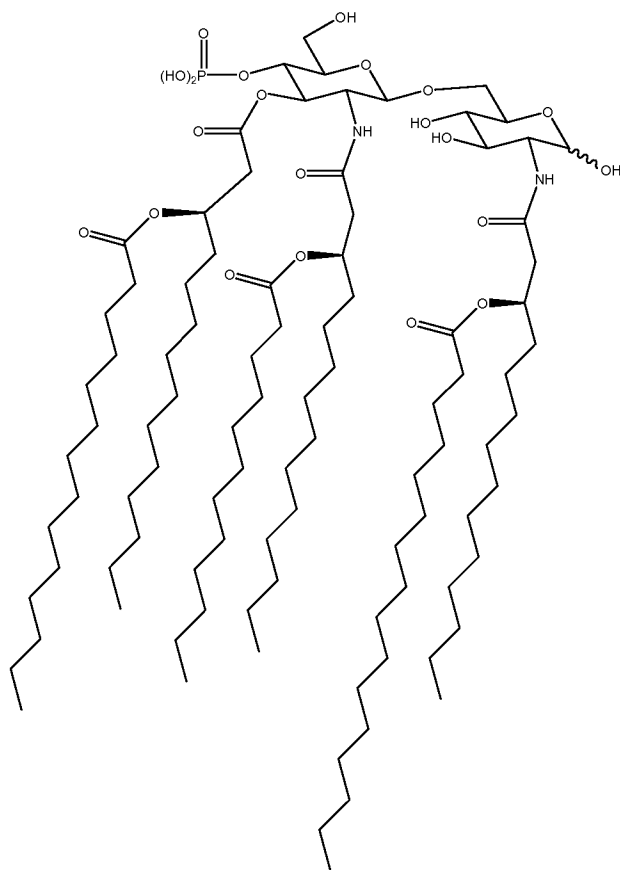
- When all of  $R^{1'}$ ,  $R^{2'}$  and  $R^{3'}$  are  $-H$  then the 3dMPL has only 3 acyl chains (one on each of positions 2, 2' and 3'). When only two of  $R^{1'}$ ,  $R^{2'}$  and  $R^{3'}$  are  $-H$  then the 3dMPL can have 4 acyl chains. When only one of  $R^{1'}$ ,  $R^{2'}$  and  $R^{3'}$  is  $-H$  then the 3dMPL can have 5 acyl chains. When none of  $R^{1'}$ ,  $R^{2'}$  and  $R^{3'}$  is  $-H$  then the 3dMPL 15 can have 6 acyl chains. The 3dMPL adjuvant used according to the invention can be a mixture of these forms, with from 3 to 6 acyl chains, but it is preferred to include 3dMPL with 6 acyl chains in the mixture, and in particular to ensure that the hexaacyl chain form makes up at least 10% by weight of the total 3dMPL *e.g.*, >20%, >30%, >40%, >50% or more. 3dMPL with 6 acyl chains has been found to be the most 20 adjuvant-active form.

Thus the most preferred form of 3dMPL for inclusion in compositions of the invention has formula (IV), shown below.

- Where 3dMPL is used in the form of a mixture then references to amounts or concentrations of 3dMPL in compositions of the invention refer to the combined 25 3dMPL species in the mixture.

- In aqueous conditions, 3dMPL can form micellar aggregates or particles with different sizes *e.g.*, with a diameter <150nm or >500nm. Either or both of these can be used with the invention, and the better particles can be selected by routine assay. Smaller particles (*e.g.*, small enough to give a clear aqueous suspension of 3dMPL) are
- 5 preferred for use according to the invention because of their superior activity (130). Preferred particles have a mean diameter less than 220nm, more preferably less than 200nm or less than 150nm or less than 120nm, and can even have a mean diameter less than 100nm. In most cases, however, the mean diameter will not be lower than 50nm. These particles are small enough to be suitable for filter sterilization. Particle diameter
- 10 can be assessed by the routine technique of dynamic light scattering, which reveals a mean particle diameter. Where a particle is said to have a diameter of  $x$  nm, there will generally be a distribution of particles about this mean, but at least 50% by number (*e.g.*, >60%, >70%, >80%, >90%, or more) of the particles will have a diameter within the range  $x \pm 25\%$ .
- 15 3dMPL can advantageously be used in combination with an oil-in-water emulsion. Substantially all of the 3dMPL may be located in the aqueous phase of the emulsion.
- The 3dMPL can be used on its own, or in combination with one or more further compounds. For example, it is known to use 3dMPL in combination with the QS21
- 20 saponin (131) (including in an oil-in-water emulsion (132)), with an immunostimulatory oligonucleotide, with both QS21 and an immunostimulatory oligonucleotide, with aluminum phosphate (133), with aluminum hydroxide (134), or with both aluminum phosphate and aluminum hydroxide.



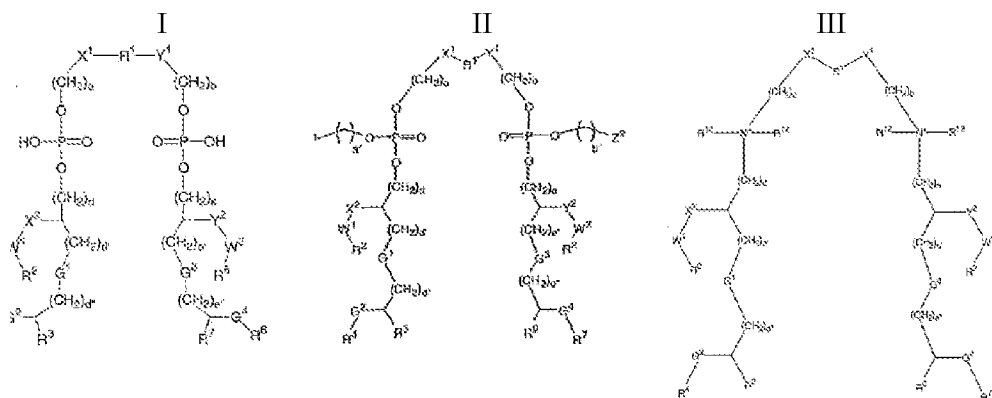


Formula (IV)

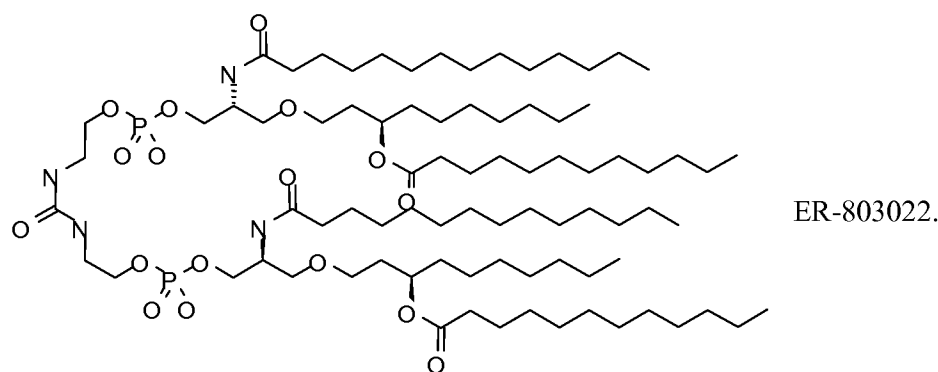
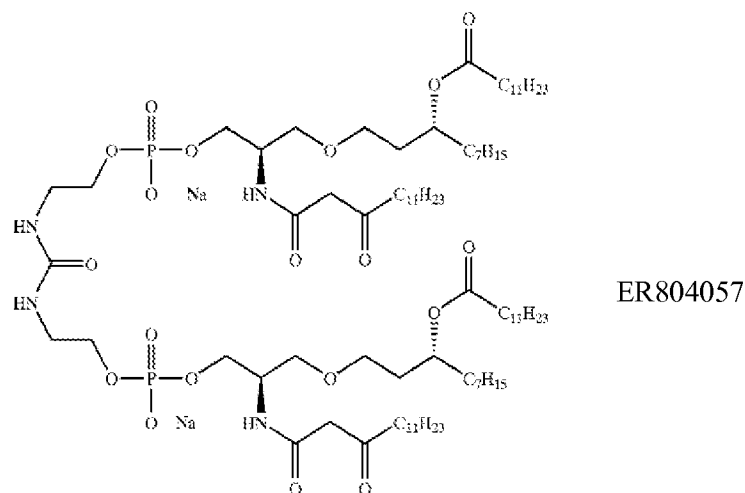
### Fatty adjuvants

Fatty adjuvants that can be used with the invention include the oil-in-water emulsions described above, and also include, for example:

- 5 • A phospholipid compound of formula I, II or III, or a salt thereof:



as defined in reference 135, such as 'ER 803058', 'ER 803732', 'ER 804053',  
ER 804058', 'ER 804059', 'ER 804442', 'ER 804680', 'ER 804764', ER  
803022 or 'ER 804057' *e.g.*:

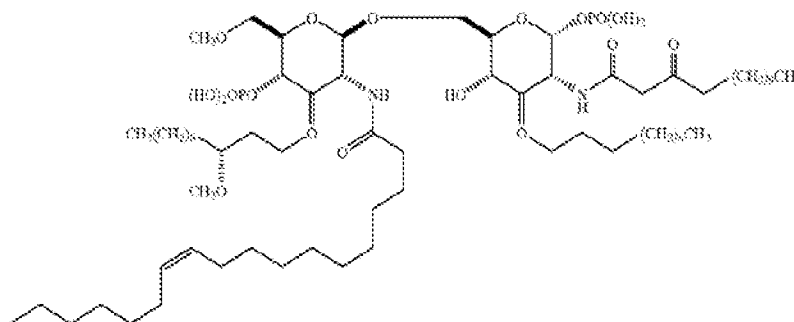


ER804057 is also called E6020. A phospholipid compound of formula I, II or III, or a salt thereof, can be used on its own, or in combination with one or more further compounds. For example, a compound of formula I, II or III, can be used in combination with an oil-in-water emulsion or a mineral-containing composition. In a particular embodiment, E6020 is used in combination with an oil-in-water emulsion (e.g. a squalene-water emulsion, such as MF59) or a mineral-containing composition (e.g., a mineral salt such as an aluminum salt or a calcium salt).

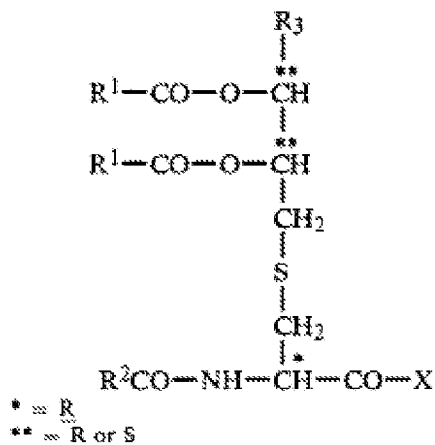
- Derivatives of lipid A from *Escherichia coli* such as OM-174 (described in refs. 136 & 137).
- A formulation of a cationic lipid and a (usually neutral) co-lipid, such as aminopropyl-dimethyl-myristeyleoxy-propanaminium bromide-diphytanoylphosphatidyl-ethanolamine ("VAXFECTIN (TM)") or aminopropyl-dimethyl-bis-dodecyloxy-propanaminium bromide-dioleoylphosphatidyl-ethanolamine ("GAP-DLRIE:DOPE"). Formulations

containing (+)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(syn-9-tetradeceneyloxy)-1-propanaminium salts are preferred (138).

- 3-O-deacylated monophosphoryl lipid A (see above).
- Compounds containing lipids linked to a phosphate-containing acyclic backbone, such as the TLR4 antagonist E5564 (139, 140):



- Lipopeptides (i.e., compounds comprising one or more fatty acid residues and two or more amino acid residues), such as lipopeptides based on glycylcysteine. Specific examples of such peptides include compounds of the following formula



in which each of  $R^1$  and  $R^2$  represents a saturated or unsaturated, aliphatic or mixed aliphatic-cycloaliphatic hydrocarbon radical having from 8 to 30, preferably 11 to 21, carbon atoms that is optionally also substituted by oxygen functions,  $R^3$  represents hydrogen or the radical  $R^1-CO-O-CH_2-$  in which  $R^1$  has the same meaning as above, and X represents an amino acid bonded by a peptide linkage and having a free, esterified or amidated carboxy group, or an amino acid sequence of from 2 to 10 amino acids of which the terminal carboxy group is in free, esterified or amidated form. In

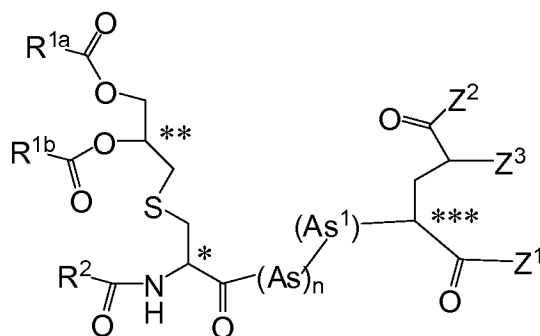
- 95 -

certain embodiments, the amino acid sequence comprises a D-amino acid, for example, D-glutamic acid (D-Glu) or D-gamma-carboxy-glutamic acid (D-Gla).

Bacterial lipopeptides generally recognize TLR2, without requiring TLR6 to participate. (TLRs operate cooperatively to provide specific recognition of various triggers, and TLR2 plus TLR6 together recognize peptidoglycans, while TLR2 recognizes lipopeptides without TLR6.) These are sometimes classified as natural lipopeptides and synthetic lipopeptides. Synthetic lipopeptides tend to behave similarly, and are primarily recognized by TLR2.

Lipopeptides suitable for use as adjuvants include compounds have the formula:

10



where the chiral center labeled \* and the one labeled \*\*\* are both in the R configuration;

15 the chiral center labeled \*\* is either in the R or S configuration;

each  $R^{1a}$  and  $R^{1b}$  is independently an aliphatic or cycloaliphatic-aliphatic hydrocarbon group having 7-21 carbon atoms, optionally substituted by oxygen functions, or one of  $R^{1a}$  and  $R^{1b}$ , but not both, is H;

$R^2$  is an aliphatic or cycloaliphatic hydrocarbon group having 1-21 carbon atoms and optionally substituted by oxygen functions;

20

n is 0 or 1;

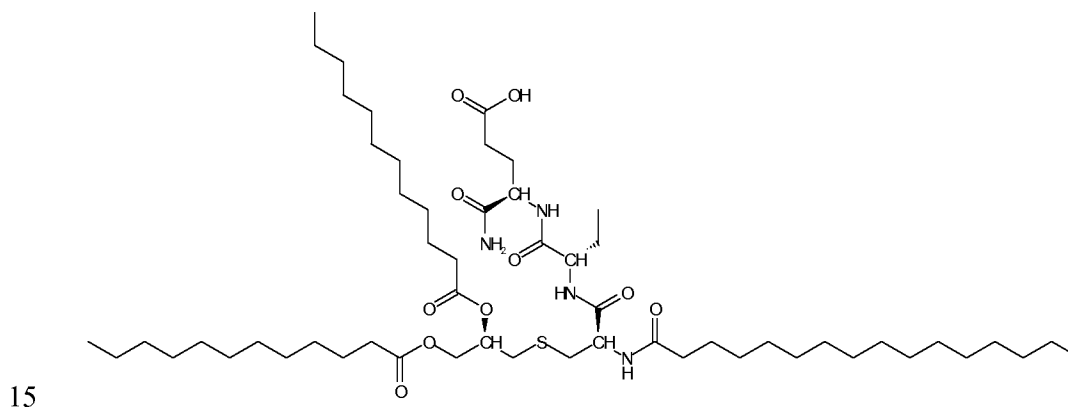
As represents either  $-O-Kw-CO-$  or  $-NH-Kw-CO-$ , where Kw is an aliphatic hydrocarbon group having 1-12 carbon atoms;

$As^1$  is a D- or L-alpha-amino acid;

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- $Z^1$  and  $Z^2$  each independently represent  $-OH$  or the N-terminal radical of a D- or L-alpha amino acid of an amino-(lower alkane)-sulfonic acid or of a peptide having up to 6 amino acids selected from the D- and L-alpha aminocarboxylic acids and amino-lower alkyl-sulfonic acids; and
- 5  $Z^3$  is H or  $-CO-Z^4$ , where  $Z^4$  is  $-OH$  or the N-terminal radical of a D- or L-alpha amino acid of an amino-(lower alkane)-sulfonic acid or of a peptide having up to 6 amino acids selected from the D and L-alpha aminocarboxylic acids and amino-lower alkyl-sulfonic acids; or an ester or amide formed from the carboxylic acid of such compounds. Suitable amides include  $-NH_2$  and
- 10  $NH(\text{lower alkyl})$ , and suitable esters include C1-C4 alkyl esters. (lower alkyl or lower alkane, as used herein, refers to  $C_1$ - $C_6$  straight chain or branched alkyls).

Such compounds are described in more detail in US 4,666,886. In one particular embodiment, the lipopeptide has the formula:



Another example of a lipopeptide species is called LP40, and is an agonist of TLR2. Akdis, et al., *Eur. J. Immunology*, 33: 2717-26 (2003).

- These are related to a known class of lipopeptides from *E. coli*, referred to as murein lipoproteins. Certain partial degradation products of those proteins called
- 20 murein lipopeptides are described in Hantke, et al., *Eur. J. Biochem.*, 34: 284-296 (1973). These comprise a peptide linked to N-acetyl muramic acid and are thus related to Muramyl peptides, which are described in Baschang, et al., *Tetrahedron*, 45(20): 6331-6360 (1989).

### Aluminum salt adjuvants

The adjuvants known as “aluminum hydroxide” and “aluminum phosphate” may be used. These names are conventional, but are used for convenience only, as  
5 neither is a precise description of the actual chemical compound which is present (*e.g.*, see chapter 9 of reference 74). The invention can use any of the “hydroxide” or “phosphate” adjuvants that are in general use as adjuvants.

The adjuvants known as “aluminum hydroxide” are typically aluminum oxyhydroxide salts, which are usually at least partially crystalline. Aluminum  
10 oxyhydroxide, which can be represented by the formula  $\text{AlO}(\text{OH})$ , can be distinguished from other aluminum compounds, such as aluminum hydroxide  $\text{Al}(\text{OH})_3$ , by infrared (IR) spectroscopy, in particular by the presence of an adsorption band at  $1070\text{cm}^{-1}$  and a strong shoulder at  $3090\text{--}3100\text{cm}^{-1}$  (chapter 9 of ref. 74). The degree of crystallinity of an aluminum hydroxide adjuvant is reflected by the width of the diffraction band at  
15 half height (WHH), with poorly-crystalline particles showing greater line broadening due to smaller crystallite sizes. The surface area increases as WHH increases, and adjuvants with higher WHH values have been seen to have greater capacity for antigen adsorption. A fibrous morphology (*e.g.*, as seen in transmission electron micrographs) is typical for aluminum hydroxide adjuvants. The pI of aluminum hydroxide adjuvants  
20 is typically about 11, *i.e.*, the adjuvant itself has a positive surface charge at physiological pH. Adsorptive capacities of between 1.8-2.6 mg protein per mg  $\text{Al}^{+++}$  at pH 7.4 have been reported for aluminum hydroxide adjuvants.

The adjuvants known as “aluminum phosphate” are typically aluminum hydroxyphosphates, often also containing a small amount of sulfate (*i.e.*, aluminum  
25 hydroxyphosphate sulfate). They may be obtained by precipitation, and the reaction conditions and concentrations during precipitation influence the degree of substitution of phosphate for hydroxyl in the salt. Hydroxyphosphates generally have a  $\text{PO}_4/\text{Al}$  molar ratio between 0.3 and 1.2. Hydroxyphosphates can be distinguished from strict  $\text{AlPO}_4$  by the presence of hydroxyl groups. For example, an IR spectrum band at  
30  $3164\text{cm}^{-1}$  (*e.g.*, when heated to  $200^\circ\text{C}$ ) indicates the presence of structural hydroxyls (ch.9 of ref. 74)

The  $\text{PO}_4/\text{Al}^{3+}$  molar ratio of an aluminum phosphate adjuvant will generally be between 0.3 and 1.2, preferably between 0.8 and 1.2, and more preferably 0.95+0.1. The aluminum phosphate will generally be amorphous, particularly for hydroxyphosphate salts. A typical adjuvant is amorphous aluminum hydroxyphosphate with  $\text{PO}_4/\text{Al}$  molar ratio between 0.84 and 0.92, included at 0.6mg  $\text{Al}^{3+}/\text{ml}$ . The aluminum phosphate will generally be particulate (*e.g.*, plate-like morphology as seen in transmission electron micrographs). Typical diameters of the particles are in the range 0.5-20 $\mu\text{m}$  (*e.g.*, about 5-10 $\mu\text{m}$ ) after any antigen adsorption. Adsorptive capacities of between 0.7-1.5 mg protein per mg  $\text{Al}^{+++}$  at pH 7.4 have been reported for aluminum phosphate adjuvants.

The point of zero charge (PZC) of aluminum phosphate is inversely related to the degree of substitution of phosphate for hydroxyl, and this degree of substitution can vary depending on reaction conditions and concentration of reactants used for preparing the salt by precipitation. PZC is also altered by changing the concentration of free phosphate ions in solution (more phosphate = more acidic PZC) or by adding a buffer such as a histidine buffer (makes PZC more basic). Aluminum phosphates used according to the invention will generally have a PZC of between 4.0 and 7.0, more preferably between 5.0 and 6.5, *e.g.*, about 5.7.

Suspensions of aluminum salts used to prepare compositions of the invention may contain a buffer (*e.g.*, a phosphate or a histidine or a Tris buffer), but this is not always necessary. The suspensions are preferably sterile and pyrogen-free. A suspension may include free aqueous phosphate ions *e.g.*, present at a concentration between 1.0 and 20 mM, preferably between 5 and 15 mM, and more preferably about 10 mM. The suspensions may also comprise sodium chloride.

The invention can use a mixture of both an aluminum hydroxide and an aluminum phosphate. In this case there may be more aluminum phosphate than hydroxide *e.g.*, a weight ratio of at least 2:1 *e.g.*, >5:1, >6:1, >7:1, >8:1, >9:1, *etc.*

The concentration of  $\text{Al}^{+++}$  in a composition for administration to a patient is preferably less than 10mg/ml *e.g.*, <5 mg/ml, <4 mg/ml, <3 mg/ml, <2 mg/ml, <1 mg/ml, *etc.* A preferred range is between 0.3 and 1mg/ml. A maximum of 0.85mg/dose is preferred.

As well as including one or more aluminum salt adjuvants, the adjuvant component may include one or more further adjuvant or immunostimulating agents. Such additional components include, but are not limited to: a benzonaphthridine compound, a 3-O-deacylated monophosphoryl lipid A adjuvant ('3d-MPL'); and/or an oil-in-water emulsion. 3d-MPL has also been referred to as 3 de-O-acylated monophosphoryl lipid A or as 3-O-desacyl-4'-monophosphoryl lipid A. The name indicates that position 3 of the reducing end glucosamine in monophosphoryl lipid A is de-acylated. It has been prepared from a heptoseless mutant of *S.minnesota*, and is chemically similar to lipid A but lacks an acid-labile phosphoryl group and a base-labile acyl group. It activates cells of the monocyte/macrophage lineage and stimulates release of several cytokines, including IL-1, IL-12, TNF- $\alpha$  and GM-CSF. Preparation of 3d-MPL was originally described in reference 129, and the product has been manufactured and sold by Corixa Corporation under the name MPL (TM). Further details can be found in refs 82 to 85.

The use of an aluminum hydroxide and/or aluminum phosphate adjuvant is useful, particularly in children, and antigens are generally adsorbed to these salts. Squalene-in-water emulsions are also preferred, particularly in the elderly. Useful adjuvant combinations include combinations of Th1 and Th2 adjuvants such as CpG and alum, or resiquimod and alum. A combination of aluminum phosphate and 3dMPL may be used. Other combinations that may be used include: alum and a benzonaphthridine compound or a SMIP, a squalene-in-water emulsion (such as MF59) and a benzonaphthridine compound or a SMIP, and E6020 and a squalene-in-water emulsion, such as MF59) or alum.

The compositions of the invention may elicit both a cell mediated immune response as well as a humoral immune response.

Two types of T cells, CD4 and CD8 cells, are generally thought necessary to initiate and/or enhance cell mediated immunity and humoral immunity. CD8 T cells can express a CD8 co-receptor and are commonly referred to as Cytotoxic T lymphocytes (CTLs). CD8 T cells are able to recognized or interact with antigens displayed on MHC Class I molecules.

CD4 T cells can express a CD4 co-receptor and are commonly referred to as T helper cells. CD4 T cells are able to recognize antigenic peptides bound to MHC class



II molecules. Upon interaction with a MHC class II molecule, the CD4 cells can secrete factors such as cytokines. These secreted cytokines can activate B cells, cytotoxic T cells, macrophages, and other cells that participate in an immune response. Helper T cells or CD4<sup>+</sup> cells can be further divided into two functionally distinct subsets: TH1  
5 phenotype and TH2 phenotypes which differ in their cytokine and effector function.

Activated TH1 cells enhance cellular immunity (including an increase in antigen-specific CTL production) and are therefore of particular value in responding to intracellular infections. Activated TH1 cells may secrete one or more of IL-2, IFN- $\gamma$ , and TNF- $\beta$ . A TH1 immune response may result in local inflammatory reactions by  
10 activating macrophages, NK (natural killer) cells, and CD8 cytotoxic T cells (CTLs). A TH1 immune response may also act to expand the immune response by stimulating growth of B and T cells with IL-12. TH1 stimulated B cells may secrete IgG2a.

Activated TH2 cells enhance antibody production and are therefore of value in responding to extracellular infections. Activated TH2 cells may secrete one or more of  
15 IL-4, IL-5, IL-6, and IL-10. A TH2 immune response may result in the production of IgG1, IgE, IgA and memory B cells for future protection.

An enhanced immune response may include one or more of an enhanced TH1 immune response and a TH2 immune response.

A TH1 immune response may include one or more of an increase in CTLs, an  
20 increase in one or more of the cytokines associated with a TH1 immune response (such as IL-2, IFN- $\gamma$ , and TNF- $\beta$ ), an increase in activated macrophages, an increase in NK activity, or an increase in the production of IgG2a. Preferably, the enhanced TH1 immune response will include an increase in IgG2a production.

A TH1 immune response may be elicited using a TH1 adjuvant. A TH1  
25 adjuvant will generally elicit increased levels of IgG2a production relative to immunization of the antigen without adjuvant. TH1 adjuvants suitable for use in the invention may include for example saponin formulations, virosomes and virus like particles, non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), immunostimulatory oligonucleotides. Immunostimulatory oligonucleotides, such as  
30 oligonucleotides containing a CpG motif, are preferred TH1 adjuvants for use in the invention.

A TH2 immune response may include one or more of an increase in one or more of the cytokines associated with a TH2 immune response (such as IL-4, IL-5, IL-6 and IL-10), or an increase in the production of IgG1, IgE, IgA and memory B cells.

Preferably, the enhanced TH2 immune response will include an increase in IgG1  
5 production.

A TH2 immune response may be elicited using a TH2 adjuvant. A TH2 adjuvant will generally elicit increased levels of IgG1 production relative to immunization of the antigen without adjuvant. TH2 adjuvants suitable for use in the invention include, for example, mineral containing compositions, oil-emulsions, and  
10 ADP-ribosylating toxins and detoxified derivatives thereof. Mineral containing compositions, such as aluminium salts are preferred TH2 adjuvants for use in the invention.

A composition may include a combination of a TH1 adjuvant and a TH2 adjuvant. Preferably, such a composition elicits an enhanced TH1 and an enhanced TH2  
15 response, i.e., an increase in the production of both IgG1 and IgG2a production relative to immunization without an adjuvant. Still more preferably, the composition comprising a combination of a TH1 and a TH2 adjuvant elicits an increased TH1 and/or an increased TH2 immune response relative to immunization with a single adjuvant (*i.e.*, relative to immunization with a TH1 adjuvant alone or immunization with a TH2  
20 adjuvant alone).

The immune response may be one or both of a TH1 immune response and a TH2 response. Preferably, immune response provides for one or both of an enhanced TH1 response and an enhanced TH2 response.

The enhanced immune response may be one or both of a systemic and a  
25 mucosal immune response. Preferably, the immune response provides for one or both of an enhanced systemic and an enhanced mucosal immune response. Preferably the mucosal immune response is a TH2 immune response. Preferably, the mucosal immune response includes an increase in the production of IgA.

### 30 **Methods of treatment, and administration**

Compositions of the invention are suitable for administration to mammals, and the invention provides a method of inducing an immune response in a mammal,

comprising the step of administering a composition (e.g., an immunogenic composition) of the invention to the mammal. The compositions (e.g., an immunogenic composition) can be used to produce a vaccine formulation for immunizing a mammal. The mammal is typically a human, and the RSV F protein ecto-domain is typically a human RSV F protein ecto-domain. However, the mammal can be any other mammal that is susceptible to infection with RSV, such as a cow that can be infected with bovine RSV. For example, the immune response may be raised following administration of a purified RSV F protein, an alphavirus particle, or self-replicating RNA.

The invention also provides a composition of the invention for use as a medicament, e.g., for use in immunizing a patient against RSV infection.

The invention also provides the use of a polypeptide as described above in the manufacture of a medicament for raising an immune response in a patient.

The immune response raised by these methods and uses will generally include an antibody response, preferably a protective antibody response. Methods for assessing antibody responses after RSV vaccination are well known in the art.

Compositions of the invention can be administered in a number of suitable ways, such as intramuscular injection (e.g., into the arm or leg), subcutaneous injection, intranasal administration, oral administration, intradermal administration, transcutaneous administration, transdermal administration, and the like. The appropriate route of administration will be dependent upon the age, health and other characteristics of the mammal. A clinician will be able to determine an appropriate route of administration based on these and other factors.

Immunogenic compositions, and vaccine formulations, may be used to treat both children and adults, including pregnant women. Thus a subject may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred subjects for receiving the vaccines are the elderly (e.g., >50 years old, >60 years old, and preferably >65 years), the young (e.g., <6 years old, such as 4 – 6 years old, <5 years old), and pregnant women. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population.

Treatment can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunization schedule and/or in a booster immunization schedule. In a multiple dose schedule the various doses may be given by

the same or different routes, *e.g.*, a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, *etc.* Administration of more than one dose (typically two doses) is particularly useful in immunologically naïve patients. Multiple doses will typically be administered at least 1 week apart (*e.g.*, about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, and the like.).P

Vaccine formulations produced using a composition of the invention may be administered to patients at substantially the same time as (*e.g.*, during the same medical consultation or visit to a healthcare professional or vaccination centre) other vaccines.

10

#### **Further aspects of the invention**

The invention also provides a polypeptide (*e.g.*, recombinant polypeptide) comprising a first domain and a second domain, wherein (i) the first domain comprises a RSV F glycoprotein ectodomain, in whole or part, and (ii) the second domain comprises a heterologous oligomerization domain. Further details are provided above. If the oligomerization domain comprises a heptad sequence (*e.g.*, the sequence from GCN described above) then it is preferably in heptad repeat phase with the HR2 sequence (if present) of the ectodomain.

The invention also provides nucleic acid (*e.g.*, DNA) encoding this polypeptide. It also provides vectors including such nucleic acids, and host cells including such vectors. The vectors may be used for, *e.g.*, recombinant expression purposes, nucleic acid immunization, *etc.*

The invention also provides a composition comprising molecules comprising RSV F glycoprotein ectodomains, wherein the ectodomains of at least 50% (*e.g.*, 50%, 60%, 70%, 80%, 85%, 90%, 95% or 100%) of the molecules are present in a pre-fusion conformation.

#### **Other viruses**

As well as being used with human RSV, the invention may be used with other members of the *Pneumoviridae* and *Paramyxoviridae*, including, but not limited to, bovine respiratory syncytial virus, parainfluenzavirus 1, parainflueznavirus 2, parainfluenzavirus 3, and parainfluenzavirus 5.

Thus the invention provides an immunogenic composition comprising a F glycoprotein from a *Pneumoviridae* or *Paramyxoviridae*, wherein the F glycoprotein is in pre-fusion conformation.

5 The invention also provides an immunogenic composition comprising a polypeptide that displays an epitope present in a pre-fusion conformation of the F glycoprotein of a *Pneumoviridae* or *Paramyxoviridae*, but absent the glycoprotein's post fusion conformation.

The invention also provides a polypeptide comprising a first domain and a second domain, wherein (i) the first domain comprises an ectodomain of the F  
10 glycoprotein of a *Pneumoviridae* or *Paramyxoviridae*, in whole or part, and (ii) the second domain comprises a heterologous oligomerization domain.

The invention also provides these polypeptides and compositions for use in immunization, *etc.*

The invention also provides a composition comprising molecules comprising  
15 RSV F glycoprotein ectodomains, wherein the ectodomains of at least 50% (e.g., 50%, 60%, 70%, 80%, 85%, 90%, 95% or 100%) of the molecules are present in a pre-fusion conformation or an intermediate conformation.

### **RSV F Protein Ecto-Domain Polypeptides**

20 Particular RSV F protein ecto-domain polypeptides are used or included in some embodiments of the invention. Some of the particular RSV F protein ecto-domain polypeptides contain altered amino acid sequences from about position 100 to about position 161. The amino acid sequences from position 100 to position 150 for several particular RSV F protein ecto-domain polypeptides are shown in FIG. 1C.  
25 Amino acid sequences of several particular RSV F protein ecto-domain polypeptides are presented herein, e.g., in Example 1.

### **General**

The term "comprising" encompasses "including" as well as "consisting" and  
30 "consisting essentially of" e.g., a composition "comprising" X may consist exclusively of X or may include something additional e.g., X + Y.

The word “substantially” does not exclude “completely” *e.g.*, a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

The term “about” in relation to a numerical value *x* means, for example,  $x \pm 10\%$ .

5 Unless specifically stated, a process comprising a step of mixing two or more components does not require any specific order of mixing. Thus components can be mixed in any order. Where there are three components then two components can be combined with each other, and then the combination may be combined with the third component, etc.

10 Where animal (and particularly bovine) materials are used in the culture of cells, they should be obtained from sources that are free from transmissible spongiform encephalopathies (TSEs), and in particular free from bovine spongiform encephalopathy (BSE). Overall, it is preferred to culture cells in the total absence of animal-derived materials.

15 Where a compound is administered to the body as part of a composition then that compound may alternatively be replaced by a suitable prodrug.

Where a cell substrate is used for reassortment or reverse genetics procedures, it is preferably one that has been approved for use in human vaccine production *e.g.*, as in Ph Eur general chapter 5.2.3.

20 Identity between polypeptide sequences is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters gap open penalty=12 and gap extension penalty=1.

25 Table 1. Phospholipids

DDPC	1,2-Didecanoyl-sn-Glycero-3-phosphatidylcholine
DEPA	1,2-Dierucoyl-sn-Glycero-3-Phosphate
DEPC	1,2-Erucoyl-sn-Glycero-3-phosphatidylcholine
DEPE	1,2-Dierucoyl-sn-Glycero-3-phosphatidylethanolamine
30 DEPG	1,2-Dierucoyl-sn-Glycero-3[Phosphatidyl-rac-(1-glycerol...)]
DLOPC	1,2-Linoleoyl-sn-Glycero-3-phosphatidylcholine
DLPA	1,2-Dilauroyl-sn-Glycero-3-Phosphate

	DLPC	1,2-Dilauroyl-sn-Glycero-3-phosphatidylcholine
	DLPE	1,2-Dilauroyl-sn-Glycero-3-phosphatidylethanolamine
	DLPG	1,2-Dilauroyl-sn-Glycero-3[Phosphatidyl-rac-(1-glycerol...)]
	DLPS	1,2-Dilauroyl-sn-Glycero-3-phosphatidylserine
5	DMG	1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine
	DMPA	1,2-Dimyristoyl-sn-Glycero-3-Phosphate
	DMPC	1,2-Dimyristoyl-sn-Glycero-3-phosphatidylcholine
	DMPE	1,2-Dimyristoyl-sn-Glycero-3-phosphatidylethanolamine
	DMPG	1,2-Myristoyl-sn-Glycero-3[Phosphatidyl-rac-(1-glycerol...)]
10	DMPS	1,2-Dimyristoyl-sn-Glycero-3-phosphatidylserine
	DOPA	1,2-Dioleoyl-sn-Glycero-3-Phosphate
	DOPC	1,2-Dioleoyl-sn-Glycero-3-phosphatidylcholine
	DOPE	1,2-Dioleoyl-sn-Glycero-3-phosphatidylethanolamine
	DOPG	1,2-Dioleoyl-sn-Glycero-3[Phosphatidyl-rac-(1-glycerol...)]
15	DOPS	1,2-Dioleoyl-sn-Glycero-3-phosphatidylserine
	DPPA	1,2-Dipalmitoyl-sn-Glycero-3-Phosphate
	DPPC	1,2-Dipalmitoyl-sn-Glycero-3-phosphatidylcholine
	DPPE	1,2-Dipalmitoyl-sn-Glycero-3-phosphatidylethanolamine
	DPPG	1,2-Dipalmitoyl-sn-Glycero-3[Phosphatidyl-rac-(1-glycerol...)]
20	DPPS	1,2-Dipalmitoyl-sn-Glycero-3-phosphatidylserine
	DPyPE	1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine
	DSPA	1,2-Distearoyl-sn-Glycero-3-Phosphate
	DSPC	1,2-Distearoyl-sn-Glycero-3-phosphatidylcholine
	DSPE	1,2-Diostearoyl-sn-Glycero-3-phosphatidylethanolamine
25	DSPG	1,2-Distearoyl-sn-Glycero-3[Phosphatidyl-rac-(1-glycerol...)]
	DSPS	1,2-Distearoyl-sn-Glycero-3-phosphatidylserine
	EPC	Egg-PC
	HEPC	Hydrogenated Egg PC
	HSPC	High purity Hydrogenated Soy PC
30	HSPC	Hydrogenated Soy PC
	LYSOPC MYRISTIC	1-Myristoyl-sn-Glycero-3-phosphatidylcholine
	LYSOPC PALMITIC	1-Palmitoyl-sn-Glycero-3-phosphatidylcholine
	LYSOPC STEARIC	1-Stearoyl-sn-Glycero-3-phosphatidylcholine
35	Milk Sphingomyelin MPPC	1-Myristoyl,2-palmitoyl-sn-Glycero 3-phosphatidylcholine
	MSPC	1-Myristoyl,2-stearoyl-sn-Glycero-3-phosphatidylcholine

	PMPC	1-Palmitoyl,2-myristoyl-sn-Glycero-3-phosphatidylcholine
	POPC	1-Palmitoyl,2-oleoyl-sn-Glycero-3-phosphatidylcholine
	POPE	1-Palmitoyl-2-oleoyl-sn-Glycero-3-phosphatidylethanolamine
	POPG	1,2-Dioleoyl-sn-Glycero-3[Phosphatidyl-rac-(1-glycerol)...]
5	PSPC	1-Palmitoyl,2-stearoyl-sn-Glycero-3-phosphatidylcholine
	SMPC	1-Stearoyl,2-myristoyl-sn-Glycero-3-phosphatidylcholine
	SOPC	1-Stearoyl,2-oleoyl-sn-Glycero-3-phosphatidylcholine
	SPPC	1-Stearoyl,2-palmitoyl-sn-Glycero-3-phosphatidylcholine

## 10 EXEMPLIFICATION

### Example 1 –RSV F polypeptides

This example provides sequences of a number of examples of polypeptides (e.g., that contain signal sequences) and nucleic acid sequences that may be used to express RSV F polypeptides of the present invention. The presented amino acid sequences include the signal peptide and contain an optional C-terminal linker and His tag (GGSAGSGHHHHHH (SEQ ID NO:90)). When these polypeptides are produced in host cells, the polypeptide will usually be processed by the cell to remove the signal peptide and, as described herein, some of the polypeptides will be cleaved, for example at unmodified furin cleavage sites. The invention includes compositions that contain, all forms of the particular RSV F protein ecto-domain polypeptides disclosed herein, including mature forms, which lack the signal peptide, forms that may be cleaved into subunits that comprise F<sub>1</sub> and F<sub>2</sub>, and forms that lack the optional C-terminal His tag. The following examples are merely illustrative of the scope of the present invention and therefore are not intended to limit the scope in any way.

25 An example of wild-type furin cleavage is RSV F wild type Truncated HIS (SEQ ID NO:84).

Examples of polypeptides that can be produced as monomers include: RSV F Furx (SEQ ID NO:45); RSV F old furx Truncated HIS (SEQ ID NO:88); RSV F Furx R113Q K123N K124N Truncated HIS (SEQ ID NO:89); RSV F delp21 furx Truncated HIS (SEQ ID NO:47); and RSV F delp23 furx Truncated HIS (SEQ ID NO:48).

Examples of polypeptides that can be produced as trimers include: RSV F N-term Furin Truncated HIS (SEQ ID NO:85); RSV F Fusion Deletion Truncated HIS (SEQ ID NO:67); and RSV F Fusion Deletion 2 Truncated HIS (SEQ ID NO:68).



Examples of polypeptides that can be produced as monomers or rosettes of trimers include: RSV F furmt Truncated HIS (SEQ ID NO:50); RSV F furdel Truncated HIS (SEQ ID NO:51); RSV F delP21 furdel Truncated HIS (SEQ ID NO:86); and RSV F delP23 furdel Truncated HIS (SEQ ID NO:49), and RSV F Factor Xa Truncated HIS (SEQ ID NO:52).

An example of a wild-type cleavage that likely produces a rosette formation is RSV F C-term Furin Truncated HIS (SEQ ID NO:87).

### full

The following polypeptide is a full-length RSV F polypeptide.

```

1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE
61 LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST PATNNRARRE LPRFMNYTLN
121 NAKKTNVTLS KKRKRRLGFL LGVGSIAIAS GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS
181 LSNGVSVLTS KVLDLKNIYD KQLLPVIVNQ SCSISNIETV IEFQQKNRNL LEITREFSVN
15 241 AGVTTTPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV
301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTDRG WYCDNAGSVS FFPQAETCKV
361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT
421 KCTASKNKRG IIKTFSSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP
481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNAGK STTNIMITTI IIVIIIVILLS
20 541 LIAVGLLLYC KARSTPVTLS KDQLSGINNI AFSN (SEQ ID NO: 21)

```

The following nucleic acid sequence is the optimized coding sequence for the foregoing polypeptide sequence.

```

1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC
61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG
25 121 AGCAAGGGCT ACCTGAGCGC CCGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG
181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG
241 CAGGAAGTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGCTGCTGAT GCAGAGCACC
301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC
361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC
30 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGTCCAAGGT GCTGCACCTG
481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC
541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC
601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG
661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC
35 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG

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781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCAGATC  
 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC  
 961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC  
 5 1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTCTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GGC GCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGCACCG CCAGCAACAA GAACCGGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 10 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GGCAGGCCA TCATCAACTT CTACGACCCC  
 1441 CTGGTGTTC CCAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TGCCGGCAAG  
 1561 AGCACCACCA ATATCATGAT CACCACAATC ATCATCGTGA TCATTGTGAT CCTGCTGTCT  
 15 1621 CTGATTGCCG TGGGCCTGCT GCTGTACTGC AAGGCCCGCA GCACCCCTGT GACCCTGTCC  
 1681 AAGGACCAGC TGTCCGGCAT CAACAATATC GCCTTCTCCA ACTGAAG (SEQ ID NO: 22)

**full HIS**

The following polypeptide includes the full-length RSV F polypeptide followed by a hexa-histidine tag.

20 1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE  
 61 LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST PATNNRARRE LPRFMNYTLN  
 121 NAKKTNVTLN KKRKRRLFLGF LLGVGSAIAS GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS  
 181 LSNVSVLTS KVLDLKNYID KQLLPIVNKQ SCSISNIETV IEFQQKNRNL LEITREFSVN  
 241 AGVTTVPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQSYSIMS IIKEEVLAYV  
 25 301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTRDRG WYCDNAGSVS FFPQAETCKV  
 361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT  
 421 KCTASNKNRG IIKTFSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP  
 481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNAGK STTNIMITTI IIVIIIVILLS  
 541 LIAVGLLLYC KARSTPVTLS KDQLSGINNI AFSNSGGSAG SGHHHHHH (SEQ ID NO: 23)

30 The following nucleic acid sequence is the optimized coding sequence for the foregoing polypeptide sequence.

1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC  
 61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG  
 121 AGCAAGGGCT ACCTGAGCGC CCTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG  
 35 181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG  
 241 CAGGAAGTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGCTGCTGAT GCAGAGCACC

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301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC  
 361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC  
 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGTCCAAGGT GCTGCACCTG  
 481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC  
 5 541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC  
 601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG  
 661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC  
 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG  
 781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCAGATC  
 10 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC  
 961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC  
 1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTCTTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 15 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GCGGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGCACCG CCAGCAACAA GAACCGGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GGCGAGCCCA TCATCAACTT CTACGACCCC  
 20 1441 CTGGTGTTC CCAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TGCCGGCAAG  
 1561 AGCACCACCA ATATCATGAT CACCACAATC ATCATCGTGA TCATTGTGAT CCTGCTGTCT  
 1621 CTGATTGCCG TGGGCCTGCT GCTGTACTGC AAGGCCCGCA GCACCCCTGT GACCCTGTCC  
 1681 AAGGACCAGC TGTCCGGCAT CAACAATATC GCCTTCTCCA ACAGCGGCGG CAGCGCCGGC  
 25 1741 TCTGGCCACC ACCACCATCA CCACTGAAG (SEQ ID NO: 24)

**full pre HIS**

The following polypeptide includes the full-length RSV F polypeptide with the trimerization domain of GCN4 (underlined) attached at the C-terminus of the RSV F polypeptide followed by a hexa-histidine tag.

30 1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE  
 61 LSNIKENKCN GTDAKVLIK QELDKYKNAV TELQLLMQST PATNNRARRE LPRFMNYTLN  
 121 NAKKTNVTLS KKRKRRLGFL LLGVGSAIAS GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS  
 181 LSNGVSVLTS KVLDLKNIYID KQLLPIVNKQ SCSISNIETV IEFQQKNNRL LEITREFSVN  
 241 AGVTPPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV  
 35 301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLRTDRG WYCDNAGSVS FFPQAETCKV  
 361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT  
 421 KCTASNKNRG IIKTFSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP

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481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNAGK STTNIMITTI IIVIIIVILLS  
 541 LIAVGLLLYC KARSTPVTLS KDQLSGINNI AFSNGSSGRM KQIEDKIEEI LSKIIYHIENE  
 601 IARIKKLIGE SGGsAGSGHH HHHH (SEQ ID NO: 25)

The following nucleic acid sequence is the optimized coding sequence for the foregoing  
 5 polypeptide sequence.

1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC  
 61 TGCTTCGCCA GCGGCCAGAA CATCACCAGG GAATTCTACC AGAGCACCTG CAGCGCCGTG  
 121 AGCAAGGGCT ACCTGAGCGC CCTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG  
 181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG  
 10 241 CAGGAAGTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGCTGCTGAT GCAGAGCACC  
 301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC  
 361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC  
 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGTCGAAGGT GCTGCACCTG  
 481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC  
 15 541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC  
 601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG  
 661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC  
 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG  
 781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCAGATC  
 20 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC  
 961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC  
 1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTCTTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 25 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GCGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGCACCG CCAGCAACAA GAACCGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GGCAGCCCCA TCATCAACTT CTACGACCCC  
 30 1441 CTGGTGTTC CAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TGCCGGCAAG  
 1561 AGCACCACCA ATATCATGAT CACCACAATC ATCATCGTGA TCATTGTGAT CCTGCTGTCT  
 1621 CTGATTGCCG TGGGCCTGCT GCTGTACTGC AAGGCCCGCA GCACCCCTGT GACCCTGTCC  
 1681 AAGGACCAGC TGTCCGGCAT CAACAATATC GCCTTCTCCA ACGGCAGCAG CGGCCGGATG  
 35 1741 AAGCAGATCG AGGACAAGAT CGAGGAAATC CTGAGCAAGA TCTACCACAT CGAGAACGAG  
 1801 ATCGCCCGGA TCAAGAAGCT GATCGCGCAA AGCGGCGGCT CTGCCGGAAG CGGCCACCAC  
 1861 CACCATCACC ACTGAAG (SEQ ID NO: 26)

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**full pre HIS 2**

The following polypeptide includes the full-length RSV F polypeptide with the trimerization domain of GCN4 (underlined) attached at the C-terminus of the RSV F polypeptide followed by a hexa-histidine tag.

```

5      1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE
      61 LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST PATNNRARRE LPRFMNYTLN
     121 NAKKTNVTLS KKRKRRLGFL LLGVGSAIAS GVAVSKVLHL EGEVNIKISA LLSTNKAVVS
     181 LSNGVSVLTS KVLDLKNIYID KQLLPVKNQ SCSISNIETV IEFQQKNRL LEITREFSVN
     241 AGVTTPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV
10    301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTRDRG WYCDNAGSVS FFPQAETCKV
     361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT
     421 KCTASNKNRG IIKTFSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP
     481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNAGK STTNIMITTI IIVIIIVILLS
     541 LIAVGLLLYC KARSTPVTLS KDQLSGINNI AFSNGSSGSG RMKQIEDKIE EILSKIYHIE
15    601 NEIARIKKLI GESGGSAGSG HHHHHH (SEQ ID NO: 27)

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The following nucleic acid sequence is the optimized coding sequence for the foregoing polypeptide sequence.

```

      1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC
      61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG
20    121 AGCAAGGGCT ACCTGAGCGC CCTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG
     181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG
     241 CAGGAAGTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGTGCTGAT GCAGAGCACC
     301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC
     361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC
25    421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGCCAAGGT GCTGCACCTG
     481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC
     541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC
     601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG
     661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC
30    721 GCCGCGGTGA CCACCCCGCT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG
     781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCAGATC
     841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG
     901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC
     961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC
35   1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG
     1081 CAGAGCAACC GGGTGTTCTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC

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1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GCGGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGCACCG CCAGCAACAA GAACCGGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 5 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GCGGAGCCCA TCATCAACTT CTACGACCCC  
 1441 CTGGTGTTC CCAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TGCCGGCAAG  
 1561 AGCACCACCA ATATCATGAT CACCACAATC ATCATCGTGA TCATTGTGAT CCTGCTGTCT  
 1621 CTGATTGCCG TGGGCCTGCT GCTGTACTGC AAGGCCCGCA GCACCCCTGT GACCCTGTCC  
 10 1681 AAGGACCAGC TGTCCGGCAT CAACAATATC GCCTTCTCCA ACGGCAGCAG CGGCAGCGGC  
 1741 CGGATGAAGC AGATCGAGGA CAAGATCGAG GAAATCCTGA GCAAGATCTA CCACATCGAG  
 1801 AACGAGATCG CCCGGATCAA GAAGCTGATC GCGGAAAGCG GCGGCTCTGC CGGAAGCGGC  
 1861 CACCACCACC ATCACCCTG AAG (SEQ ID NO: 28)

**ecto HIS**

- 15 The following polypeptide includes the ecto domain of the RSV F polypeptide followed by a hexa-histidine tag.

1 MELLILKANA ITTILTAVT FCFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE  
 61 LSNIKENKCN GTDAKVLIK QELDKYKNAV TELQLLMQST PATNNRRARRE LPRFMNYTLN  
 121 NAKKTNVTL S KKRKRRLGFL LGVGSIAIAS GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS  
 20 181 LSNQSVLTS KVLDLKNYID KQLLPVKNQ SCSISNIETV IEFQQKNRL LEITREFSVN  
 241 AGVTTTPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQSYSIMS IIKEEVLAYV  
 301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTRDRG WYCDNAGSVS FFPQAETCKV  
 361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT  
 421 KCTASKNRNG IIKTFSSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP  
 25 481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNSGG SAGSGHHHHH H  
 (SEQ ID NO: 29)

The following nucleic acid sequence is the optimized coding sequence for the foregoing polypeptide sequence.

1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC  
 30 61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG  
 121 AGCAAGGGCT ACCTGAGCGC CCTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG  
 181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG  
 241 CAGGAACTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGCTGCTGAT GCAGAGCACC  
 301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC  
 35 361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC  
 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGTCCAAGGT GCTGCACCTG

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481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC  
 541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC  
 601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG  
 661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC  
 5 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG  
 781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCAGATC  
 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC  
 961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC  
 10 1021 TGGTACTGCG ACAACGCCCG CAGCGTGAGC TTCTTCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTCTTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GCGGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGCACCG CCAGCAACAA GAACCGGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 15 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GGCGAGCCCA TCATCAACTT CTACGACCCC  
 1441 CTGGTGTTC CCAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TAGCGGCGGC  
 1561 AGCGCCGGCT CTGGCCACCA CCACCATCAC CACTGAAG (SEQ ID NO: 30)

## 20 **ecto pre HIS**

The following polypeptide includes the ecto domain of the RSV F polypeptide with the trimerization domain of GCN4 (underlined) inserted into the RSV F polypeptide up stream of where the TM domain of the RSV protein would have been (beginning at a.a. 517) followed by a hexa-histidine tag.

25 1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE  
 61 LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST PATNNRARRE LPRFMNYTLN  
 121 NAKKTNVTLS KKRKRRLGFL LLGVGSAIAS GVAVSKVLHL EGEVNIKISA LLSTNKAVVS  
 181 LSNGVSVLTS KVLDLKNYID KQLLPVINKQ SCSISNIETV IEFQQKNRNL LEITREFSVN  
 241 AGVTPPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV  
 30 301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTDRG WYCDNAGSVS FFPQAETCKV  
 361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT  
 421 KCTASNKNRG IIKTFSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP  
 481 LVFSPDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNDKI EEILSKIYHI ENEIARIKKL  
 541 IGESGGSAGS GHHHHHH (SEQ ID NO: 31)

35 The following nucleic acid sequence is the optimized coding sequence for the foregoing polypeptide sequence.

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1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC  
 61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG  
 121 AGCAAGGGCT ACCTGAGCGC CCTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG  
 181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG  
 5 241 CAGGAAGTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGTGCTGTGAT GCAGAGCACC  
 301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC  
 361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC  
 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGTCCAAGGT GCTGCACCTG  
 481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC  
 10 541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC  
 601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG  
 661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC  
 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG  
 781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCAGATC  
 15 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC  
 961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC  
 1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTTCTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 20 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GCGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGACCG CCAGCAACAA GAACCGGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GCGAGCCCA TCATCAACTT CTACGACCCC  
 25 1441 CTGGTGTTC CCAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TGACAAGATC  
 1561 GAGGAAATCC TGAGCAAGAT CTACCACATC GAGAACGAGA TCGCCCGGAT CAAGAAGCTG  
 1621 ATCGGCGAAA GCGGCGGCTC TGCCGGAAGC GGCCACCACC ACCATCACCA CTGAAG  
 (SEQ ID NO: 32)

### 30 full pre HA HIS

The following polypeptide includes the full-length RSV F polypeptide with the post-fusion trimerization domain of the influenza hemagglutinin polypeptide (underlined) attached at the C-terminus of the RSV F polypeptide followed by a hexa-histidine tag.

1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE  
 35 61 LSNIKENKCN GTDAKVLIK QELDKYKNAV TELQLLMQST PATNNRARRR LPRFMNYTLN  
 121 NAKKTNVTLS KKRKRRLGFL LLGVGSAIAS GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS  
 181 LSNVSVLTS KVLDLKNYID KQLLPIVNKQ SCSISNIETV IEFQQKNRNL LEITREFSVN



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241 AGVTTTPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV  
 301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTDRG WYCDNAGSVS FFPQAETCKV  
 361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT  
 421 KCTASNKNRG IIKTFSSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP  
 5 481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNAGK STTNIMITTI IIVIIVILLS  
 541 LIAVGLLLYC KARSTPVTLS KDQLSGINNI AFSNGSSGNE KFHQIEKEFS EVEGRIQDLE  
 601 KSGGSAGSGH HHHHH (SEQ ID NO: 33)

The following nucleic acid sequence is the optimized coding sequence for the foregoing polypeptide sequence.

10 1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC  
 61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG  
 121 AGCAAGGGCT ACCTGAGCGC CCTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG  
 181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG  
 241 CAGGAACTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGCTGCTGAT GCAGAGCACC  
 15 301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC  
 361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC  
 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGTCCAAGGT GCTGCACCTG  
 481 GAAGCGGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC  
 541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC  
 20 601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG  
 661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC  
 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG  
 781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCAGATC  
 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 25 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC  
 961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC  
 1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTTCTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 30 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GCGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGACCG CCAGCAACAA GAACCGGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GGCGAGCCCA TCATCAACTT CTACGACCCC  
 1441 CTGGTGTTC CCAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 35 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TGCCGGCAAG  
 1561 AGCACCACCA ATATCATGAT CACCACAATC ATCATCGTGA TCATTGTGAT CCTGCTGTCT  
 1621 CTGATTGCCG TGGGCCTGCT GCTGTACTGC AAGGCCGCA GCACCCCTGT GACCCTGTCC  
 1681 AAGGACCAGC TGTCCGGCAT CAACAATATC GCCTTCTCCA ACGGACGAG CGGCAATGAG

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1741 AAGTTCCACC AGATCGAGAA AGAATTCAGC GAGGTGGAGG GCCGATCCA GGACCTGGAA  
 1801 AAGAGCGGCG GCTCTGCCGG AAGCGGCCAC CACCACCATC ACCACTGAAG  
 (SEQ ID NO: 34)

**ecto pre HA HIS**

- 5 The following polypeptide includes the ecto domain of the RSV F polypeptide with the post-fusion trimerization domain of the influenza hemagglutinin polypeptide (underlined) inserted into the RSV F polypeptide up stream of where the TM domain of the RSV protein would have been (beginning at a.a. 517) followed by a hexa-histidine tag.

10 1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE  
 61 LSNIKENKCN GTDAKVLIK QELDKYKNAV TELQLLMQST PATNNRARRE LPRFMNYTLN  
 121 NAKKTNVTLS KKRKRRLGFG LLGVGSAIAS GVAVSKVLHL EGEVNIKISA LLSTNKAVVS  
 181 LSNGVSVLTS KVLDLKNIYD KQLLPVINKQ SCSISNIETV IEFQQKNNRL LEITREFSVN  
 241 AGVTPPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV  
 15 301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTDRG WYCDNAGSVS FFPQAETCKV  
 361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT  
 421 KCTASNKNRG IIKTFSSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP  
 481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNEKF HQIEKEFSEV EGRIQDLEKS  
 541 GGSAGSGHHH HHH (SEQ ID NO: 35)

- 20 The following nucleic acid sequence is the optimized coding sequence for the foregoing polypeptide sequence.

1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC  
 61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG  
 121 AGCAAGGGCT ACCTGAGCGC CCTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG  
 25 181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG  
 241 CAGGAAGTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGTGCTGAT GCAGAGCACC  
 301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC  
 361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC  
 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGCCAAGGT GCTGCACCTG  
 30 481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC  
 541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC  
 601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG  
 661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC  
 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG  
 35 781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGACGATC  
 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC

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961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC  
 1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTTCTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 5 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GCGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGCACCG CCAGCAACAA GAACCGGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GCGGAGCCCA TCATCAACTT CTACGACCCC  
 1441 CTGGTGTTC CCAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 10 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TGAGAAGTTC  
 1561 CACCAGATCG AGAAAGAATT CAGCGAGGTG GAGGGCCGGA TCCAGGACCT GGAAAAGAGC  
 1621 GCGGCTCTG CCGGAAGCGG CCACCACCAC CATCACCCT GAAG (SEQ ID NO: 36)

**fullΔHRB HIS**

The following polypeptide includes the full-length RSV F polypeptide with the  
 15 HRB domain deleted followed by a hexa-histidine tag.

1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE  
 61 LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST PATNNRARRR LPRFMNYTLN  
 121 NAKKTNVTLN KKRKRRLGFL LLGVGSAIAS GVAVSKVLHL EGEVNIKISA LLSTNKAVVS  
 181 LSNVSVLTS KVLDLKNYID KQLLPVINKQ SCSISNIETV IEFQQKNRNL LEITREFSVN  
 20 241 AGVTPPVSTY MLTNSELLSL INDMPTINDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV  
 301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTRDRG WYCDNAGSVS FFPQAETCKV  
 361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT  
 421 KCTASNNKRG IIKTFSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPNIMITTI  
 481 IIVIIIVILLS LIAVGLLLYC KARSTPVTLS KDQLSGINNI AFSNMGGSHH HHHH  
 25 (SEQ ID NO: 37)

The following nucleic acid sequence is the optimized coding sequence for the  
 foregoing polypeptide sequence.

1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC  
 61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG  
 30 121 AGCAAGGGCT ACCTGAGCGC CTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG  
 181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG  
 241 CAGGAAGTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGCTGCTGAT GCAGAGCACC  
 301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACCTA CACCCTGAAC  
 361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC  
 35 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGTCCAAGGT GCTGCACCTG  
 481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC  
 541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC  
 601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG

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661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC  
 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG  
 781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCGAGATC  
 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 5 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC  
 961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGGC  
 1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTCTTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 10 1201 GACGTGAGCA GCTCCGTGAT CACCTCCCTG GCGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGCACCG CCAGCAACAA GAACCGGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GGCGAGCCCA ATATCATGAT CACCACAATC  
 1441 ATCATCGTGA TCATTGTGAT CCTGCTGTCT CTGATTGCCG TGGGCTGCT GCTGTACTGC  
 15 1501 AAGGCCCGCA GCACCCCTGT GACCTGTCC AAGGACCAGC TGTCCGGCAT CAACAATATC  
 1561 GCCTTCTCCA ACATGGGGG TTCTCATCAT CATCATCATC ATTGAAG  
 (SEQ ID NO: 38)

**ecto**

20 The following polypeptide includes just the ecto domain of the RSV F polypeptide.

1 MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE  
 61 LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST PATNNRRARRE LPRFMNYTLN  
 121 NAKKTNVTLS KKRKRRLGFL LLGVGSAIAS GVAVSKVLHL EGEVNIKISA LLSTNKAVVS  
 25 181 LSNGVSVLTS KVLDLKNYID KQLLPVINKQ SCSISNIETV IEFQQKNRNL LEITREFSVN  
 241 AGVTPPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV  
 301 VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTRDRG WYCDNAGSVS FFPQAETCKV  
 361 QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT DVSSSVITSL GAIVSCYGKT  
 421 KCTASNKNRG IIKTFSNGCD YVSNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP  
 30 481 LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHNVNAGK STTN (SEQ ID NO: 39)

The following nucleic acid sequence is the optimized coding sequence for the foregoing polypeptide sequence.

1 ATGGAAGTGC TGATCCTGAA GGCCAACGCC ATCACCACCA TCCTGACCGC CGTGACCTTC  
 61 TGCTTCGCCA GCGGCCAGAA CATCACCGAG GAATTCTACC AGAGCACCTG CAGCGCCGTG  
 35 121 AGCAAGGGCT ACCTGAGCGC CCTGCGGACC GGCTGGTACA CCAGCGTGAT CACCATCGAG  
 181 CTGTCCAACA TCAAAGAAAA CAAGTGCAAC GGCACCGACG CCAAGGTGAA ACTGATCAAG  
 241 CAGGAAGTGG ACAAGTACAA GAACGCCGTG ACCGAGCTGC AGCTGCTGAT GCAGAGCACC  
 301 CCCGCCACCA ACAACCGGGC CAGAAGAGAG CTGCCCCGGT TCATGAACTA CACCCTGAAC

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361 AACGCCAAGA AAACCAACGT GACCCTGAGC AAGAAGCGGA AGCGGCGGTT CCTGGGCTTC  
 421 CTGCTGGGCG TGGGCAGCGC CATCGCCAGC GGGGTGGCCG TGTCCAAGGT GCTGCACCTG  
 481 GAAGGCGAGG TGAACAAGAT CAAGTCCGCC CTGCTGTCCA CCAACAAGGC CGTGGTGTCC  
 541 CTGAGCAACG GCGTGAGCGT GCTGACCAGC AAGGTGCTGG ATCTGAAGAA CTACATCGAC  
 5 601 AAGCAGCTGC TGCCCATCGT GAACAAGCAG AGCTGCAGCA TCAGCAACAT CGAGACCGTG  
 661 ATCGAGTTCC AGCAGAAGAA CAACCGGCTG CTGGAAATCA CCCGGGAGTT CAGCGTGAAC  
 721 GCCGGCGTGA CCACCCCGT GAGCACCTAC ATGCTGACCA ACAGCGAGCT GCTGTCCCTG  
 781 ATCAATGACA TGCCCATCAC CAACGACCAG AAAAAGCTGA TGAGCAACAA CGTGCAGATC  
 841 GTGCGGCAGC AGAGCTACTC CATCATGAGC ATCATCAAAG AAGAGGTGCT GGCCTACGTG  
 10 901 GTGCAGCTGC CCCTGTACGG CGTGATCGAC ACCCCCTGCT GGAAGCTGCA CACCAGCCCC  
 961 CTGTGCACCA CCAACACCAA AGAGGGCAGC AACATCTGCC TGACCCGGAC CGACCGGGC  
 1021 TGGTACTGCG ACAACGCCGG CAGCGTGAGC TTCTTCCCCC AAGCCGAGAC CTGCAAGGTG  
 1081 CAGAGCAACC GGGTGTCTTG CGACACCATG AACAGCCTGA CCCTGCCCTC CGAGGTGAAC  
 1141 CTGTGCAACG TGGACATCTT CAACCCCAAG TACGACTGCA AGATCATGAC CTCCAAGACC  
 15 1201 GACGTGAGCA GCTCCGTGAT CACCTCCTG GGCGCCATCG TGAGCTGCTA CGGCAAGACC  
 1261 AAGTGACCCG CCAGCAACAA GAACCGGGC ATCATCAAGA CCTTCAGCAA CGGCTGCGAC  
 1321 TACGTGAGCA ACAAGGGCGT GGACACCGTG AGCGTGGGCA ACACACTGTA CTACGTGAAT  
 1381 AAGCAGGAAG GCAAGAGCCT GTACGTGAAG GGCAGGCCA TCATCAACTT CTACACCCC  
 1441 CTGGTGTTC CCAGCGACGA GTTCGACGCC AGCATCAGCC AGGTCAACGA GAAGATCAAC  
 20 1501 CAGAGCCTGG CCTTCATCCG GAAGAGCGAC GAGCTGCTGC ACAATGTGAA TGCCGGCAAG  
 1561 AGCACCACCA ATTGAAG (SEQ ID NO: 40)

RSV F Full Length

25 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARELPRFMNYTLNNAKKTNTVLSKKRKRFLGF  
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVINKQ  
 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDGRWYCDNAGSVS  
 30 FFPQAETCKVQSNRVFCDTMNSLTLPEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
 KCTASNKNRGIKTFSGNCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
 SISQVNEKINQSLAFIRKSDELHNVNAGKSTTNIMITTTIIIVIIIVILLSLIAVGLLLYCKARSTPVTLS  
 KDQLSGINNIAFSN (SEQ ID NO:41)

35 RSV F Cleavage Enterokinase idealized

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARELPRFMNYTLNNAKKTNTVLSKKRKRFLGF  
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVINKQ  
 40 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDGRWYCDNAGSVS

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FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQINEKINQILAFIRKIDELLHNINAGKSTTNGSGSGDDDDDKSGSGGIMITTTIIIVIIIVILLSLIAV  
GLLLYCKARSTPVTLSKDQLSGINNIAFSN (SEQ ID NO:42)

5

RSV F Cleavage Thrombin idealized

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSSKKRRRFLGF  
10 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVNVKQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDGRWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
15 SISQINEKINQILAFIRKIDELLHNINAGKSTTNGSGSGLVPRGSGSGIMITTTIIIVIIIVILLSLIAVGL  
LLYCKARSTPVTLSKDQLSGINNIAFSN (SEQ ID NO:43)

RSV F Cleavage FactorXa idealized

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSSKKRRRFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVNVKQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDGRWYCDNAGSVS  
25 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQINEKINQILAFIRKIDELLHNINAGKSTTNGSGSGIEGRGSGSGIMITTTIIIVIIIVILLSLIAVGL  
LLYCKARSTPVTLSKDQLSGINNIAFSN (SEQ ID NO:44)

30 RSV F furx Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPRFMNYTLNNAKKTNVTLSSQNQNQNFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVNVKQ  
35 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDGRWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO:45)

40

RSV F furx R113Q, K123N, K124N Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNNAANTNVTLSSQNQNQNFLGF  
45 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVNVKQ

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SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVS  
 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
 KCTASNKNRGIKTFSGNGCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
 5 SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:46)

RSV F furx R113Q, K123Q, K124Q Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 10 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNNAQQTNVTLNQNNQNFLGF  
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLPVKNQ  
 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVS  
 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
 15 KCTASNKNRGIKTFSGNGCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
 SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:93)

RSV F delP21 furx Truncated HIS

20 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQN-----  
 QNNQNFLGFLLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYID  
 KQLLPVKNQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQ  
 25 KKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRG  
 WYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSL  
 GAIVSCYGKTKCTASNKNRGIKTFSGNGCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDP  
 LVFPDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID  
 NO:47) (the symbol "-" indicates that the amino acid at this position  
 30 is deleted)

RSV F delP23 furx Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 35 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQN-----  
 QNNQNFLGFLLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQ  
 LLPVKNQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKK  
 LMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWY  
 CDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGA  
 40 IVSCYGKTKCTASNKNRGIKTFSGNGCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLV  
 FPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:48)  
 (the symbol "-" indicates that the amino acid at this position is  
 deleted)

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RSV F delP23 furdel Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNRARQ-----  
 5 QQQRFLGFLLGVSASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQ  
 LLPIVNKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKK  
 LMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWY  
 CDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGA  
 IVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLV  
 10 FPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:49)  
 (the symbol "-" indicates that the amino acid at this position is  
 deleted)

RSV F furmt Truncated HIS

15 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNRARKELPRFMNYTLNNAKKTNVTLSSKKRKKKFLGF  
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLP  
 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 20 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGK  
 KCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
 SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:50)

25 RSV F furdel Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNRARQELPRFMNYTLNNAKKTNVTLSSK---  
 RFLGFLLGVSASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLP  
 30 IVNKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMS  
 NNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDN  
 AGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVS  
 CYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLVFP  
 DEFDAISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:51)  
 35 (the symbol "-" indicates that the amino acid at this position is  
 deleted)

RSV F Factor Xa Truncated HIS

40 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNIEGRELPRFMNYTLNNAKKTNVTLSSKIEGRFLGF  
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLP  
 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS



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FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIKTFSSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO:52)

## 5 RSV F Short linker Foldon HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSSKKRKRFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVNVKQ  
10 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIKTFSSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGSGYIPEAPRDGQAYVRKDGWVLLSTFLGGSAGSG  
15 HHHHHH (SEQ ID NO:53)

## RSV F Long linker Foldon HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
20 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSSKKRKRFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVNVKQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
25 KCTASNKNRGIKTFSSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNNKNDDKSGSYIPEAPRDGQAYVRKDGWVLLSTFLG  
GSAGSGHHHHH (SEQ ID NO:54)

## RSV\_F\_ecto\_pre\_his

30 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSSKKRKRFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPVNVKQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
35 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIKTFSSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELLHNVNDKIEEILSKIYHIENEIARIKKLIGESGGSAGSGHHHHH  
(SEQ ID NO:55)

40

## ECTO PRE HA HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSSKKRKRFLGF

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LLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLPVINKQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNL CNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
5 KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELLHNVNEKFHQIEKEFSEVEGRIQDLEKSGGSAGSGHHHHHH (SEQ  
ID NO:56)

RSV F ECTO Furx GCN HIS

10

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNNANNTNVTLSONQNQNFLGF  
LLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLPVINKQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
15 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNL CNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELLHNVN DKIEEILSKIYHIENEIARIKKLIGESGGSAGSGHHHHHH  
(SEQ ID NO:57)

20

RSV F ECTO delp21 GCN HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQN-----  
25 QNQNQNFLGFLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYID  
KQLLPVINKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQ  
KKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRG  
WYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNL CNVDIFNPKYDCKIMTSKTDVSSSVITSL  
GAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDP  
30 LVFPDEFDASISQVNEKINQSLAFIRKSDELLHNVN  
DKIEEILSKIYHIENEIARIKKLIGESGGSAGSGHHHHHH (SEQ ID NO:58) (the symbol "-  
" indicates that the amino acid at this position is deleted)

RSV F ECTO delp23 Furx GCN HIS

35

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQN-----  
QNQNFLGFLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQ  
LLPIVINKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKK  
40 LMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWY  
CDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNL CNVDIFNPKYDCKIMTSKTDVSSSVITSLGA  
IVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLV  
FPSDEFDASISQVNEKINQSLAFIRKSDELLHNVN

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DKIEEILSKIYHIENEIARIKKLIGESGGSAGSGHHHHHH (SEQ ID NO:59) (the symbol "-" indicates that the amino acid at this position is deleted)

RSV F ECTO delp23 Furdel GCN HIS

5

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDKAVKLIKQELDKYKNAVTELQLLMQSTPATNNRARQ-----  
QQQRFLGFLLGVSASIAAGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSTKVLDLKNYIDKQ  
LLPIVKNQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKK  
10 LMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRDRGWY  
CDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGA  
IVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLV  
FPSDEFDASISQVNEKINQSLAFIRKSDELLHNVN

15 DKIEEILSKIYHIENEIARIKKLIGESGGSAGSGHHHHHH (SEQ ID NO:60) (the symbol "-" indicates that the amino acid at this position is deleted)

RSV F Full Length Furx

20 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDKAVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNANNTNVTLNQNNQNFLGF  
LLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSTKVLDLKNYIDKQLLPIVKNQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
25 KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELLHNVN  
AGKSTTNIMITTIIIVIIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN (SEQ ID  
NO:61)

30 RSV F Full Length delp21

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDKAVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQN-----  
QNQNQNFLGFLLGVSASIAAGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSTKVLDLKNYID  
35 KQLLPIVKNQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQ  
KKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRDRG  
WYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSL  
GAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDP  
LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVN  
40 AGKSTTNIMITTIIIVIIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN (SEQ ID  
NO:62) (the symbol "-" indicates that the amino acid at this position  
is deleted)

RSV F Full Length p23 Furx GCN HIS

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MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQN-----  
 QNQNFLGLFLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLKNYIDKQ  
 5 LLPIVINKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKK  
 LMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWY  
 CDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGA  
 IVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLV  
 FPSDEFDASISQVNEKINQSLAFIRKSDELLHNVN  
 10 AGKSTTNIMITTIIIVIIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN (SEQ ID  
 NO:63) (the symbol "-" indicates that the amino acid at this position  
 is deleted)

RSV F Full Length p23 Furdel GCN HIS

15 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARQ-----  
 QQQRFLGLFLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLKNYIDKQ  
 LLPIVINKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKK  
 20 LMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWY  
 CDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGA  
 IVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLV  
 FPSDEFDASISQVNEKINQSLAFIRKSDELLHNVN  
 AGKSTTNIMITTIIIVIIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN (SEQ ID  
 25 NO:64) (the symbol "-" indicates that the amino acid at this position  
 is deleted)

RSV F N-term Furin Furx Truncated HIS

30 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNNAQQTNVTLSSKKRRRFLGF  
 LLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLKNYIDKQLLPIVINKQ  
 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
 35 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
 KCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLVFPDEFDA  
 SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO:65)

RSV F C-term Furin Furx Truncated HIS

40 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELQPQFMNYTLNNAQQTNVTLSSQNQNFLGF  
 LLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLKNYIDKQLLPIVINKQ  
 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI

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VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIKTFSTNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO:66)

5

RSV F Fusion Deletion 1 Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDKAVKLIKQELDKYKNAVTELQLLMQSTPATNNRARELPRFMNYTLNNAKKTNVTLSSKKRKRSAIA  
10 SGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLNNGVSVLTSSKVLDLKNYIDKQLLPVNVKQSCSISNIET  
VIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIM  
SIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCK  
VQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNR  
GIIKTFSTNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDASISQVNEKI  
15 NQSLAFIRKSDELHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO:67)

RSV F Fusion Deletion 2 Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
20 GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARELPRFMNYTLNNAKKTNVTLSSKKRKRGVGS  
AIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLNNGVSVLTSSKVLDLKNYIDKQLLPVNVKQSCSISN  
IETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSY  
SIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAE  
TCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASN  
25 KNRGIKTFSTNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDASISQVN  
EKINQSLAFIRKSDELHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO:68)

RSV F Furx Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDKAVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNNAQQTNVTLSSQNQNQNFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLNNGVSVLTSSKVLDLKNYIDKQLLPVNVKQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
35 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIKTFSTNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
SISQVNEKINQSLAFIRKSDELHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO:69)

RSV F Furx Truncated

40

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDKAVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNNAQQTNVTLSSQNQNQNFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLNNGVSVLTSSKVLDLKNYIDKQLLPVNVKQ  
SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI

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VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPSPDEFDA  
SISQVNEKINQSLAFIRKSDELHNVNAGKSTTN (SEQ ID NO:70)

5

RSV F delP23 furdel Truncated No HIS (For CHO cells)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARQ-----

10

QQQRFLGFLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQ  
LLPIVKNQSCSISNIETVIEFQQKNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKK  
LMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWY  
CDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGA  
IVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLV

15

FPSDEFDASISQVNEKINQSLAFIRKSDELHNVNAGKSTTN (SEQ ID NO:71) (the symbol  
"-" indicates that the amino acid at this position is deleted)

RSV F (Wt) Truncated HIS

20

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSSKKRKRFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLPIVKNQ  
SCSISNIETVIEFQQKNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
25 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPSPDEFDA  
SISQVNEKINQSLAFIRKSDELHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO: 84)

RSV F old furx Truncated HIS

30

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELQRFMNYTLNNAANTNVTLSSQNQNQNFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLPIVKNQ  
SCSISNIETVIEFQQKNRLLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
35 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVS  
FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPSPDEFDA  
SISQVNEKINQSLAFIRKSDELHNVNAGKSTTNGGSAGSGHHHHH (SEQ ID NO:88)

40

RSV F Furx R113Q K123N K124N Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
GTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNNAQQTNVTLSSQNQNQNFLGF  
LLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLPIVKNQ

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SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVS  
 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
 KCTASNKNRGIKTFSGCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
 5 SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:89)

RSV F N-term Furin Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 10 GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNRARELPQFMNYTLNNAQQTNVTLNQNNQNLGFL  
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLPVKNQ  
 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVS  
 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
 15 KCTASNKNRGIKTFSGCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
 SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:85)

RSV F delP21 furdel Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 20 GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNRARQ-----  
 QNQQRFLGLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYID  
 KQLLPVKNQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQ  
 KKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRG  
 25 WYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSL  
 GAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDP  
 LVFPDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID  
 NO:86)

30 RSV F C-term Furin Truncated HIS

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCN  
 GTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNQAQNELPQFMNYTLNNAQQTNVTLSSKKRRRFLGF  
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSSKVLDLKNYIDKQLLPVKNQ  
 35 SCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI  
 VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVS  
 FFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT  
 KCTASNKNRGIKTFSGCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPDEFDA  
 SISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNGGSAGSGHHHHHH (SEQ ID NO:87)

40

#### Example 2 – Expression and Purification of RSV F constructs

The RSV F ECTO and truncated constructs, lacking the transmembrane domain and cytoplasmic tail region with either wild-type furin cleavage sites or harboring

knock-out mutations to the furin cleavage sites and with or without prefusion stabilization mutations, were cloned into a pFastBac baculovirus expression vector (Invitrogen). Several of these constructs contain a C-terminal flexible linker followed by a His6-tag sequence used for chelating purification. The production of high-titer  
5 baculovirus stocks were passaged in Sf9 insect cells. Proteins were expressed by infecting either Sf9, Tn5 or High Five insect cells with the required baculovirus and harvesting the media supernatant two or three days post infection, monitored by western blot using an anti-RSV F or anti-6HIS antibody.

Large scale expression media was concentrated/purified by one of two general  
10 strategies for eliminating the deleterious Effect of the ferritin present in insect cell media from corrupting the chelating resin. The first approach was to concentrate the approximately 10-20 liters of insect expression media down to approximately 300 mls using a GE Healthcare Hollotube fiber concentration column. Copper sulfate was added to this concentrated mixture to a final concentration of 500  $\mu$ M and the resulting  
15 solution was loaded onto 5 ml HiTrap chelating columns. The bound HIS-tagged protein was then eluted from the column with 25 mM Tris pH 7.5, 300 mM NaCl and a gradient of imidazole.

In the second purification strategy,  $\text{CuCl}_2$  was added to media supernatant to a final concentration of 500  $\mu$ M. To each 1 liter of media, four milliliters of chelation  
20 resin (Chelating Resin, BioRad) was added and the slurry was rocked for at least thirty minutes at 4 degrees centigrade and the resin and media were separated by a gravity column. The resin was washed with ten-times column volume of equilibration buffer (25 mM Tris pH 7.5, 300 mM NaCl) and the F protein was eluted with ten-times column volume of elution buffer (equilibration buffer with 250 mM imidazole). The  
25 elution was dialyzed against 25 mM Tris buffer pH 7.5, and the resulting solution was loaded onto a 5 ml Hitrap chelation column charged with  $\text{NiSO}_4$  and eluted with 25 mM Tris pH 7.5, 300 mM NaCl and a gradient of imidazole.

Elutions from the imidazole gradient in either case were evaluated using anti-6HIS western and coomassie gels. Fractions containing pure constructs were collected,  
30 dialyzed against different buffer/saline solutions and were concentrated for subsequent analysis using Millipore Centrprep Concentrators and/or Vivaspin concentration units.



We have also developed a size-exclusion purification protocol capable of further purifying monodispersed RSV trimers from rosettes (below).

#### **SEC analysis of RSV F proteins:**

5           A documented feature of other paramyxovirus fusion proteins stabilized in their prefusion conformation is that, even when cleaved so that the fusion peptide is exposed, they do not form rosettes as is observed for the postfusion conformation. A simple size-exclusion chromatography analysis allows for identification of a protein and determination of whether a protein is forming rosettes. Two methods were developed,  
10   HPLC-SEC and FPLC-SEC, which also serves as an efficient purification step.

          HPLC-SEC was performed using a Biorad SEC column (18 mm) with a 25 mM Tris pH 7.5, 300 mM NaCl mobile phase. Using Biorad HPLC-SEC standards to calibrate the system, we found that the RSV rosettes (representing cleaved-postfusion conformations) elute in the column void volume of the analysis, while RSV  
15   monodispersed trimers (presumed trimers from subsequent EM analysis) elute with an apparent molecular weight of approximately 100 kDa.

          FPLC-SEC was performed on a GE Healthcare FPLC using a 16/60 Superdex 200 column with 25 mM Tris pH 7.5, 300 mM NaCl mobile phase. Using GE Healthcare High molecular weight standards to calibrate the system, we found that the  
20   RSV rosettes elute in the column void volume of the analysis, while RSV monodispersed trimers elute with an apparent molecular weight of approximately 100 kDa.

#### **Electron Microscopy (EM) of RSV F proteins.**

25           Protein solutions of approximately 50 micrograms per ml RSV F constructs were absorbed onto glow-discharged carbon coated grids and were negatively stained with 2% sodium phosphotungstate (pH 7.0) or 0.75% Urynal-formate (unquantified low pH). The grids were observed on a Technai Spirit or JOEL 1230 transmission electron microscope operating between 80-120 kV with a magnification between 20,000 to  
30   150,000 depending on required resolution.

Construct	Conformation by EM
RSV F ECTO HIS	Predominately rosettes
RSV F Furdel ECTO (cleaved)	Predominately rosettes
RSV F Delp23 Furdel Truncated (uncleaved)	Trimers observed
RSV F Fusion Peptide Deletion 1 Truncated (uncleaved)	Timers
RSV F Delp23 Furdel Truncated (cleaved by Trypsin after purification)	Predominantly rosettes with some trimers
RSV F Delp23 Furdel Truncated (cleaved by Trypsin after purification in presence of nanolipid disk)	Asymmetric rosettes with apparent nanolipid disk at the center of rosette

#### Example 3 – Detection of pre-fusion and post-fusion RSV F

A number of methods are available to determine the conformation of the RSV F protein to assay whether a modification to the RSV F polypeptide or added molecule disfavors the post-fusion conformation. Examples include liposome association, conformation specific monoclonal antibodies (including as used in FACS, ELISA, etc.), electron microscopy, differential protease sensitivity between the conformations, gel filtration chromatography, analytical ultracentrifugation, dynamic light scattering, deuterium exchange NMR experiments, mass spectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry, tryptophan spectroscopy, and X-ray crystallography.

#### **Liposome Association**

Liposome association may be used to assay the conformation of the RSV F protein. Soluble forms of the RSV F protein in the pre-fusion conformation will not associate with liposomes while the post-fusion conformation will associate with liposomes.

Liposomes may be prepared as follows: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, and

cholesterol in chloroform (available from Avanti Polar Lipids) are mixed at an 8:2:5 molar ratio. The chloroform is evaporated under argon. A lipid film will form that is dried under vacuum overnight and resuspended in PBS at 40 mM total lipid. After five freeze-thaw cycles, the lipids are vortexed and extruded 21 times through two 100- $\mu$ m  
5 filters by using a miniextruder (available from Avanti Polar Lipids).

Once the liposomes have been prepared, the liposome association assay may be performed. For each sample to be tested, 2  $\mu$ g of the RSV F polypeptide to test is cleaved with 25 milliunits of trypsin (available from Worthington Biochemical) in 100 mM phosphate buffer (pH 7.1) for 30 min at 25°C. After cleavage, 40 pg of soybean  
10 trypsin inhibitor (available from Worthington Biochemical) is added to each sample to end the reaction. The samples are pretreated at 60°C for 30 min which would induce a conformational shift from the pre-fusion to the post-fusion forms in native isolated RSF F protein. Liposomes (40  $\mu$ l per sample) and PBS are added (80  $\mu$ l final volume), and the samples are incubated at 60°C for 30 min. Sucrose is added to a final concentration  
15 of 50% (500  $\mu$ l final volume). The samples are overlaid with 500  $\mu$ l each of 40% sucrose, 25% sucrose, and PBS and are spun in a TLS55 rotor at 49,000 rpm for 3 h at 25°C. Fractions (500  $\mu$ l) are collected from the top of the gradients. Proteins are solubilized in 0.5% Triton X-100 and precipitated by using 12.5% vol/vol trichloroacetic acid. Polypeptides are separated by SDS/PAGE and transferred to  
20 PVDF membranes. Blots are probed with anti-RSV F monoclonal antibodies.

### **Electron Microscopy**

Electron microscopy was used to assay the conformational distribution of RSV F polypeptides. RSV F polypeptides in the pre-fusion form have a “ball and stem”  
25 shape with a length of ~12nm. In contrast, RSV F polypeptides in the post-fusion form have a “golf tee” shape with a length of ~16 nm. In addition, the fusion peptides at the narrow end of the “golf tees” aggregate to form a rosette structures. Thus, electron microscopy may be used to assay the distribution of conformations in a sample of RSV F polypeptides owing to the readily distinguishable shapes.

30

### **Example 4 RSV F ectodomain trimers and rosettes**

The RSV F protein ecto-domain constructs, encoding polypeptides that lack the transmembrane domain and cytoplasmic tail region with either wild-type furin cleavage sites or harboring knock-out mutations to the furin cleavage sites and/or fusion peptide mutations mutations, were cloned into a pFastBac baculovirus expression vector  
5 (Invitrogen). Several of these constructs contain a C-terminal flexible linker followed by a HIS<sub>6</sub>-tag sequence used for chelating purification. The production of high-titer baculovirus stocks were obtained by passage in Sf9 insect cells. Proteins were expressed by infecting either Sf9, Tn5 or High Five insect cells with the required baculovirus and harvesting the conditioned media supernatant two or three days post  
10 infection. Protein production was monitored by western blot using an anti-RSV F or anti- HIS<sub>6</sub> antibody.

Large scale expression media was concentrated/purified using one of two general strategies for eliminating the deleterious effect of the ferritin present in insect cell media, which can corrupt the chelating resin. The first approach was to concentrate  
15 the approximately 10-20 liters of insect expression media down to approximately 300 mls using a GE Healthcare Hollotube fiber concentration column. Copper sulfate was added to this concentrated mixture to a final concentration of 500  $\mu$ M, and the resulting solution was loaded onto 5 ml HiTrap chelating columns. The bound HIS-tagged protein was then eluted from the column with 25 mM Tris pH 7.5, 300 mM NaCl and a  
20 gradient of imidazole.

In the second purification strategy, CuCl<sub>2</sub> was added to media supernatant to a final concentration of 500  $\mu$ M. To each 1 liter of media, approximately four to ten milliliters of chelating resin (Chelating Resin, BioRad) was added, and the slurry was rocked for at least thirty minutes at 4 degrees centigrade, and the resin and media were  
25 separated using a gravity column. The resin was washed with approximately ten times the column volume of equilibration buffer (25 mM Tris pH 7.5, 300 mM NaCl), and the F protein ecto-domain was eluted with approximately ten-times the column volume of elution buffer (equilibration buffer with 250 mM imidazole). The elution was dialyzed against 25 mM Tris buffer pH 7.5, and the resulting solution was loaded onto a 5 ml  
30 Hitrap chelation column charged with NiSO<sub>4</sub>. Bound protein was eluted with 25 mM Tris pH 7.5, 300 mM NaCl and a gradient of imidazole.

Elutions from the imidazole gradient in either case were evaluated using anti-HIS<sub>6</sub> western blots and/or Coomassie-stained SDS-PAGE gels. Fractions containing pure constructs were collected, dialyzed against different buffer/saline solutions and were concentrated for subsequent analysis using Millipore Centriprep concentrators  
5 and/or Vivaspin concentration units. In some instances, monomers, trimers or rosettes were further purified using size exclusion chromatography.

### **SEC Analysis and Purification of RSV F Ecto-Domains**

Size exclusion chromatography was used to purify and analyze RSV F protein  
10 ecto-domain monomers, trimers and rosettes. This method also allowed uncleaved RSV F protein ecto-domains to be purified away from host cell or media derived lipid and lipoprotein contaminants. Two methods were developed, HPLC-SEC and FPLC-SEC, which may also serve as an efficient purification step.

HPLC-SEC was performed using a Biorad SEC column (18 mm) with a 25 mM  
15 Tris pH 7.5, 300 mM NaCl mobile phase. Using Biorad HPLC-SEC standards to calibrate the system, we found that the RSV rosettes (representing cleaved, postfusion conformations) elute in the column void volume of the analysis, while RSV F monomers elute with an apparent molecular weight of approximately 75-85 kDa.

FPLC-SEC was performed on a GE Healthcare FPLC using a 16/60 Superdex  
20 200 column with 25 mM Tris pH 7.5, 300 mM NaCl as a mobile phase. Using GE Healthcare High molecular weight standards to calibrate the system, we found that the RSV rosettes elute in the column void volume of the analysis, while RSV monodispersed trimers elute with an apparent molecular weight of approximately 140-160 kDa and RSV F monomers elute with an apparent molecular weight of  
25 approximately 75-85 kDa.

For purification, the FPLC-SEC method was used and 1 ml fractions were collected.

### **Trypsin Cleavage of Furdel or Delp23 Furdel Constructs to Form Postfusion**

#### **30 Rosettes**

In general, trypsin digestion of Delp23 Furdel monomers is done with 1:1000 trypsin:RSV F by weight, or 10-15 BAEE units of trypsin for 1 mg of RSV F antigen.

In a typical reaction, trypsin from bovine plasma (Sigma Aldrich, T8802: 10,000-15,000 BAEE units/mg trypsin) was diluted to a 1 mg/ml concentration in 25 mM Tris pH 7.5, 300 mM NaCl. A 1 mg/ml solution of RSV F protein ecto-domain polypeptide (diluted in 25 mM Tris pH 7.5, 300 mM NaCl) was treated with one microliter of  
5 trypsin solution (final mass ratio 0.001:1 trypsin:RSV F or approximately 10-15 BAEE units of trypsin to each milligram of RSV F) for 1 hour at 37 °C. Typically, progress of the cleavage reaction was monitored by SDS-PAGE gel. The cleavage reaction was stopped using a trypsin inhibitor. The cleaved RSV F protein was further purified by size exclusion chromatography.

10 On occasion, 1:100 volume of immobilized trypsin inhibitor (Sigma) or 1 microliter of 1 mM soybean trypsin inhibitor was added to the cleavage solution and the mixture was incubated at room temperature for approximately 15-30 minutes with gentle rocking to stop the trypsin reaction. The inhibitor resin was separated from the protein solution using microcentrifuge columns. The resulting solution was purified by  
15 SEC purification.

#### **Electron Microscopy (EM) of RSV F proteins.**

RSV F protein ecto-domain polypeptides (approximately 50 micrograms per ml), were absorbed onto glow-discharged carbon-coated grids and were negatively  
20 stained with 2% sodium phosphotungstate (pH 7.0) or 0.75% uranyl-formate (unquantified low pH). The grids were observed on a Technai Spirit or JOEL 1230 transmission electron microscope operating between 80-120 kV with a magnification between 20,000 to 150,000 depending on required resolution.

#### **25 Phospholipid Assay**

This assay is based on the Wako Pure Chemical Industries, Ltd. assay Phospholipids C choline oxidase – DAOS method (Cat. No 433-36201). The assay protocol is modified only to reduce the amount of material used in the assay, and to decrease the sample dilution in the reaction relative to the general protocol from the  
30 vendor. To determine the lipid content of the RSV F sample, generate the color reagent by dissolving one bottle of Color Reagent with one bottle of Buffer (color reagent is stable for 1 week at 4°C). Dilute the 300 mg/dL (3 mg/ml) phospholipid standard to

1.5, 1.0, 0.75, 0.5 and 0.25 mg/ml with distilled water. For each standard, a water blank and sample reaction, add to a microcentrifuge tube 10 µl of color reagent and 2 µl of either standard, distilled water (0 mg/ml standard) or sample. Centrifuge reactions briefly to ensure proper mixing and incubate the tubes for 15 min at 37°C. Record the absorbance at 595 nm for each standard point and generate a standard curve. Record the 595 nm absorbance for each sample and calculate the phospholipids concentration from the prepared calibration curve.

### Immunogenicity in Cotton Rats

- 10 Immunogenicity of RSV F protein ectodomain polypeptides in the forms of monomers (uncleaved delp21 furx), rosettes of trimers (cleaved delp23 furdel), and trimers (fusion peptide deletion) were determined in cotton rats (*Sigmodon hispidus*) in two studies. In study 1 (FIGS. 8A and 8B), 10 cotton rats per group were vaccinated intramuscularly with 10 µg of monomers or rosettes (each adsorbed to aluminum hydroxide) on days 0 and 21. Serum anti- RSV F protein IgG and RSV neutralizing antibody titers were measured 2 weeks after the 1<sup>st</sup> vaccination (2wp1) and 2 weeks after the 2<sup>nd</sup> vaccination (2wp2), or 3 weeks after the 1<sup>st</sup> vaccination (3wp1) and 2 weeks after the 2<sup>nd</sup> vaccination (2wp2). Anti- RSV F protein IgG (Figure 8A) was determined by ELISA using RSV F protein coated plates and horse radish peroxidase-conjugated chicken anti-cotton rat IgG detection antibody. Data are presented as log<sub>10</sub> geometric mean titers (GMT) + standard deviations of individual cotton rats. RSV neutralization titers (FIG. 8B) were measured by plaque reduction neutralization test (PRNT). In brief, dilutions of heat-inactivated serum were preincubated with RSV Long, and then inoculated on HEp-2 cells in 12-well plates. After a 2 hour infection the inoculum was removed and cells were overlaid with agarose. Plaques were enumerated 5 days later by neutral red staining. The neutralization titer is defined as the reciprocal of the serum dilution producing at least a 60% reduction in number of plaques per well, relative to controls (no serum). Data are presented as log<sub>10</sub> GMT + standard error of 2 pools of 5 cotton rats per group.
- 20
- 25
- 30 In study 2 (FIG. 8C), 9 cotton rats per group were immunized intramuscularly with the indicated doses of monomers, trimers, or rosettes (each adsorbed to aluminum hydroxide). Serum anti- RSV F protein IgG titers were measured 2wp1 as above.

## Results

The soluble RSV F ecto-domain (with un-mutated furin cleavage sites) was expressed, but could not be purified from lipid and lipoprotein impurities derived from the host cells or culture media using size exclusion chromatography. These RSV F ecto-domain polypeptides elute in the SEC column void volume along with the lipid and lipoprotein contaminants.

Several constructs were prepared for making RSV F ecto-domain polypeptides that contain mutations to the furin cleavage sites, including the Furdel constructs. See, FIG. 1. Polypeptides produced by expression of the Furdel constructs were secreted from the cells as an ~65 kDa uncleaved species. The Furdel mutation also prevents fusion peptide exposure, which in turn prevents rosette formation. As a result, soluble RSV F Furdel migrated in the included volume of a Superdex 200 preparatory column, resulting in separation from both the lipid debris, which eluted in the void volume, and insect protein impurity. These results show that RSV F ecto-domain polypeptides in which the furin cleavage sites have been mutated are produced as uncleaved polypeptide that can be purified by SEC. In addition, analysis of the uncleaved RSV F retention time was consistent with the polypeptides being monomers rather than trimers.

Whether the RSV F furdel polypeptides were monomers, trimers or a mixture of monomers and trimers was assessed further using analytical ultracentrifugation. Analytical ultracentrifugation studies were performed using protein purified from the monomer peak from the SEC purification. Sedimentation velocity data of the uncleaved RSV F showed a step pattern suggesting two species in solution. Analysis of the sedimentation velocity experiment showed that the uncleaved RSV F ecto-domain had a high population of monomer and a minor population of apparent trimer in the solution. Equilibrium run data was collected and attempts to fit the data to either an ideal monomer model or a monomer-trimer equilibrium model were performed. However, the residuals of the fits are poor, particularly toward the bottom of the cell where the protein concentration is higher. These observations suggested that the uncleaved RSV F ecto-domain polypeptides are predominantly a monomer with a



smaller population which self associates (potentially as trimers) or aggregates at higher concentrations.

Further analysis of select RSV F protein ectodomain polypeptides was conducted using size exclusion (SEC) chromatography. FIGS. 6A-6D. The principle peaks containing monomers, trimers or rosettes of trimers are indicated by an asterisk in FIGS. 6A-6D, with the retention time of the Superdex P200 16/60 column (GE Healthcare) is indicated in milliliters. On a calibrated column, the approximate retention times of 47 mls, 65 mls and 77 mls correspond to the column void volume, the retention of F trimers and the retention of the monomers, respectively. In FIG. 6A, the uncleaved Delp23 Furdel ( $\Delta p23$  Furdel) construct was purified from the monomer peak. When the uncleaved Delp23 Furdel RSV F antigen was treated with trypsin, the protein formed rosettes, which migrated on SEC in the void volume (FIG. 6B). The cleaved trimer species of RSV F fusion peptide deletion was purified from the trimer peak at approximately 65 mls retention time (FIG. 6C) while the uncleaved Delp21 Furx construct ( $\Delta p21$  Furx) was purified from the monomer peak at approximately 77 mls (FIG. 6D).

Several RSV F protein ecto-domain polypeptides in uncleaved form or after trypsin cleavage were assessed by EM. The RSV F Furdel and delp23 Furdel constructs have arginine residues remaining in the furin cleavage site. These arginines are susceptible to trypsin cleavage. Upon cleavage, the uncleaved  $F_0$  species was converted to the  $F_1/F_2$  species, in which the fusion peptide is exposed. EM analysis confirmed that following trypsin cleavage the uncleaved RSV ecto-domains formed rosettes of trimers by virtue of their fusion peptides, as has been observed for related fusion proteins. The results are presented in the Table 3, and show that uncleaved RSV F protein ecto-domain polypeptides can be cleaved to form rosettes of trimers. The fusion peptide deleted construct, which is cleaved by furin, formed monodispersed trimers. See, also, FIGS. 7A-7D. Advantageously, producing rosettes of trimers in this way results in rosettes of trimers that are substantially free of lipid debris and lipoproteins.

The results of the immunogenicity studies showed that RSV F protein ectodomain polypeptides in the form of monomers (uncleaved delp21 furx), rosettes of trimers (cleaved delp23 furdel), and trimers (fusion peptide deletion) were

immunogenic in cotton rats (*Sigmodon hispidus*), and induced neutralizing antibodies. FIG. 8A-8C.

Table 3	
Construct	Conformation by EM
RSV F wild type ecto-domain (cleaved in host cell during expression)	Rosettes of trimers associated with lipid debris
Trypsin-cleavable Furdel (purified monomer peak - uncleaved)	Variable. Some preparations show monodispersed trimers; others show little material visible by EM of negatively-stained material
Trypsin-cleavable Furdel (purified monomer peak – trypsin cleaved after purification)	Rosettes of trimers
Trypsin-cleavable delp23 furdel (purified monomer peak - uncleaved)	Variable. Some preparations show monodispersed trimers; others show little material visible by EM of negatively-stained material
Trypsin-cleavable delp23 Furdel (purified monomer peak – trypsin cleaved after purification)	Rosettes of trimers
Cleaved Fusion Peptide Deletion (purified monomer peak)	Monodispersed trimers

## 5 Example 5 – Methods for Making RSV F Subunit Antigens in Insect or CHO Cells

### **RSV F antigen purification from insect cells:**

RSV F ectodomain subunits, including Delp21 Furx, Delp23 Furdel and Fusion Peptide Deletion constructs, were expressed in HiFive insect cells (Invitrogen) using the pFAST Bac baculovirus system. The RSV F subunit was purified from large scale expressions, 10-25 liters, via a two step chelating method that reduced the deleterious effect of the ferritin contaminant present in insect cell media, which can corrupt the

chelating resin.  $\text{CuSO}_4$  was added to media supernatant to a final concentration of 500  $\mu\text{M}$ . Approximately ten to twenty milliliters of chelating resin (Chelating Resin, BioRad) was added to each 1 liter of media, the slurry was rocked for at least thirty minutes at 4°C, and the resin and media were separated using a gravity column. The resin was washed with approximately two-times the resin volume of equilibration buffer (25 mM Tris pH 7.5, 300 mM NaCl), and the F protein ecto-domain was eluted with approximately two-times the column volume of elution buffer (equilibration buffer with 250 mM imidazole). The elution was dialyzed against 25 mM Tris buffer pH 7.5, 300 mM NaCl and the resulting solution was loaded onto a 5 ml Hitrap chelation column charged with  $\text{NiSO}_4$  (GE Healthcare). Bound protein was eluted with 25 mM Tris pH 7.5, 300 mM NaCl and a gradient of imidazole.

Elutions from the imidazole gradient in both cases were evaluated using anti-HIS<sub>6</sub> western blots and/or Coomassie-stained SDS-PAGE gels. Fractions containing pure constructs were collected and concentrated to approximately 1 mg/ml using Millipore Centrprep concentrators and/or Vivaspin concentration units for subsequent analysis/purification by size exclusion chromatography.

### SEC analysis and purification of RSV F ectodomains

Size exclusion chromatography (SEC) was used to purify and analyze RSV F protein ectodomain uncleaved monomers and cleaved trimers. This method also allowed uncleaved RSV F protein ectodomains to be purified away from host cell or media derived lipid and lipoprotein contaminants. In the case of clean-rosette generation, the uncleaved Delp23 Furdel construct was initially purified as a monomer and subsequently protease treated and re-purified using SEC to purify homogeneous rosettes (see below). Two methods were developed for analysis of RSV F oligamerization, HPLC-SEC and FPLC-SEC, which may also serve as an efficient purification step.

HPLC-SEC was performed using a Biorad SEC column (18 mm) with a 25 mM Tris pH 7.5, 300 mM NaCl mobile phase. Using Biorad HPLC-SEC standards to calibrate the system, we found that the RSV rosettes (representing cleaved, postfusion conformations) elute in the column void volume of the analysis while RSV F monomers elute with an apparent molecular weight of approximately 75-85 kDa.

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FPLC-SEC was performed on a GE Healthcare FPLC using a 16/60 Superdex 200 column with 25 mM Tris pH 7.5, 300 mM NaCl as a mobile phase. Using GE Healthcare High molecular weight standards to calibrate the system, we found that the RSV rosettes elute in the column void volume of the analysis, while RSV monodispersed trimers elute with an apparent molecular weight of approximately 140-160 kDa and RSV F monomers elute with an apparent molecular weight of approximately 75-85 kDa. For purification of RSV uncleaved Delp21 Furx or Delp23 Furdel (monomers) or Fusion Peptide Deletion (trimer) 0.5-2 mls of approximately 1 mg/ml chelation purified material was loaded on to an equilibrated Superdex P200 16/60 column with a flow rate between 0.5-2 mls/min and relevant fractions were collected.

#### **Trypsin cleavage of Delp23 Furdel constructs to form postfusion rosettes**

Trypsin from bovine plasma (Sigma Aldrich, T8802: 10,000-15,000 BAEE units/mg trypsin) was diluted to a 1 mg/ml concentration in 25 mM Tris pH 7.5, 300 mM NaCl. A 1 mg/ml solution of RSV F protein ecto-domain polypeptide (diluted in 25 mM Tris pH 7.5, 300 mM NaCl) was treated with one microliter of trypsin solution (final mass ratio 0.001:1 trypsin:RSV F or approximately 10-15 BAEE units of trypsin to each milligram of RSV F) for 1 hour at 37 °C. Progress of the cleavage reaction was monitored by SDS-PAGE gel. The cleavage reaction was stopped using a trypsin inhibitor (Gibco Soy Bean Trypsin Inhibitor using equal mass of inhibitor to trypsin). It was found that an incubation period was required between the cleavage step and subsequent rosette purification to allow higher efficiency of monomer to rosette conversion. A one to 6 hour incubation period at 37°C was given to provide higher rosette formation efficiency. The cleaved RSV F protein was further purified from unconverted monomer species using size exclusion chromatography (as described above) where homogeneous rosettes can be collected in the column void volume fractions.

#### **RSV F antigen purification from CHO cells:**

RSV F Fusion Peptide Deletion constructs, which do not contain a HIS-tag, were purified by cation purification. CHO material containing expressed RSV F trimer

antigen was concentrated to approximately one-tenth the original volume on a GE Healthcare hollow fiber cartridge concentration system (MWCO 10,000 kDa). The concentrated solution was then buffer exchanged four times with an equivalent volume of 25 mM Sodium Acetate pH 6.0, 25 mM NaCl. The resulting solution, containing concentrated RSV F trimer in the acetate/saline buffer, was loaded onto a pre-charged GE Healthcare HiTrap CM column which had been equilibrated with acetate/saline buffer. The protein was eluted from the column using a step gradient of 25 mM acetate buffer containing either 25, 150, 250, 500 or 1000 mM NaCl (the 250 mM and 500 mM NaCl fractions containing the bulk of the eluted material). This material could be further purified using a SEC purification similar to the protocol above.

#### Example 6 – Immunogenicity of RSV F subunits in cotton rats

The immunogenicity and protective capacity of RSV-F trimer (RSV-F-fusion-peptide-deletion-trunc) and rosette (RSV-F-delp23-furdel-trunc,cleaved) subunits, each formulated with alum or MF59, was evaluated in the cotton rat model. The antigen used for ELISA in this study was RSV-F-fusion-peptide-deletion-trunc (Table 4). Neutralization was against infectious RSV, strain Long (Table 5). All combinations were immunogenic, eliciting high titer RSV-F-specific IgG and RSV neutralizing antibody responses that were boosted by a second vaccination, and afforded protection from nasal RSV challenge.

### **Methods**

#### Vaccination and challenge of cotton rats

Female cotton rats (*Sigmodon hispidus*) were obtained from Harlan Laboratories. Groups of animals were immunized intramuscularly (i.m., 100 µl) with the indicated vaccines on days 0 and 21. Serum samples were collected 3 weeks after the first immunization (3wp1) and 2 weeks after the second immunization (2wp2). Immunized or unvaccinated control animals were challenged intranasally (i.n.) with  $1 \times 10^5$  pfu RSV Long 4 weeks after the final immunization. Blood collection and RSV challenge were performed under anesthesia with 3% isoflurane using a precision vaporizer.

#### RSV F-specific ELISA

Individual serum samples were assayed for the presence of RSV F-specific IgG by enzyme-linked immunosorbent assay (ELISA). ELISA plates (MaxiSorp 96-well, Nunc) were coated overnight at 4°C with 1 µg/ml purified RSV F (fusion-peptide deletion-trunc) in PBS. After washing (PBS with 0.1% Tween-20), plates were blocked with Superblock Blocking Buffer in PBS (Thermo Scientific) for at least 1.5 hours at 37°C. The plates were then washed, serial dilutions of serum in assay diluent (PBS with 0.1% Tween-20 and 5% goat serum) from experimental or control cotton rats were added, and plates were incubated for 2 hours at 37°C. After washing, plates were incubated with horse radish peroxidase (HRP)-conjugated chicken anti-cotton rat IgG (Immunology Consultants Laboratory, Inc, diluted 1:5,000 in assay diluent) for 1 hour at 37°C. Finally, plates were washed and 100 µl of TMB peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc) was added to each well. Reactions were stopped by addition of 100 µl of 1M H<sub>3</sub>PO<sub>4</sub>, and absorbance was read at 450 nm using a plate reader. For each serum sample, a plot of optical density (OD) versus logarithm of the reciprocal serum dilution was generated by nonlinear regression (GraphPad Prism). Titers were defined as the reciprocal serum dilution at an OD of approximately 0.5 (normalized to a standard, pooled sera from RSV-infected cotton rats with a defined titer of 1:2500, that was included on every plate).

#### 20        Micro neutralization assay

Serum samples were tested for the presence of neutralizing antibodies by microneutralization assay. Two-fold serial dilutions of heat inactivated (HI)-serum (in PBS with 5% HI-fetal bovine serum(FBS)) were added to an equal volume of RSV, strain Long previously titrated to give approximately 115 PFU/25 µl. Serum/virus mixtures were incubated for 2 hours at 37°C and 5% CO<sub>2</sub>, to allow virus neutralization to occur, and then 25 µl of this mixture (containing approximately 115 PFU) was inoculated on duplicate wells of HEp-2 cells in 96 well plates. After 2 hours at 37° and 5% CO<sub>2</sub>, the cells were overlayed with 0.75% Methyl Cellulose/ EMEM 5% HI-FBS and incubated for 42 hours. The number of infectious virus particles was determined by detection of syncytia formation by immunostaining followed by automated counting.

The neutralization titer is defined as the reciprocal of the serum dilution producing at least a 60% reduction in number of syncytia per well, relative to controls (no serum).

#### Viral load

5           Viral load in the lung was determined by plaque assay. Specifically, lungs were harvested 5 days post RSV infection and one right lobe was placed into 2.5 ml Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) with 25% sucrose and disrupted with a tissue homogenizer. Cell-free supernatants from these samples were stored at -80°C. To assay for infectious virus, dilutions of clarified lung homogenate  
10 (in PBS with 5% HI-FBS) were inoculated on confluent HEp-2 cell monolayers in a volume of 200 µl/well of a 12-well plate. After 2 hours with periodic gentle rocking (37°C, 5% CO<sub>2</sub>), the inoculum was removed, and cells were overlaid with 1.5 ml of 1.25% SeaPlaque agarose (Lonza) in Eagle's Minimal Essential Medium (EMEM, Lonza) supplemented with 5% HI-FBS, glutamine, and antibiotics. After 3-4 days of  
15 incubation, cells were again overlaid with 1 ml of 1.25% agarose in EMEM (Sigma) containing 0.1% neutral red (Sigma). Plaques were counted one day later with the aid of a light box.

An alternative method for determining viral load is quantitative real-time PCR  
20 (qRT-PCR). Viral load can be determined by qRT-PCR using oligonucleotide primers specific for the RSV-F gene as described (I. Borg *et al*, Eur Respir J 2003; 21:944-51) with some modifications. Briefly, RNA is isolated from 140 µl of clarified lung homogenate, or from a known number of plaque forming units (PFU) of RSV (determined by plaque assay, and diluted in lung homogenate from uninfected animals),  
25 using the RNeasy kit (Qiagen) with a final elution volume of 100 µl H<sub>2</sub>O. cDNA synthesis and PCR is performed in a single tube using the SuperScript III Platinum One-Step Quantitative RT-PCR kit (Invitrogen) with 5 µL of eluted RNA, 10 µM of each primer, and 50µM of the probe (primers and probes from Integrated DNA Technologies). Forward primer: TTGGATCTGCAATCGCCA (SEQ ID NO:72).  
30 Reverse primer: CTTTGTGATCTTGTTCACTTCTCCTTCT (SEQ ID NO:73). Probe: 5'-carboxyfluorescein (FAM)-TGGCACTGCTGTATCTAAGGTCCTGCACT-

tetramethylcarboxyrhodamine(TAMRA)-3' (SEQ ID NO:74). Amplification and detection is performed with an ABI Prism 7900HT or 7500 (Applied Biosystems). A threshold cycle value (Ct) is defined for each sample as the cycle number at which the fluorescent signal first becomes detectable above a set threshold. PFU equivalents for each sample is then determined based on a standard curve of Ct verses the logarithm of defined copy number of viral RNA.

### Results

The cotton rat as a model has been used extensively in the study of RSV pathogenesis and immunity because of the many similarities between RSV-induced disease in cotton rats and humans. Two important parallels are the efficacy of neutralizing antibodies, and the enhanced lung histopathology associated with formalin-inactivated RSV vaccination. Cotton rats are also more susceptible to RSV infection than other small animals such as mice.

To evaluate the immunogenicity of our RSV-F subunit vaccines, groups of female cotton rats were vaccinated intramuscularly with various doses of trimers (RSV-F-fusion-peptide-deletion-trunc) or rosettes (RSV-F-delp23-furdel-trunc, cleaved), each formulated with either alum or MF59. In all cases, a single immunization was sufficient to induce both F-specific and neutralizing antibody in the serum when measured three weeks after the first vaccination (3wp1). All cotton rats were given a homologous booster immunization three weeks after the first, and this resulted in a significant increase in F-specific IgG and neutralizing antibody when measured two weeks later (2wp2). Generally, the immunogenicity of rosettes was equal to or greater than that of trimers, MF59 formulation enhanced titers more than alum formulation, and higher protein doses yielded higher titers, although there were some exceptions.

To determine the protective capacity of the subunit vaccines, all cotton rats were infected four weeks after the second vaccination with RSV by the nasal route and the viral load in the lung was measured five days later by plaque assay. In all cases, subunit vaccination conferred protection from challenge, as pulmonary viral loads in vaccinated cotton rats were greater than three orders of magnitude lower than unimmunized, but challenged control animals.



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Table 4: F-specific serum IgG titers

Serum collected	Protein dose (µg)	F-specific serum IgG titer <sup>a</sup>			
		alum		MF59	
		trimer	rosette	trimer	rosette
3wp1	10	20276	36841	10251	22415
	1	18341	20802	3712	28610
	0.1	2698	6896	1065	8293
2wp2	10	103670	97174	130016	156144
	1	142331	102405	177441	299501
	0.1	11581	34354	50238	111099

<sup>a</sup> geometric mean titer for individual cotton rats (7-8 per group)

trimer immunogen was RSV-F-fusion-peptide-deletion-trunc

rosette immunogen was RSV-F-delp23-furdel-trun, cleaved.

Table 4A: Lung viral titer 5 days post RSV challenge<sup>a</sup>

vaccination	Protein dose (μg)	viral titer <sup>b</sup>
none	-	822760
trimer/alum	10	546
	1	636
	0.1	903
rosette/alum	10	305
	1	341
	0.1	548
trimer/MF59	10	360
	1	301
	0.1	456
rosette/MF59	10	244
	1	257
	0.1	716

<sup>a</sup> intranasal challenge with  $1 \times 10^5$  plaque-forming units(pfu) of RSV Long

<sup>b</sup> pfu/gram lung 5 days post challenge

Geometric mean titers of 7-8 individual cotton rats/group.

If an individual animal had a titer of <203 (limit of detection) it was assigned a titer of 100

Table 5: RSV serum neutralization titer

Serum collected	Protein dose (μg)	RSV serum neutralization titer <sup>a</sup>			
		alum		MF59	
3wp1	10	trimer	rosette	trimer	rosette
	1	628	1050	578	229
	0.1	208	633	165	205
2wp2	10	57	200	51	65
	1	3669	4015	3983	3436
	0.1	3369	2844	5728	3940
		744	1902	2414	2093

<sup>a</sup> 60% syncytia reduction neutralization titers

geometric mean titer for two pools of 3-4 cotton rats per group

Example 7 – RSV RNA vaccine**RNA synthesis**

Plasmid DNA encoding alphavirus replicon (FIG. 4, SEQ ID NO:77) served as a template for synthesis of RNA in vitro. For these experiments the full length surface fusion glycoprotein of RSV (RSV-F) was used (FIG. 4). Upon delivery of the replicons to eukaryotic cells, the positive-stranded RNA was translated to produce four non-structural proteins, which together replicated the genomic RNA and transcribed abundant subgenomic mRNAs encoding the heterologous gene product. Due to the lack of expression of the alphavirus structural proteins, replicons are incapable of inducing the generation of infectious particles. A bacteriophage (T7 or SP6) promoter upstream of the alphavirus cDNA facilitates the synthesis of the replicon RNA in vitro and the hepatitis delta virus (HDV) ribozyme immediately downstream of the poly(A)-tail generates the correct 3'-end through its self-cleaving activity.

Following linearization of the plasmid DNA downstream of the HDV ribozyme with a suitable restriction endonuclease, run-off transcripts were synthesized in vitro using T7 or SP6 bacteriophage derived DNA-dependent RNA polymerase. Transcriptions were performed for 2 hours at 37°C in the presence of 7.5 mM (T7 RNA polymerase) or 5 mM (SP6 RNA polymerase) of each of the nucleoside triphosphates (ATP, CTP, GTP and UTP) following the instructions provided by the manufacturer (Ambion, Austin, TX). Following transcription, the template DNA was digested with TURBO DNase (Ambion, Austin, TX). The replicon RNA was precipitated with LiCl and reconstituted in nuclease-free water. Uncapped RNA was capped post-transcriptionally with Vaccinia Capping Enzyme (VCE) using the ScriptCap m<sup>7</sup>G Capping System (Epicentre Biotechnologies, Madison, WI) as outlined in the user manual. Post-transcriptionally capped RNA was precipitated with LiCl and reconstituted in nuclease-free water. The concentration of the RNA samples was determined by measuring the optical density at 260 nm. Integrity of the in vitro transcripts was confirmed by denaturing agarose gel electrophoresis.

**Lipid nanoparticle (Liposome) Formulation RV01(01)**

1,2-dilinoyleoxy-N,N-dimethyl-3-aminopropane (DlinDMA) was synthesized using a previously published procedure [Heyes, J., Palmer, L., Bremner, K.,

MacLachlan, I. Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids. *Journal of Controlled Release*, 107: 276-287 (2005)]. 1, 2-Diastearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Genzyme. Cholesterol was obtained from Sigma-Aldrich (St. Louis, MO). 1, 2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG DMG 2000), 1, 2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) was obtained from Avanti Polar Lipids (Alabaster, AL).

Fresh lipid stock solutions in ethanol were prepared. 37 mg of DlinDMA, 11.8 mg of DSPC, 27.8 mg of Cholesterol and 8.07 mg of PEG DMG 2000 were weighed and dissolved in 7.55 mL of ethanol. The freshly prepared lipid stock solution was gently rocked at 37 °C for about 15 minutes to form a homogenous mixture. Then, 755 µL of the stock was added to 1.245 mL ethanol to make a working lipid stock solution of 2 mL. This amount of lipid was used to form LNPs with 250 µg RNA at a 8:1 N:P (Nitrogen to Phosphate) ratio. The protonatable nitrogen on DlinDMA (the cationic lipid) and phosphates on the RNA are used for this calculation. Each µg of self-replicating RNA molecule was assumed to contain 3 nmoles of anionic phosphate, each µg of DlinDMA was assumed to contain 1.6 nmoles of cationic nitrogen. A 2 mL working solution of RNA was also prepared from a stock solution of ~ 1 µg/µL in 100 mM citrate buffer (pH 6) (Teknova, Hollister, CA)). Three 20 mL glass vials (with stir bars) were rinsed with RNase Away solution (Molecular BioProducts, San Diego, CA) and washed with plenty of MilliQ water before use to decontaminate the vials of RNases. One of the vials was used for the RNA working solution and the others for collecting the lipid and RNA mixes (as described below). The working lipid and RNA solutions were heated at 37 °C for 10 minutes before being loaded into 3cc luer-lok syringes (BD Medical, Franklin Lakes, NJ). 2 mL of citrate buffer (pH 6) was loaded in another 3 cc syringe. Syringes containing RNA and the lipids were connected to a T mixer (PEEK™ 500 µm ID junction, IDEX Health Science, Oak Harbor, WA) using FEP tubing ([fluorinated ethylene-propylene] 2mm ID x 3mm OD, IDEX Health Science, Oak Harbor, WA). The outlet from the T mixer was also FEP tubing (2mm ID x 3mm). The third syringe containing the citrate buffer was connected to a separate piece of tubing (2mm ID x 3mm OD). All syringes were then driven at a flow rate of 7 mL/min

using a syringe pump (kdScientific, model no. KDS-220, Holliston, MA). The tube outlets were positioned to collect the mixtures in a 20 mL glass vial (while stirring). The stir bar was taken out and the ethanol/aqueous solution was allowed to equilibrate to room temperature for 1 hour. 4 ml of the mixture was loaded into a 5 cc syringe (BD Medical), which was connected to a piece of FEP tubing (2mm ID x 3mm OD, Idex Health Science, Oak Harbor, WA) and in another 5 cc syringe connected to an equal length of FEP tubing, an equal amount of 100 mM citrate buffer (pH 6) was loaded. The two syringes were driven at 7mL/min flow rate using the syringe pump and the final mixture collected in a 20 mL glass vial (while stirring). Next, the mixture collected from the second mixing step (liposomes) were passed through a Mustang Q membrane (an anion-exchange support that binds and removes anionic molecules, obtained from Pall Corporation, AnnArbor, MI, USA). Before passing the liposomes, 4 mL of 1 M NaOH, 4 mL of 1 M NaCl and 10 mL of 100 mM citrate buffer (pH 6) were successively passed through the Mustang membrane. Liposomes were warmed for 10 minutes at 37 °C before passing through the mustang filter. Next, liposomes were concentrated to 2 mL and dialyzed against 10-15 volumes of 1X PBS (from Teknova) using the Tangential Flow Filtration (TFF) system before recovering the final product. The TFF system and hollow fiber filtration membranes were purchased from Spectrum Labs (Rancho Dominguez, CA) and were used according to the manufacturer's guidelines. Polysulfone hollow fiber filtration membranes (part number P/N: X1AB-100-20P) with a 100 kD pore size cutoff and 8 cm<sup>2</sup> surface area were used. For *in vitro* and *in vivo* experiments, formulations were diluted to the required RNA concentration with 1X PBS (from Teknova).

#### 25           **Method of preparing cationic emulsion 17 (CNE17)**

Squalene, sorbitan trioleate (Span 85), and polyoxy- ethylene sorbitan monololeate (Tween 80) were obtained from Sigma (St. Louis, MO, USA). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Lipoid (Ludwigshafen Germany). Cationic nanoemulsions (CNEs) were prepared similarly to charged MF59 as previously described with minor modifications Ott, *et al.* Journal of Controlled Release, 79(1-3):1-5 (2002)). Briefly, oil soluble components (ie. Squalene, span 85, cationic lipids, lipid surfactants) were combined in a beaker, lipid components

were dissolved in chloroform (CHCl<sub>3</sub>) or dichloromethane (DCM). The resulting lipid solution was added directly to the oil plus span 85. The solvent was allowed to evaporate at room temperature for 2 hours in a fume hood prior to combining the aqueous phase and homogenizing the sample using an IKA T25 homogenizer at 24K RPM in order to provide a homogeneous feedstock. The primary emulsions were passed three to five times through a Microfluidizer M110S or M110PS homogenizer with an ice bath cooling coil at a homogenization pressure of approximately 15k – 20k PSI (Microfluidics, Newton, MA). The 20ml batch samples were removed from the unit and stored at 4°C. The table below describes the composition of CNE17.

Table 6: Composition of CNE17

CNE	Cationic Lipid (+)	mg/ml +Lipid	Surfactant	Squalene	Buffer/water
CNE17	DOTAP (in DCM)	1.40	0.5% SPAN 85 0.5% Tween 80	4.3%	10mM citrate buffer pH 6.5

#### *RNA complexation*

The number of nitrogens in solution were calculated from the cationic lipid concentration, DOTAP for example has 1 nitrogen that can be protonated per molecule. The RNA concentration was used to calculate the amount of phosphate in solution using an estimate of 3 nmols of phosphate per microgram of RNA. By varying the amount of RNA : Lipid the N/P ratio can be modified. RNA was complexed to CNE17 at a nitrogen / phosphate ratios (N/P) of 10:1. Using these values the RNA was diluted to the appropriate concentration in RNase free water and added directly into an equal volume of emulsion while vortexing lightly. The solution was allowed to sit at room temperature for approximately 2 hours. Once complexed the resulting solution was diluted to the required concentration prior to administration.

#### **Electroporation**

Electroporation was a very effective method for the delivery of pDNA vaccines and this technique was used to deliver self-replicating RNA. Mice were anesthetized under isofluorane, both hind legs were closely shaven to expose the area on the limb to be treated. A dose of 30 ul of vaccine was injected to the calf muscle of the hind limb

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using a 1/2cc insulin syringe. The muscle was electroporated using the Elgen® DNA Delivery System (Inovio, San Diego). The instrument parameters are as follows: 60V, 2 pulses each at 60ms. Another dose was similarly delivered to the second limb, followed by electroporation.

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#### **Viral replicon particles (VRP)**

To compare RNA vaccines to traditional RNA-vectored approaches for achieving in vivo expression of reporter genes or antigens, we utilized viral replicon particles (VRPs) produced in BHK cells by the methods described by Perri et al. In this system, the antigen (or reporter gene) replicons consisted of alphavirus chimeric replicons (VCR) derived from the genome of Venezuelan equine encephalitis virus (VEEV) engineered to contain the 3' terminal sequences (3' UTR) of Sindbis virus and a Sindbis virus packaging signal (PS) (see Fig. 2 of Perri et al). These replicons were packaged into VRPs by co-electroporating them into baby hamster kidney (BHK) cells along with defective helper RNAs encoding the Sindbis virus capsid and glycoprotein genes (see Fig. 2 of Perri et al). The VRPs were then harvested and titrated by standard methods and inoculated into animals in culture fluid or other isotonic buffers. Perri S, Greer CE, Thudium K, Doe B, Legg H, Liu H, Romero RE, Tang Z, Bin Q, Dubensky TW, Jr. et al (2003) An alphavirus replicon particle chimera derived from venezuelan equine encephalitis and sindbis viruses is a potent gene-based vaccine delivery vector. J Virol 77: 10394-10403

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#### **RSV F trimer subunit vaccine**

The RSV F trimer is a recombinant protein comprising the ectodomain of RSV F with a deletion of the fusion peptide region preventing association with other trimers. The resulting construct forms a homogeneous trimer, as observed by size exclusion chromatography, and has an expected phenotype consistent with a postfusion F conformation as observed by electron microscopy. The protein was expressed in insect cells and purified by virtue of a HIS-tagged in fusion with the construct's C-terminus followed by size exclusion chromatography using conventional techniques. The resulting protein sample exhibits greater than 95% purity. For the in vivo evaluation of the F-subunit vaccine, 100 µg/mL trimer protein was adsorbed on 2 mg/mL alum using

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10 mM Histidine buffer, pH 6.3 and isotonicity adjusted with sodium chloride to 150 mM. F-subunit protein was adsorbed on alum overnight with gentle stirring at 2-8°C. The pH and osmolality of the final vaccine was targeted as 6.5-7.5 and 240-360 mOsm/kg. The vaccine was characterized for protein adsorption by SDS-PAGE  
5 (Invitrogen Corporation, USA) and for endotoxin content by LAL assay (Charles River Laboratories, USA). The vaccine was mixed by gentle inversion prior to immunization.

### **Murine immunogenicity studies**

Groups of 10 female BALB/c mice aged 8-10 weeks and weighing about 20  
10 grams were immunized at day 0 and day 21 with bleeds taken at days 14, 35 and 49. All animals were injected in the quadriceps in the two hind legs each getting an equivalent volume (50µl per site) for a total of 100 µl of vaccine to deliver 10 µg antigen dose. When measurement of T cell responses was required, spleens were harvested at day 35 or 49.

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### **Vaccination and challenge of cotton rats**

Female cotton rats (*Sigmodon hispidus*) were obtained from Harlan Laboratories. All experiments were approved and performed according to Novartis Animal Care and Use Committee. Groups of animals were immunized intramuscularly (i.m., 100 µl)  
20 with the indicated vaccines on days 0 and 21. Serum samples were collected 2 weeks after each immunization. Immunized or unvaccinated control animals were challenged intranasally (i.n.) with  $1 \times 10^5$  PFU RSV 4 weeks after the final immunization. Blood collection and RSV challenge were performed under anesthesia with 3% isoflurane using a precision vaporizer.

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### **Mouse T cell function assays**

#### **Intracellular cytokines immunofluorescence assay**

Two to five spleens from identically vaccinated BALB/c mice were pooled and single cell suspensions were prepared for culture. Two antigen-stimulated cultures and  
30 two unstimulated cultures were established for each splenocyte pool. Antigen-stimulated cultures contained  $1 \times 10^6$  splenocytes, RSV F peptide 85-93 ( $1 \times 10^{-6}$  M), RSV F peptide 249-258 ( $1 \times 10^{-6}$  M), RSV F peptide 51-66 ( $1 \times 10^{-6}$  M), anti-CD28 mAb



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(1 mcg/mL), and Brefeldin A (1:1000). Unstimulated cultures did not contain RSV F peptides, and were otherwise identical to the stimulated cultures. After culturing for 6 hours at 37 °C, cultures were processed for immunofluorescence. Cells were washed and then stained with fluorescently labeled anti-CD4 and anti-CD8 monoclonal  
5 antibodies (mAb). Cells were washed again and then fixed with Cytofix/cytoperm for 20 minutes. The fixed cells were then washed with Perm-wash buffer and then stained with fluorescently labeled mAbs specific for IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-5. Stained cells were washed and then analyzed on an LSR II flow cytometer. FlowJo software was used to analyze the acquired data. The CD4+8- and CD8+4- T cell subsets were  
10 analyzed separately. For each subset in a given sample the % cytokine-positive cells was determined. The % RSV F antigen-specific T cells was calculated as the difference between the % cytokine-positive cells in the antigen-stimulated cultures and the % cytokine-positive cells in the unstimulated cultures. The 95% confidence limits for the % antigen-specific cells were determined using standard methods (Statistical Methods,  
15 7<sup>th</sup> Edition, G.W. Snedecor and W.G. Cochran).

#### **Secreted cytokines assay**

The cultures for the secreted cytokines assay were similar to those for the intracellular cytokines immunofluorescence assay except that Brefeldin A was omitted.  
20 Culture supernatants were collected after overnight culture at 37 °C, and were analyzed for multiple cytokines using mouse Th1/Th2 cytokine kits from Meso Scale Discovery. The amount of each cytokine per culture was determined from standard curves produced using purified, recombinant cytokines supplied by the manufacturer.

#### **RSV F-specific ELISA**

Individual serum samples were assayed for the presence of RSV F-specific IgG by enzyme-linked immunosorbent assay (ELISA). ELISA plates (MaxiSorp 96-well, Nunc) were coated overnight at 4°C with 1  $\mu$ g/ml purified RSV F (delp23-furdel-trunc  
30 uncleaved) in PBS. After washing (PBS with 0.1% Tween-20), plates were blocked with Superblock Blocking Buffer in PBS (Thermo Scientific) for at least 1.5 hours at 37°C. The plates were then washed, serial dilutions of serum in assay diluent (PBS with 0.1% Tween-20 and 5% goat serum) from experimental or control cotton rats were

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added, and plates were incubated for 2 hours at 37°C. After washing, plates were incubated with horse radish peroxidase (HRP)-conjugated chicken anti-cotton rat IgG (Immunology Consultants Laboratory, Inc, diluted 1:5,000 in assay diluent) for 1 hour at 37°C. Finally, plates were washed and 100 µl of TMB peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc) was added to each well. Reactions were stopped by addition of 100 µl of 1M H<sub>3</sub>PO<sub>4</sub>, and absorbance was read at 450 nm using a plate reader. For each serum sample, a plot of optical density (OD) versus logarithm of the reciprocal serum dilution was generated by nonlinear regression (GraphPad Prism). Titers were defined as the reciprocal serum dilution at an OD of approximately 0.5 (normalized to a standard, pooled sera from RSV-infected cotton rats with a defined titer of 1:2500, that was included on every plate).

#### Micro neutralization assay

Serum samples were tested for the presence of neutralizing antibodies by a plaque reduction neutralization test (PRNT). Two-fold serial dilutions of HI-serum (in PBS with 5% HI-FBS) were added to an equal volume of RSV Long previously titrated to give approximately 115 PFU/25 µl. Serum/virus mixtures were incubated for 2 hours at 37°C and 5% CO<sub>2</sub>, to allow virus neutralization to occur, and then 25 µl of this mixture (containing approximately 115 PFU) was inoculated on duplicate wells of HEP-2 cells in 96 well plates. After 2 hours at 37°C and 5% CO<sub>2</sub>, the cells were overlaid with 0.75% Methyl Cellulose/ EMEM 5% HI-FBS and incubated for 42 hours. The number of infectious virus particles was determined by detection of syncytia formation by immunostaining followed by automated counting. The neutralization titer is defined as the reciprocal of the serum dilution producing at least a 60% reduction in number of syncytia per well, relative to controls (no serum).

#### Viral load

Viral load in the lung was determined by plaque assay. Specifically, lungs were harvested 5 days post RSV infection and one right lobe was placed into 2.5 ml Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) with 25% sucrose and disrupted with a tissue homogenizer. Cell-free supernatants from these samples were

stored at -80°C. To assay for infectious virus, dilutions of clarified lung homogenate (in PBS with 5% heat-inactivated fetal bovine serum, HI-FBS) were inoculated on confluent HEp-2 cell monolayers in a volume of 200 µl/well of a 12-well plate. After 2 hours with periodic gentle rocking (37°C, 5% CO<sub>2</sub>), the inoculum was removed, and  
5 cells were overlaid with 1.5 ml of 1.25% SeaPlaque agarose (Lonza) in Eagle's Minimal Essential Medium (EMEM, Lonza) supplemented with 5% HI-FBS, glutamine, and antibiotics. After 3-4 days of incubation, cells were again overlaid with 1 ml of 1.25% agarose in EMEM (Sigma) containing 0.1% neutral red (Sigma). Plaques were counted one day later with the aid of a light box.

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### **Cotton rat lung pathology**

Five days after RSV challenge lungs were harvested and 4 lobes from each animal were collected and fixed with 10% neutral buffered formalin (NBF) by gentle intratracheal instillation followed by immersion fixation. Tissues were processed  
15 routinely to prepare hematoxylin & eosin-stained sections for microscopic examination. Findings were evaluated using a modification of previously published criteria [Prince GA, et al., 2001] for the following parameters: peribronchiolitis, alveolitis, bronchitis, perivascular cellular infiltrates, and interstitial pneumonitis. Lesions were graded on a 4-point semiquantitative scale. Minimal (+) change contained one or a few small foci; mild (++) change was composed of small- to medium-size foci; moderate (+++) change  
20 contained frequent and/or moderately-sized foci; and marked (++++ change showed extensive to confluent foci affecting most/all of the tissue.

### **Example 7**

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#### **A – Cotton rat RSV challenge study (CRIS14)**

The A317 replicon, which expresses the surface fusion glycoprotein of RSV (RSV-F) was used for this experiment. Cotton rats (*Sigmodon hispidus*), 8 animals per group, were given bilateral intramuscular vaccinations (50 µL per leg) on days 0 and 21 with naked self-replicating RNA (A317, 1 µg or 10 µg), self-replicating RNA  
30 formulated in LNP [RV01(01), A317, 0.1 µg or 1 µg), VRPs (5x10<sup>6</sup> IU) expressing RSV-F, F-trimer/alum subunit (10 µg), or formalin inactivated RSV vaccine (5200 FI-pfu). Serum was collected for antibody analysis on days 14 (2wp1) and 35 (2wp2). All

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animals were challenged with  $1 \times 10^5$  pfu RSV intranasally on day 49 and lungs were collected on day 54 (5dpc) for determination of viral load and lung pathology.

## Results

5 Table 7: F-specific serum IgG titers on day 14 and 35

vaccine	dose	F-specific IgG	F-specific IgG
		2wp1	2wp2
A317	10 $\mu$ g	198	1599
A317	1 $\mu$ g	78	526
CNE17	1 $\mu$ g	408	4918
CNE17	0.1 $\mu$ g	325	2512
RV01(01)	1 $\mu$ g	531	4351
RV01(01)	0.1 $\mu$ g	134	1033
VRP	$5 \times 10^6$ IU	961	5864
F-trimer/alum	10 $\mu$ g	3526	111893
FI-RSV	5200 FI-pfu	17	2074
none		5	5

10 Table 7. F-specific serum IgG titers of cotton rats (*Sigmodon hispidus*), 8 animals per group, after intramuscular vaccinations on days 0 and 21. Serum was collected for antibody analysis on days 14 (2wp1) and 35 (2wp2), all animals were challenged with  $1 \times 10^5$  pfu RSV intranasally on day 49. Lungs were collected on day 54 (5dpc) for determination of viral load and lung pathology. Data are represented as geometric mean titers of 8 individual cotton rats per group. If an individual animal had a titer of <25 (limit of detection) it was assigned a titer of 5.

Table 8: RSV serum neutralization titers on days 14 and 35

vaccine	dose	PRNT60	PRNT60
		2wp1	2wp2
A317	10 $\mu$ g	78	240
A317	1 $\mu$ g	58	70
CNE17	1 $\mu$ g	91	269
CNE17	0.1 $\mu$ g	63	145
RV01(01)	1 $\mu$ g	103	667
RV01(01)	0.1 $\mu$ g	46	130
VRP	$5 \times 10^6$ IU	149	683
F-trimer/alum	10 $\mu$ g	142	>5120
FI-RSV	5200 FI-pfu	28	38
none		30	<20

- Table 8. RSV serum neutralization titers of cotton rats (*Sigmodon hispidus*), 8 animals per group, after intramuscular vaccinations on days 0 and 21. Serum was collected for analysis on days 14 (2wp1) and 35 (2wp2). Data are represented as 60% plaque reduction neutralization titers. Geometric mean titer of 2 pools of 4 cotton rats per group. If an individual animal had a titer of <25 (limit of detection) it was assigned a titer of 5.

Table 9: Lung viral titers 5 days post RSV challenge

vaccine	dose	pfu/g lung 5dpc
A317	10 µg	397
A317	1 µg	659
CNE17	1 µg	414
CNE17	0.1 µg	572
RV01(01)	1 µg	445
RV01(01)	0.1 µg	716
VRP	5x10 <sup>6</sup> IU	359
F-trimer/alum	10 µg	190
FI-RSV	5200 FI-pfu	5248
none (challenged)		728618

- Table 9: Lung viral titers 5 days post RSV challenge of cotton rats (*Sigmodon hispidus*), 8 animals per group, after intramuscular vaccinations on days 0 and 21. All animals were challenged with 1x10<sup>5</sup> pfu RSV intranasally on day 49. Lungs were collected on day 54 (5dpc) for determination of viral load and lung pathology. Data are represented as plaque forming units per gram lung as determined by plaque assay. Geometric mean titers of 8 individual cotton rats per group. In an individual animal had a titer of <200 (limit of detection) it was assigned a titer of 100.

Table 10: Lung alveolitis scores 5 days post RSV challenge

vaccine	dose	# of cotton rats with indicated alveolitis score				
		0	1	2	3	4
A317I	10 µg	8				
A317I	1 µg	8				
CNE17	1 µg	8				
CNE17	0.1 µg	7	1			
RV01(01)	1 µg	6	2			
RV01(01)	0.1 µg	8				
VRP	5x10 <sup>6</sup> IU	3	4	1		
F-trimer/alum	10 µg	7	1			
FI-RSV	5200 FI-pfu	1	4	3		
none (challenged)		5	3			

- Table 10. Lung alveolitis 5 days post RSV challenge of cotton rats (*Sigmodon hispidus*), 8 animals per group, after intramuscular vaccinations on days 0 and 21. All animals were challenged with 1x10<sup>5</sup> pfu RSV intranasally on day 49. Lungs were collected on day 54 (5dpc) for determination of viral load and lung pathology. Lesions

were graded on a 4-point semiquantitative scale. Minimal (1) change contained one or a few small foci; mild (2) change was composed of small- to medium-size foci; moderate (3) change contained frequent and/or moderately-sized foci; and marked (4) change showed extensive to confluent foci affecting most/all of the tissue.

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### Conclusions

One objective of this study was to determine the immunogenicity and protective capacity of replicon RNA in the cotton rat RSV model. Another objective was to evaluate the effect of Liposomes and CNE17 formulations on vaccine immunogenicity and efficacy. Unformulated replicon RNA induced serum F-specific IgG and RSV neutralizing antibodies after one vaccination, and this response was boosted by a second vaccination. Liposomes and CNE17 formulations were similarly effective in this model, boosting F-specific IgG titers to 1 µg replicon RNA approximately 8-fold and neutralization titers by 4-10-fold (CNE17 and Liposomes, respectively) after the second vaccination. All replicon RNA vaccines provided protection from a nasal RSV challenge, reducing the lung viral load great than 3 order of magnitude when measured 5 days later. The magnitude and protective capacity of the immune response generated by 1 µg replicon RNA formulated with Liposomes was within 2-fold the response elicited by  $5 \times 10^6$  VRPs. The alum adjuvanted trimer subunit elicited the highest total anti-F IgG ELISA titers, elicited the highest neutralization titers, and elicited the greatest degree of protection from RSV titers in the lung on challenge of any of the vaccine preparations tested in this study.

### Example 7B - RSV-F immunogenicity study (10-1001)

The A317 replicon that expresses the surface fusion glycoprotein of RSV (RSV-F) was used for this experiment. BALB/c mice, 10 animals per group, were given bilateral intramuscular vaccinations (50 µL per leg) on days 0 and 21 with VRP's expressing RSV-F ( $1 \times 10^6$  IU), naked self-replicating RNA (A317, 1 µg), self-replicating RNA delivered using electroporation (A317, 10 µg), self-replicating RNA formulated in liposomes [RV01(01), A317, 0.1 µg or 1 µg) and self-replicating RNA formulated with CNE17 (A317, 0.1 µg or 1 µg) . Serum was collected for antibody analysis on days 14 (2wp1), 35 (2wp2) and 49 (4wp2). Spleens were harvested from 5 mice per group at day 49 (4wp2) for T cell analysis.

## Results

Table 11: F-specific serum IgG titers on day 14

	1 µg A317	0.1 µg RV01(01)	1 µg RV01(01)	0.1 µg CNE17	1 µg CNE17	10 µg A317 + EP	1E6 IU VRP
	529	14385	19299	2429	3373	5	6041
	1530	10713	19170	2060	4417	88	4912
	2734	12756	13860	2012	1927	964	12923
	2503	11546	17352	1887	3597	7235	7075
	5539	15300	22094	3174	5731	2558	6829
	1033	14072	21213	3904	2852	5105	4885
	5110	18274	17915	1481	3739	9806	3680
	1106	7873	15659	2345	4904	2787	9813
	1493	17175	6669	3084	3824	2576	8631
	3456	19730	13259	2497	3004	1858	6314
<b>GMT</b>	<b>1980</b>	<b>13731</b>	<b>15903</b>	<b>2398</b>	<b>3590</b>	<b>1180</b>	<b>6685</b>

Table 11. (10-1001) F-specific serum IgG titers of mice, 10 animals per group, 14 days after intramuscular vaccination. Data are represented as titers for individual mice and the geometric mean titers of 10 individual mice per group. If an individual animal had a titer of <25 (limit of detection) it was assigned a titer of 5.

Table 12: F-specific serum IgG titers on day 35

	1 µg A317	0.1 µg RV01(01)	1 µg RV01(01)	0.1 µg CNE17	1 µg CNE17	10 µg A317 + EP	1E6 IU VRP
	958	128208	227021	48079	8473	14612	813045
	12518	191729	212911	17589	58556	22805	365485
	4839	315786	303987	8522	12053	32156	961601
	10128	218147	335071	10985	20395	24090	349215
	18451	225622	155893	30801	51514	31053	297526
	9805	182693	519162	13372	26348	18105	207652
	19154	185342	169332	5137	80686	23918	1580066
	4490	82744	489441	47173	21014	9091	900889
	14674	190010	131361	78232	61076	21006	822285
	15223	553164	254500	24135	25499	9835	587121
<b>GMT</b>	<b>8532</b>	<b>201892</b>	<b>253687</b>	<b>20767</b>	<b>29111</b>	<b>19117</b>	<b>579033</b>

Table 12. (10-1001) F-specific serum IgG titers of mice, 10 animals per group, after intramuscular vaccinations on days 0 and 21. Serum was collected for antibody analysis on day 35 (2wp2). Data are represented as titers for individual mice and the geometric mean titers of 10 individual mice per group. If an individual animal had a titer of <25 (limit of detection) it was assigned a titer of 5.

Table 13: F-specific serum IgG titers on day 49

	1 µg A317	0.1 µg RV01(01)	1 µg RV01(01)	0.1 µg CNE17	1 µg CNE17	10 µg A317 + EP	1E6 IU VRP
	1248	140407	133787	52747	34245	30388	366771
	12441	155669	182995	29352	128030	20768	209400
	4967	203059	211020	10857	17016	53763	360615
	14536	134253	488698	28800	57250	28373	191475
	31556	370726	158816	44613	76576	34318	139148
	13815	184738	185184	20314	42357	35736	63839
	20495	141644	103026	4546	101445	34611	192101
	4800	76711	312096	27476	47285	10138	177858
	19159	143275	139811	68386	55865	23958	130218
	26836	479594	230331	24360	52871	13624	174378
<b>GMT</b>	<b>10947</b>	<b>177168</b>	<b>194350</b>	<b>24891</b>	<b>53615</b>	<b>25888</b>	<b>180420</b>

5 Table 13. (10-1001) F-specific serum IgG titers of mice, 10 animals per group, after intramuscular vaccinations on days 0 and 21. Serum was collected for antibody analysis on days 49 (4wp2). Data are represented as titers for individual mice and the geometric mean titers of 10 individual mice per group. If an individual animal had a titer of <25 (limit of detection) it was assigned a titer of 5.

Table 14: RSV serum neutralization titers on day 35

A317, 1µg	RV01(01) 0.1µg	RV01(01) 1µg	CNE17 0.1µg	CNE17 1µg	VRP 1E6 IU
2wp2	2wp2	2wp2	2wp2	2wp2	2wp2
NA	143	106	NA	NA	265
NA	272	62	NA	NA	73
NA	294	<40	NA	NA	77
NA	814	334	NA	NA	140
NA	67	86	NA	NA	290
NA	420	125	NA	NA	134
NA	<40	566	NA	NA	466
NA	104	292	NA	NA	127
NA	241	<40	NA	NA	75
NA	223	44	NA	NA	77
<b>GMT</b>	<b>NA</b>	<b>176</b>	<b>96</b>	<b>NA</b>	<b>139</b>

10 Table 14: (10-1001) RSV serum neutralization titers of mice, 10 animals per group, after intramuscular vaccinations on days 0 and 21. Serum was collected for analysis on day 35 (2wp2). Data are represented as 60% plaque reduction neutralization titers of individual mice and the geometric mean titer of 10 individual mice per group. If an



individual animal had a titer of <40 (limit of detection) it was assigned a titer of 20.  
NA = not assayed.

Table 15: RSV serum neutralization titers on day 49

	A317, 1µg	RV01(01) 0.1µg	RV01(01) 1µg	CNE17 0.1µg	CNE17 1µg	VRP 1E6 IU
	4wp2	4wp2	4wp2	4wp2	4wp2	4wp2
	<40	194	82	<40	<40	161
	<40	272	165	<40	70	64
	<40	142	<40	<40	<40	126
	<40	881	442	<40	76	151
	<40	61	108	42	57	194
	<40	426	156	52	<40	123
	<40	78	814	<40	<40	1033
	<40	<40	291	173	<40	174
	<40	246	103	<40	<40	122
	<40	574	396	<40	<40	76
<b>GMT</b>	<b>&lt;40</b>	<b>231</b>	<b>215</b>	<b>29</b>	<b>29</b>	<b>155</b>

- 5 Table 15: (10-1001) RSV serum neutralization titers of mice, 10 animals per group, after intramuscular vaccinations on days 0 and 21. Serum was collected for analysis on day 49 (4wp2). Data are represented as 60% plaque reduction neutralization titers of individual mice and the geometric mean titer of 10 individual mice per group. If an individual animal had a titer of <40 (limit of detection) it was assigned a titer of 20.
- 10 NA = not assayed.

Table 16: T cell responses at day 49

4wp2 splenic CD4 T cell responses	CD4+CD8- splenic T cells: % cytokine-positive and specific for RSV F51-66 peptide			
	IFN $\gamma$ +	IL2+	IL5+	TNF $\alpha$ +
VRP 1E6 IU	<b>0.07</b> $\pm$ 0.06	0.04 $\pm$ 0.05	0.00 $\pm$ 0.02	<b>0.10</b> $\pm$ 0.04
1 µg A317	0.00 $\pm$ 0.05	<b>0.05</b> $\pm$ 0.04	0.00 $\pm$ 0.01	<b>0.03</b> $\pm$ 0.02
RV01(01) 1 µg	0.04 $\pm$ 0.06	<b>0.07</b> $\pm$ 0.05	0.00 $\pm$ 0.01	<b>0.09</b> $\pm$ 0.03
RV01(01) 0.1 µg	<b>0.06</b> $\pm$ 0.05	<b>0.08</b> $\pm$ 0.04	0.00 $\pm$ 0.01	<b>0.10</b> $\pm$ 0.03
CNE17 1 µg	0.00 $\pm$ 0.05	0.04 $\pm$ 0.04	0.00 $\pm$ 0.01	<b>0.05</b> $\pm$ 0.02
CNE17 0.1 µg	0.00 $\pm$ 0.05	0.02 $\pm$ 0.04	0.00 $\pm$ 0.01	<b>0.02</b> $\pm$ 0.02
10 µg vA317 + EP	0.02 $\pm$ 0.06	<b>0.04</b> $\pm$ 0.04	0.01 $\pm$ 0.01	<b>0.05</b> $\pm$ 0.03
none	0.04 $\pm$ 0.06	0.00 $\pm$ 0.05	0.00 $\pm$ 0.02	0.00 $\pm$ 0.01

Table 16. (10-1001) Frequencies of RSV F-specific CD4+ splenic T cells on day 49 (4wp2). Shown are net (antigen-specific) cytokine-positive frequency (%)  $\pm$  95%

confidence half-interval. Net frequencies shown in **bold** indicate stimulated responses that were statistically significantly > 0.

Table 17: T cell responses at day 49

4wp2 splenic CD8 T cell responses	CD8+CD4- splenic T cells: % cytokine-positive and specific for RSV F peptides F85-93 and F249-258			
	IFN $\gamma$ +	IL2+	IL5+	TNF $\alpha$ +
VRP 1E6 IU	<b>3.48</b> $\pm$ 0.29	<b>1.21</b> $\pm$ 0.18	-0.03 $\pm$ 0.05	<b>3.31</b> $\pm$ 0.28
1 $\mu$ g A317	<b>0.74</b> $\pm$ 0.15	<b>0.46</b> $\pm$ 0.11	-0.03 $\pm$ 0.04	<b>0.70</b> $\pm$ 0.14
RV01(01) 1 $\mu$ g	<b>3.69</b> $\pm$ 0.28	<b>1.43</b> $\pm$ 0.18	-0.01 $\pm$ 0.04	<b>3.44</b> $\pm$ 0.27
RV01(01) 0.1 $\mu$ g	<b>2.52</b> $\pm$ 0.23	<b>1.10</b> $\pm$ 0.15	0.03 $\pm$ 0.03	<b>2.31</b> $\pm$ 0.22
CNE17 1 $\mu$ g	<b>1.25</b> $\pm$ 0.17	<b>0.60</b> $\pm$ 0.12	0.01 $\pm$ 0.03	<b>1.15</b> $\pm$ 0.16
CNE17 0.1 $\mu$ g	<b>0.89</b> $\pm$ 0.15	<b>0.49</b> $\pm$ 0.11	-0.03 $\pm$ 0.04	<b>0.83</b> $\pm$ 0.14
10 $\mu$ g A317 + EP	<b>0.85</b> $\pm$ 0.15	<b>0.53</b> $\pm$ 0.11	0.01 $\pm$ 0.04	<b>0.72</b> $\pm$ 0.15
none	0.01 $\pm$ 0.07	0.00 $\pm$ 0.05	-0.02 $\pm$ 0.05	0.02 $\pm$ 0.06

- 5 Table 17. (10-1001) Frequencies of RSV F-specific CD8+ splenic T cells on day 49 (4wp2). Shown are net (antigen-specific) cytokine-positive frequency (%)  $\pm$  95% confidence half-interval. Net frequencies shown in **bold** indicate stimulated responses that were statistically significantly > 0.

## 10 Conclusions

- Liposome formulation significantly enhanced immunogenicity, as determined by increased F-specific IgG titers (8-30-fold increase), neutralization titers, and CD4 and CD8 T cell responses, relative to the naked RNA control. Surprisingly, the F-specific IgG titers and neutralization titers for RV01(01) at both the 0.1 and 1.0  $\mu$ g doses were equivalent to the VRP ( $1 \times 10^6$  IU). T cell responses for the LNP formulation were equivalent at the higher dose to the VRP ( $1 \times 10^6$  IU). Formulation of the self-replicating RNA with CNE17 significantly enhanced immunogenicity, as determined by increased F-specific IgG titers (2-5-fold increase), neutralization titers, and CD4 and CD8 T cell responses, relative to the naked RNA control. Electroporation of RNA enhanced immunogenicity relative to the naked RNA control, but was significantly lower than Liposome delivery.

### Example 7C - RSV-F immunogenicity study (10-1018)

- The A317 replicon that expresses the surface fusion glycoprotein of RSV (RSV-F) was used for this experiment. BALB/c mice, 8 animals per group, were given bilateral intramuscular vaccinations (50  $\mu$ L per leg) on days 0 and 21 with VRP's

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expressing RSV-F ( $1 \times 10^6$  IU), naked self-replicating RNA (A306, 1, 0.1, 0.01  $\mu\text{g}$ ) and self-replicating RNA formulated in liposomes (RV01(01) using method 1 (A317, 10.0, 1.0, 0.1, 0.01  $\mu\text{g}$ ). Serum was collected for antibody analysis on days 14 (2wp1) and (2wp2). Spleens were harvested from 5 mice per group at day 49 (4wp2) for T cell analysis.

### Results

RV01(01) liposome formulation had a Z average particle diameter of 158 nm with a polydispersity index of 0.14, the encapsulation efficiency was 96%. F-specific serum IgG titers on day 14 and 35 are shown in tables 18 and 19 and T cell responses at day 49 are shown in tables 20 and 21.

Table 18: F-specific serum IgG titers on day 14 and 35

	VRP 1E6 IU		A317					
			1 µg		0.1 µg		0.01 µg	
	2wp1	2wp2	2wp1	2wp2	2wp1	2wp2	2wp1	2wp2
	6334	39539	772	4687	5	2334	143	1377
	1500	14895	5	142	5	161	5	333
	5450	38252	109	2972	5	145	5	5
	1835	12831	5	3674	5	97	5	5
	2277	30326	5	5003	5	1077	5	175
	2818	33437	663	8258	221	457	5	5
	2405	22551	257	845	5	1558	5	456
2137	19179	5	1765	5	5	5	180	
GMT	2735	24427	41	2144	8	259	8	73

Table 18: (10-1018) F-specific serum IgG titers of mice, 8 animals per group, after intramuscular vaccinations on days 0 and 21. Serum was collected for antibody analysis on days 14 (2wp1) and 35 (2wp2). Data are represented as individual mice and the geometric mean titers of 8 individual cotton rats per group. If an individual animal had a titer of <25 (limit of detection) it was assigned a titer of 5.

Table 19: F-specific serum IgG titers on day 14 and 35

RV01(01)								
10 µg		1 µg		0.1 µg		0.01 µg		
2wp1	2wp2	2wp1	2wp2	2wp1	2wp2	2wp1	2wp2	
5880	82689	7255	45018	4072	22174	619	2872	
6126	42529	3009	22288	3974	27730	474	3603	
8069	76263	5385	23561	3272	15328	914	2692	
5966	108234	4148	53675	3968	24456	2011	11542	
8590	57912	4210	37004	4950	13014	903	4684	
7172	74162	2179	24179	2856	13694	1575	6720	
8072	122796	1640	5994	4073	17849	438	16514	
8706	83601	5725	28760	3797	17342	1058	13665	
<i>GMT</i>	<i>7235</i>	<i>77338</i>	<i>3783</i>	<i>25790</i>	<i>3826</i>	<i>18325</i>	<i>879</i>	<i>6235</i>

Table 19: Continued from 23A. (10-1018) F-specific serum IgG titers of mice, 8 animals per group, after intramuscular vaccinations on days 0 and 21. Serum was collected for antibody analysis on days 14 (2wp1) and 35 (2wp2). Data are represented as individual animals and the geometric mean titers (GMT) of 8 individual cotton rats per group. If an individual animal had a titer of <25 (limit of detection) it was assigned a titer of 5.

Table 20: T cell responses at day 49

4wp2 splenic T cell responses	CD4+CD8- splenic T cells: % cytokine-positive and specific for RSV F51-66 peptide			
	IFN $\gamma$ +	IL2+	IL5+	TNF $\alpha$ +
VRP 1E6 IU	0.00 $\pm$ 0.02	<b>0.07</b> $\pm$ 0.02	0.00 $\pm$ 0.01	<b>0.07</b> $\pm$ 0.03
1 µg A317	0.01 $\pm$ 0.01	<b>0.03</b> $\pm$ 0.02	0.00 $\pm$ 0.01	<b>0.03</b> $\pm$ 0.02
0.1 µg A317	0.00 $\pm$ 0.01	0.01 $\pm$ 0.01	0.00 $\pm$ 0.00	<b>0.01</b> $\pm$ 0.01
0.01 µg A317	0.00 $\pm$ 0.00	<b>0.01</b> $\pm$ 0.01	0.01 $\pm$ 0.01	0.00 $\pm$ 0.01
RV01(01), 10 µg	<b>0.02</b> $\pm$ 0.01	<b>0.05</b> $\pm$ 0.02	0.00 $\pm$ 0.00	<b>0.06</b> $\pm$ 0.02
RV01(01), 1 µg	<b>0.03</b> $\pm$ 0.02	<b>0.08</b> $\pm$ 0.02	0.00 $\pm$ 0.01	<b>0.09</b> $\pm$ 0.02
RV01(01), 0.1 µg	<b>0.02</b> $\pm$ 0.01	<b>0.03</b> $\pm$ 0.01	0.00 $\pm$ 0.01	<b>0.03</b> $\pm$ 0.02
RV01(01), 0.01 µg	0.00 $\pm$ 0.00	<b>0.02</b> $\pm$ 0.02	0.01 $\pm$ 0.01	<b>0.02</b> $\pm$ 0.02
none	0.00 $\pm$ 0.00	0.00 $\pm$ 0.01	0.00 $\pm$ 0.01	0.01 $\pm$ 0.01

Table 20. Frequencies of RSV F-specific CD4+ splenic T cells on day 49 (Expt. 10-1018, 4wp2). Shown are net (antigen-specific) cytokine-positive frequency (%)  $\pm$  95% confidence half-interval. Net frequencies shown in **bold** indicate stimulated responses that were statistically significantly > 0.

Table 21: T cell responses at day 49

4wp2 splenic T cell responses	CD8+CD4- splenic T cells: % cytokine-positive and specific for RSV F peptides F85-93 and F249-258			
	IFN $\gamma$ +	IL2+	IL5+	TNF $\alpha$ +
VRP 1E6 IU	<b>2.45</b> $\pm$ 0.21	<b>0.58</b> $\pm$ 0.10	0.00 $\pm$ 0.01	<b>2.64</b> $\pm$ 0.21
1 $\mu$ g A317	<b>1.68</b> $\pm$ 0.17	<b>0.45</b> $\pm$ 0.09	0.00 $\pm$ 0.02	<b>1.75</b> $\pm$ 0.18
0.1 $\mu$ g A317	<b>0.21</b> $\pm$ 0.07	<b>0.08</b> $\pm$ 0.04	0.01 $\pm$ 0.02	<b>0.30</b> $\pm$ 0.08
0.01 $\mu$ g A317	<b>0.06</b> $\pm$ 0.05	<b>0.05</b> $\pm$ 0.03	0.01 $\pm$ 0.02	<b>0.16</b> $\pm$ 0.06
RV01(01), 10 $\mu$ g	<b>3.32</b> $\pm$ 0.23	<b>0.69</b> $\pm$ 0.11	0.00 $\pm$ 0.02	<b>3.90</b> $\pm$ 0.25
RV01(01), 1 $\mu$ g	<b>1.81</b> $\pm$ 0.17	<b>0.59</b> $\pm$ 0.10	0.00 $\pm$ 0.02	<b>2.04</b> $\pm$ 0.20
RV01(01), 0.1 $\mu$ g	<b>0.91</b> $\pm$ 0.12	<b>0.32</b> $\pm$ 0.07	0.00 $\pm$ 0.01	<b>1.06</b> $\pm$ 0.14
RV01(01), 0.01 $\mu$ g	<b>0.58</b> $\pm$ 0.10	<b>0.33</b> $\pm$ 0.08	0.00 $\pm$ 0.01	<b>0.64</b> $\pm$ 0.11
none	0.01 $\pm$ 0.02	0.01 $\pm$ 0.01	0.00 $\pm$ 0.01	0.00 $\pm$ 0.05

Table 21. F-specific splenic CD8<sup>+</sup> T cell frequencies on day 49 (Expt. 10-1018, 4wp2). Shown are net (antigen-specific) cytokine-positive frequency (%)  $\pm$  95% confidence half-interval. Net frequencies shown in **bold** indicate stimulated responses that were statistically significantly > 0.

### Conclusions

Liposome formulation significantly enhanced immunogenicity, as determined by increased F-specific IgG titers and T cell frequencies, relative to the naked RNA controls. The F-specific IgG titers and CD8 T cell frequencies for RV01(01) at the 10  $\mu$ g RNA dose were enhanced relative to the VRP group (1x10<sup>6</sup> IU).

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- 20 132. WO95/17210.
133. WO96/26741.
134. WO93/19780.
135. WO03/011223.
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138. US Patent No. 6,586,409.

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140. US2005/0215517.

The entire teachings of all documents cited herein are hereby incorporated  
5 herein by reference.

## CLAIMS

What is claimed is:

1. An immunogenic composition comprising one or more respiratory syncytial virus F (RSV F) polypeptides in which amino acids 100-150 are replaced with the amino acid sequence of SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7; SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:91 or SEQ ID NO:92.
2. The immunogenic composition of claim 1, wherein amino acids 100-150 of said RSV F are replaced with the amino acid sequence of SEQ ID NO:12.
3. The immunogenic composition of claim 1, wherein amino acids 100-150 of said RSV F are replaced with the amino acid sequence of SEQ ID NO:9, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7; SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13 or SEQ ID NO:92.
4. The immunogenic composition of claim 1, wherein amino acids 100-150 are replaced with the amino acid sequence of SEQ ID NO:9.
5. The immunogenic composition of claim 1 or claim 2, wherein the RSV F is in the form of a monomer, trimer, or combination of monomers and trimers.
6. The immunogenic composition of claim 3 or 4, wherein RSV F is in the form of a monomer, trimer, rosette or any combination thereof.
7. The immunogenic composition of any one of claims 1-6, wherein said RSV F polypeptide is a soluble polypeptide comprising the RSV-F ectodomain.
8. The immunogenic composition of any one of claims 1-6, wherein said RSV F comprises amino acids 23-99 and 151-524 of SEQ ID NO:1 or SEQ ID NO:2.

9. An immunogenic composition comprising a polypeptide selected from the group consisting of SEQ ID NO:49, SEQ ID NO:68, SEQ ID NO:71, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, 5 SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID 10 NO:67, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:93, any of the foregoing sequences in which the signal peptide and/or HIS tag is omitted, and combinations thereof.

15 10. The immunogenic composition of claim 9, wherein said polypeptide is SEQ ID NO:68 or SEQ ID NO:68 in which the signal peptide, and optionally the HIS tag, is omitted.

20 11. The immunogenic composition of claim 9, wherein said polypeptide is selected from the group consisting of SEQ ID NO:49, SEQ ID NO:71, and any of the foregoing sequences in which the signal peptide, and optionally the HIS tag, is omitted.

25 12. The immunogenic composition of any one of claims 1-11, further comprising an adjuvant.

30 13. The immunogenic composition of claim 12, wherein the adjuvant is selected from the group consisting of: an aluminum salt, a squalene-in-water emulsion, a benzonaphthylridine compound, a phospholipid compound, a small molecule immunopotentiator and combinations of any of the foregoing.

14. A recombinant respiratory syncytial virus F polypeptide (RSV F) in which amino acids 100-150 are replaced with the amino acid sequence of SEQ ID

NO:9, SEQ ID NO:12, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:91 or SEQ ID NO:92.

5           15.     The recombinant RSV F of claim 14, wherein amino acids 100-150 of said RSV F are replaced with the amino acid sequence of SEQ ID NO:12.

            16.     The recombinant RSV F of claim 15, wherein amino acids 100-150 of said RSV F are replaced with the amino acid sequence of SEQ ID NO:9, SEQ ID NO:3,  
10   SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7; SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13 or SEQ ID NO:92.

            17.     The recombinant RSV F of claim 14, wherein amino acids 100-150 are replaced with the amino acid sequence of SEQ ID NO:9.

15

            18.     The recombinant RSV F of claim 14 or 15, wherein the RSV F is in the form of a monomer, trimer, or combination of monomers and trimers.

            19.     The recombinant RSV F of claim 16 or 17, wherein the RSV F is in the  
20   form of a monomer, trimer, rosette of trimers or combinations thereof.

            20.     A recombinant polypeptide selected from the group consisting of SEQ ID NO:49, SEQ ID NO:68, SEQ ID NO:71, SEQ ID NO: 25, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:93, any of the foregoing

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sequences in which the signal peptide and/or HIS tag is omitted, and combinations thereof.

21. The recombinant polypeptide of any one of claims 14-20, further  
5 comprising a heterologous oligomerization domain, an epitope, or a signal peptide.

22. The recombinant polypeptide of claim 21, wherein said heterologous  
oligomerization domain is selected from the group comprising a trimerization domain  
from influenza hemagglutinin, from SARS spike, or from HIV gp41, NadA, modified  
10 GCN4, or ATCase.

23. An isolated nucleic acid encoding the polypeptide of any of claims 14-  
21.

15 24. The isolated nucleic acid of claim 23, which is a self-replicating RNA  
molecule.

25. An immunogenic composition comprising the self-replicating RNA  
molecule of claim 24.  
20

26. The immunogenic composition of claim 25, further comprising a  
delivery system.

27. A method of inducing an immune response in a subject to RSV F  
25 comprising administering an immunogenic composition of any one of claims 1-14 to  
the subject.

28. A method of inducing an immune response in a subject to RSV F  
comprising administering an immunogenic composition of claim 25 to the subject.  
30

29. A method for producing a composition comprising cleaved RSV F  
protein ecto-domain polypeptides, the method comprising:



a) providing uncleaved RSV F protein ecto-domain polypeptides that contain a protease cleavage site that when cleaved produces F<sub>1</sub> and F<sub>2</sub> subunits, the amino acid sequence of said uncleaved soluble RSV F protein ecto-domain polypeptides comprising altered furin cleavage sites at positions 106-109 and 133-136, and said uncleaved soluble RSV F protein ecto-domain polypeptides are obtained from a cell that produces them uncleaved; and

b) cleaving the provided soluble RSV F protein ecto-domain polypeptides with a protease that cleaves said protease cleavage site, thereby producing a composition comprising cleaved soluble RSV F protein ecto-domain polypeptides.

10

30. The method of claim 29, wherein in step b) the provided uncleaved RSV F protein ecto-domain polypeptides are cleaved at one or more of positions from about position 101 to about position 161.

15 31. The method of claim 30, wherein the provided uncleaved RSV F protein ecto-domain polypeptides are cleaved at one or more of positions from about position 106 to about position 142.

20 32. The method of any one of claims 29-32, wherein the uncleaved RSV F protein ecto-domain polypeptides provided in a) are purified.

33. The method of any one of claims 29-32, wherein the uncleaved RSV F protein ecto-domain polypeptides provided in a) comprise an intact fusion peptide.

25 34. The method of claim 33, wherein the cleaving in step b) results in the formation of rosettes of trimers.

35. The method of claim 34, further comprising c) purifying said rosettes of trimers.

30 36. The method of claims 35, wherein purifying the rosettes of trimers comprises size exclusion chromatography.

37. The method of any one of claims 29-32, wherein the uncleaved RSV F protein ecto-domain polypeptides provided in a) comprise an altered fusion peptide.
38. The method of claim 37, wherein at least a portion of the fusion peptide is  
5 deleted in the uncleaved RSV F protein ecto-domain polypeptides.
39. The method of claim 37, wherein amino acids 137-153, amino acids 137-146, amino acids 137-145, or amino acids 137-142 are deleted from the fusion peptide.
- 10 40. The method of any one of claims 37-39, wherein the cleaving in step b) results in the formation of trimers.
41. The method of claim 40, further comprising c) purifying said trimers.
- 15 42. The method of claims 41, wherein purifying the trimers comprises size exclusion chromatography.
43. The method of any one of claims 29-42, wherein the uncleaved RSV F protein ecto-domain polypeptides provided in a) are expressed in insect cells, mammalian cells,  
20 avian cells, yeast cells, Tetrahymena cells or combinations thereof.
44. The method of claims 29-43, wherein the uncleaved RSV F protein ecto-domain polypeptides provided in a) are provided in cell culture conditioned media.
- 25 45. The method of claim 44, wherein said cell culture conditioned media is selected from the group consisting of insect cell culture conditioned media, mammalian cell culture conditioned media and combinations thereof.
46. The method of any one of claims 29-36 and 41, wherein the rosettes of trimers  
30 comprise F<sub>1</sub> and F<sub>2</sub> fragments of at least one polypeptide selected from the group consisting of Furdel and Delp23 furdel.

47. The method of any one of claims 29-46, wherein the cleaved RSV F protein ecto-domain polypeptides are substantially free of lipids and lipoproteins.
48. A composition comprising cleaved RSV F protein ecto-domain polypeptides  
5 produced using the method of any one of claims 29-47.
49. The composition of claim 48, wherein the composition is an immunogenic composition.
- 10 50. A method for producing a composition comprising uncleaved RSV F protein ecto-domain polypeptide monomers, trimers or a combination of monomers and trimers, the method comprising:
- a) providing a biological material that contains uncleaved RSV F protein ecto-domain polypeptides, the amino acid sequence of said uncleaved soluble RSV F protein  
15 ecto-domain polypeptides comprising altered furin cleavage sites at positions 106-109 and 133-136, and said uncleaved soluble RSV F protein ecto-domain polypeptides are secreted from a cell that produces them uncleaved; and
- b) purifying uncleaved RSV F protein ecto-domain polypeptide monomers or trimers from the biological material, thereby producing the composition.  
20
51. The method of claim 50, wherein the biological material is selected from the group consisting of insect cell culture conditioned medium or cell lysate, mammalian cell culture conditioned medium or cell lysate, avian cell culture conditioned medium or cell lysate, yeast cell conditioned medium or cell lysate, Tetrahymena cell culture  
25 conditioned medium or cell lysate, and combinations thereof.
52. The method of claim 50 or 51, wherein in b) uncleaved RSV F protein ecto-domain polypeptide trimers are purified.
- 30 53. The method of claim 50 or 51, wherein in b) uncleaved RSV F protein ecto-domain polypeptide monomers are purified.

54. The method of claim 50, wherein purifying in b) comprises size exclusion chromatography.
55. The method of any one of claims 50-54, wherein the uncleaved RSV F protein  
5 ecto-domain polypeptides further comprise an altered fusion peptide.
56. The method of claim 50, wherein said soluble uncleaved RSV F protein ecto-  
domain polypeptides comprise at least one polypeptide selected from the group  
consisting of furmt, furdel, Delp21 furx, Delp23 furx, Delp21 furdel, Delp23 furdel,  
10 and the factor Xa construct.
57. The method of any one of claims 50-56, wherein the composition comprising  
uncleaved RSV F protein ecto-domain polypeptides is substantially free of lipids and  
lipoproteins.  
15
58. A composition comprising soluble uncleaved RSV F protein ecto-domain  
polypeptide monomers or trimers produced using the method of any one of claims 50-  
57.
- 20 59. The composition of claim 58, wherein the composition is an immunogenic  
composition.
60. A method for producing a composition comprising uncleaved RSV F protein  
ecto-domain polypeptide monomers, trimers or a combination of monomers and  
25 trimers, the method comprising:  
a) providing a biological material that contains uncleaved RSV F protein ecto-domain  
polypeptides, wherein the amino acid sequence of the uncleaved RSV F protein ecto-  
domain polypeptide contains altered furin cleavage sites, lysine and arginine residues  
present between about position 101 and about position 161 are deleted or replaced with  
30 an amino acid that is not lysine or arginine, the RSV F protein ecto-domain  
polypeptides are obtained from a host cell that produces them uncleaved between about

position 101 and about position 161, and the RSV F protein ecto-domain polypeptides are not cleaved between about position 101 and about position 161; and

b) purifying uncleaved RSV F protein ecto-domain polypeptide monomers, trimers or a combination of monomers and trimers from the biological material.

5

61. The method of claim 60, wherein the biological material is selected from the group consisting of insect cell culture conditioned medium or cell lysate, mammalian cell culture conditioned medium or cell lysate, avian cell culture conditioned medium or cell lysate, yeast cell conditioned medium or cell lysate, Tetrahymena cell culture  
10 conditioned medium or cell lysate, and combinations thereof.

62. The method of claim 60 or 61, wherein in b) uncleaved RSV F protein ecto-domain polypeptide trimers are purified.

15 63. The method of claim 60 or 61, wherein in b) uncleaved RSV F protein ecto-domain polypeptide monomers are purified.

64. The method of claim 60 or 61, wherein in b) uncleaved RSV F protein ecto-domain polypeptide monomers and uncleaved RSV F protein ecto-domain polypeptide  
20 trimers are purified.

65. The method of any one of claims 60-64, wherein purifying in b) comprises size exclusion chromatography.

25 66. The method of any one of claims 60-64, wherein the uncleaved RSV F protein ecto-domain polypeptides further comprise an altered fusion peptide.

67. The method of claim 60, wherein said uncleaved RSV F protein ecto-domain polypeptides comprise at least one polypeptide selected from the group consisting of  
30 Furx, Furx R113Q K123N K124N, Delp21 furx and Delp23 furx.

68. The method of any one of claims 60-67, wherein the composition comprising uncleaved RSV F protein ecto-domain polypeptides is substantially free of lipids and lipoproteins.
- 5 69. A composition comprising soluble uncleaved RSV F protein ecto-domain polypeptides produced using the method of any one of claims 60-68.
70. The composition of claim 69, wherein the composition is an immunogenic composition.
- 10 71. A method for producing a composition comprising cleaved RSV F protein ecto-domain polypeptide monomers, trimers or a combination of monomers and trimers, the method comprising:
- 15 a) providing biological material that contains cleaved RSV F protein ecto-domain polypeptides that contain an altered fusion peptide; and
- b) purifying cleaved RSV F protein ecto-domain polypeptides from the biological material.
- 20 72. The method of claim 71, wherein the RSV F protein ecto-domain polypeptide comprises an amino acid sequence in which about amino acids 137-152 are deleted, about amino acids 137-153 are deleted, about amino acids 137-145 are deleted, about amino acids 137-146 are deleted or about amino acids 137-142 are deleted.
- 25 73. The method of claim 71 or 72, wherein the biological material is selected from the group consisting of insect cell culture conditioned medium or cell lysate, mammalian cell culture conditioned medium or cell lysate, avian cell culture conditioned medium or cell lysate, yeast cell conditioned medium or cell lysate, Tetrahymena cell culture conditioned medium or cell lysate, and combinations thereof.
- 30 74. The method of any one of claims 71-73, wherein in b) cleaved RSV F protein ecto-domain polypeptide trimers are purified.

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75. The method of any one of claims 71-73, wherein in b) cleaved RSV F protein ecto-domain polypeptide monomers are purified.

76. The method of any one of claims 71-73, wherein in b) cleaved RSV F protein  
5 ecto-domain polypeptide monomers and cleaved RSV F protein ecto-domain polypeptide trimers are purified.

77. The method of any one of claims 71-73, wherein purifying in b) comprises size exclusion chromatography.

10

78. The method of claim 71, wherein said uncleaved RSV F protein ecto-domain polypeptides comprise at least the Fusion Peptide deletion polypeptide.

79. The method of any one of claims 71-78, wherein the composition comprising  
15 uncleaved RSV F protein ecto-domain polypeptides is substantially free of lipids and lipoproteins.

80. A composition comprising soluble uncleaved RSV F protein ecto-domain polypeptides produced using the method of any one of claims 71-79.

20

81. The composition of claim 80, wherein the composition is an immunogenic composition.

82. A method for producing a composition comprising RSV F protein ecto-domain  
25 polypeptides, the method comprising:

a) providing RSV F protein ecto-domain polypeptides that comprises an altered furin cleavage site at positions 133-136, and said soluble RSV F protein ecto-domain polypeptides are secreted from a cell that produces them in the form of an F<sub>2</sub> fragment that is associated with a subunit that comprises F<sub>1</sub> but is uncleaved at position 136/137;  
30 and

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b) cleaving the provided RSV F protein ecto-domain polypeptides with a protease that cleaves RSV F protein ecto-domain at a site between positions 101 and 161, thereby producing said composition.

5 83. The method of claim 82, wherein the RSV F protein ecto-domain polypeptides provided in a) are purified.

84. The method claim 82 or 83, wherein the RSV F protein ecto-domain polypeptides provided in a) are expressed in insect cells, mammalian cells, avian cells,  
10 yeast cells, Tetrahymena cells or combinations thereof.

85. The method of any one of claims 82-84, wherein the RSV F protein ecto-domain polypeptides provided in a) are provided in cell culture conditioned media, cell extract or a combination thereof.

15

86. The method of claim 85, wherein the RSV F protein ecto-domain polypeptides are provided in cell culture conditioned media is selected from the group consisting of insect cell culture conditioned media, mammalian cell culture conditioned media, avian cell culture conditioned media, yeast cell culture conditioned media, Tetrahymena cell  
20 culture conditioned media and combinations thereof.

87. The method of any one of claims 82-86, wherein the RSV F protein ecto-domain polypeptides include at least the C-term Furin polypeptide.

25 88. The method of any one of claims 82-87, wherein the cleaved RSV F protein ecto-domain polypeptides produced in b) are substantially free of lipids and lipoproteins.

89. A composition comprising cleaved RSV F protein ecto-domain polypeptides  
30 produced using the method of any one of claims 82-88.



90. The composition of claim 89, wherein the composition is an immunogenic composition.

91. A method for producing a composition comprising RSV F protein ecto-domain polypeptides, the method comprising:

- 5 a) providing biological material that contains RSV F protein ecto-domain polypeptides that comprise an altered furin cleavage site at positions 133-136, and said soluble RSV F protein ecto-domain polypeptides are secreted from a cell that produces them in the form of an F<sub>2</sub> fragment that is associated with a subunit that comprises F<sub>1</sub>
- 10 but is uncleaved at position 136/137, with the proviso that the altered furin cleavage site is not deletion of amino acids 131-134; and
- b) purifying the RSV F protein ecto-domain polypeptides from the biological material, thereby producing the composition.

15 92. The method of claim 91, wherein the biological material is selected from the group consisting of insect cell culture conditioned medium or cell lysate, mammalian cell culture conditioned medium or cell lysate, avian cell culture conditioned medium or cell lysate, yeast cell conditioned medium or cell lysate, Tetrahymena cell culture conditioned medium or cell lysate, and combinations thereof.

20

93. The method of claim 91 or 92, wherein in b) RSV F protein ecto-domain polypeptide monomers, trimers or a combination of monomers and trimers are purified.

94. The method of claim 93, wherein purifying in b) comprises size exclusion

25 chromatography.

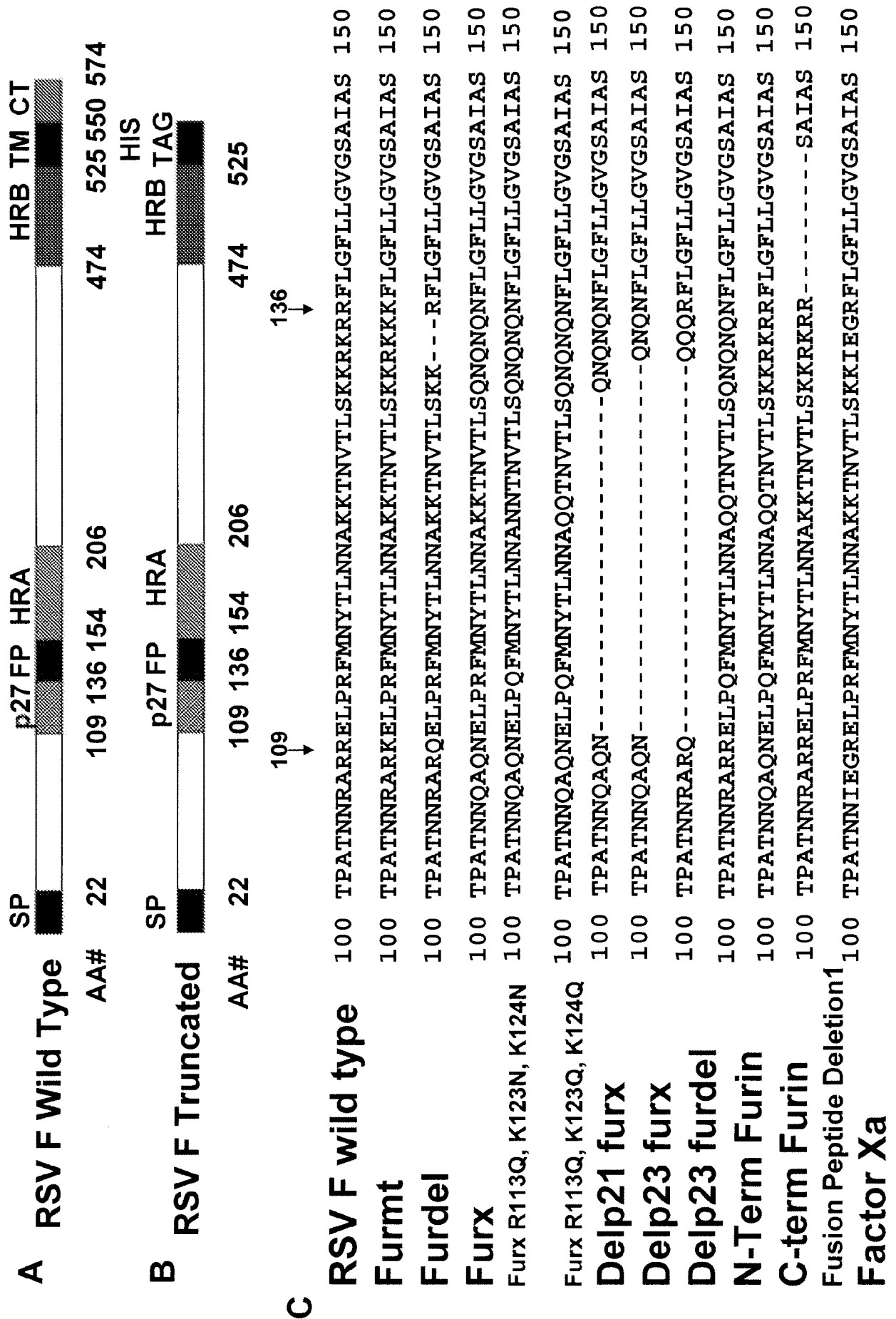
95. The method of any one of claims 91-94, wherein the RSV F protein ecto-domain polypeptides include at least the C-term Furin polypeptide.

30 96. The method of any one of claims 91-95, wherein the composition comprising RSV F protein ecto-domain polypeptides is substantially free of lipids and lipoproteins.

97. A composition comprising RSV F protein ecto-domain polypeptide produced using the method of any one of claims 92-96.

98. The composition of claim 97, wherein the composition is an immunogenic  
5 composition.

Figure 1



# Constructs to Cleave from the Cell Surface

RSV F Wildtype	488	FDASISQVNEKINQSLAFIRKSDLLHNVNAGKSTTN-----	TM-C
Idealized Ent	488	FDASISQINEKINQILAFIRKIDELLHNINAGKSTTNGSGSGDDDDKGGSG-----	TM-C
GCN Ent	488	FDASISQVNEKINQSLAFIRKSDLLHNVNDKIEEILSKIYHIENEIARIKKLIGEGSGDDDDKGGSG	TM-C
Idealized FacXa	488	FDASISQINEKINQILAFIRKIDELLHNINAGKSTTNGSGGIEGRGSG-----	TM-C
GCN Thr	488	FDASISQVNEKINQSLAFIRKSDLLHNVNDKIEEILSKIYHIENEIARIKKLIGEGSGGIEGRGSG	TM-C
Idealized Thr	488	FDASISQINEKINQILAFIRKIDELLHNINAGKSTTNGSGGLVPRGSG-----	TM-C
GCN Thr	488	FDASISQVNEKINQSLAFIRKSDLLHNVNDKIEEILSKIYHIENEIARIKKLIGEGSGGLVPRGSG-	TM-C

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TM-C = Transmembrane domain + C-terminal tail =  
 IMITTTIIIVIIISLIIVGLLYCKARSTPVTLSKDQLSGINNIAFSN  
 Ent = Enterokinase cleavage site - DDDDK  
 FacXa = Factor Xa cleavage site - IEGR  
 Thr = Thrombin cleavage site - LVPR

Figure 2

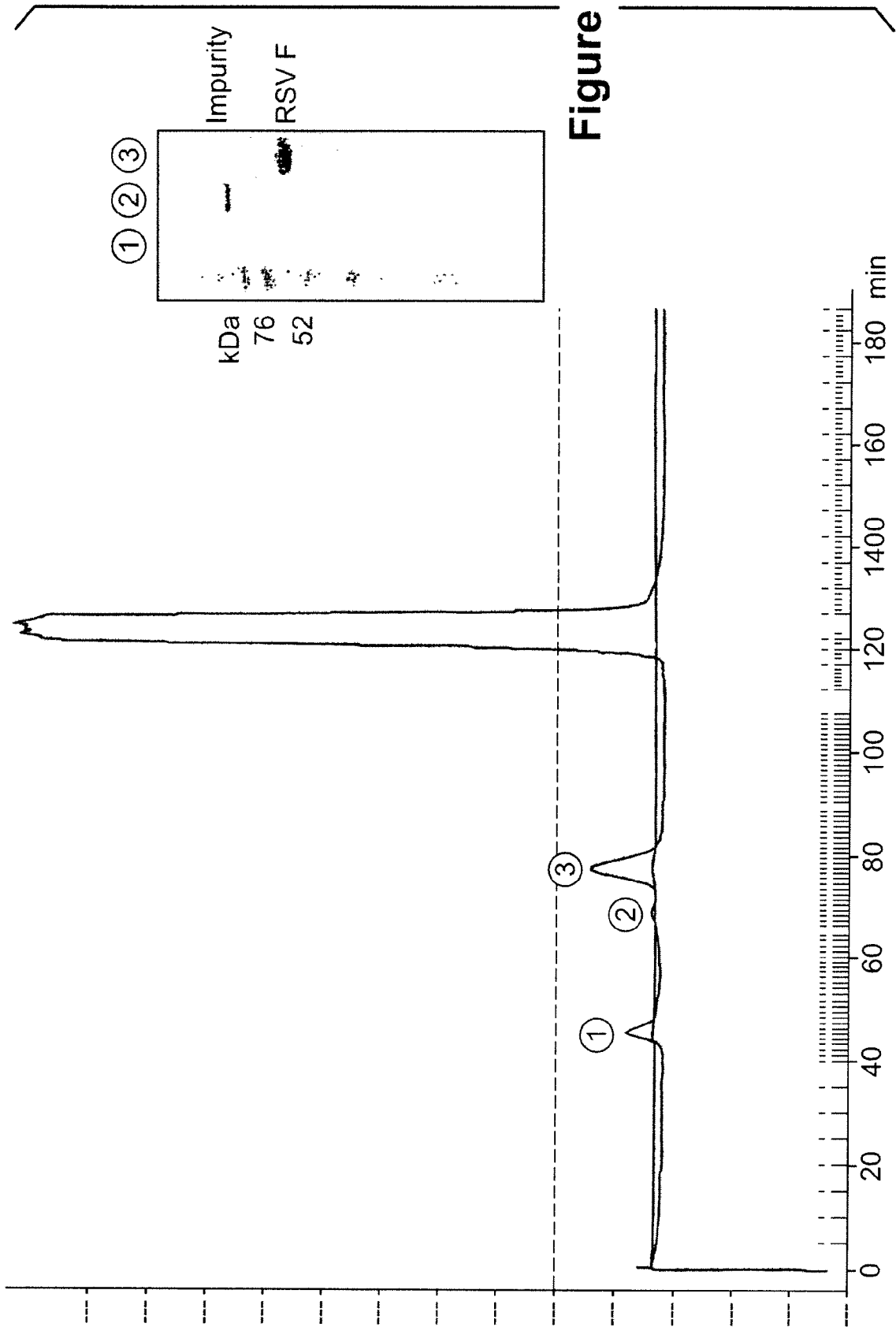


Figure 3

ATAGGCGGCGCATGAGAGAAGCCCAGACCAATTACCTACCCAAAATGGAGAAAGTTCACGTTGACATCGA  
GGAAGACAGCCCATTCTCAGAGCTTTGCAGCGGAGCTTCCCGCAGTTTGAGGTAGAAGCCAAGCAGGTC  
ACTGATAATGACCATGCTAATGCCAGAGCGTTTTTCGCATCTGGCTTCAAAACTGATCGAAACGGAGGTGG  
ACCCATCCGACACGATCCTTGACATTGGAAGTGCGCCCCGCCGAGAATGTATTCTAAGCACAAGTATCA  
TTGTATCTGTCCGATGAGATGTGCGGAAGATCCGGACAGATTGTATAAGTATGCAACTAAGCTGAAGAAA  
AACTGTAAGGAAATAACTGATAAGGAATTGGACAAGAAAAATGAAGGAGCTCGCCGCCGTCTAGAGCGACC  
CTGACCTGGAAACTGAGACTATGTGCCTCCACGACGACGAGTCGTGTGCTACGAAGGGCAAGTCGCTGT  
TTACCAGGATGTATACGCGGTTGACGGACCGACAAGTCTCTATCACCAGCCAATAAGGGAGTTAGAGTC  
GCCTACTGGATAGGCTTTGACACCACCCCTTTTATGTTTAAGAACTTGGCTGGAGCATATCCATCATACT  
CTACCAACTGGGCCGACGAAACCGTGTTAACGGCTCGTAACATAGGCCTATGCAGCTCTGACGTTATGGA  
GCGGTCACGTAGAGGGATGTCCATTCTTAGAAAGAAGTATTTGAAACCATCCAACAATGTTCTATTCTCT  
GTTGGCTCGACCATCTACCACGAGAAGAGGGACTTACTGAGGAGCTGGCACCTGCCGTCTGTATTTCACT  
TACGTGGCAAGCAAAATTACACATGTCGGTGTGAGACTATAGTTAGTTGCGACGGGTACGTCGTTAAAAG  
AATAGCTATCAGTCCAGGCCTGTATGGGAAGCCTTCAGGCTATGCTGCTACGATGCACCGCGAGGGATTC  
TTGTGCTGCAAAGTGACAGACACATTGAACGGGGAGAGGGTCTCTTTTCCCGTGTGCACGTATGTGCCAG  
CTACATTGTGTGACCAAATGACTGGCATACTGGCAACAGATGTCAGTGCGGACGACGCGCAAAAACCTGCT  
GGTTGGGCTCAACCAGCGTATAGTCGTCAACGGTCGCACCCAGAGAAACACCAATACCATGAAAAATTAC  
CTTTTGCCCGTAGTGGCCCAGGCATTTGCTAGGTGGGCAAGGAATATAAGGAAGATCAAGAAGATGAAA  
GGCCACTAGGACTACGAGATAGACAGTTAGTCATGGGGTGTGTTGGGCTTTTAGAAGGCACAAGATAAC  
ATCTATTTATAAGCGCCCGGATACCCAAACCATCATCAAAGTGAACAGCGATTTCCACTCATTCTGTGCTG  
CCCAGGATAGGCAGTAACACATTGGAGATCGGGCTGAGAACAAAGAATCAGGAAAATGTTAGAGGAGCACA  
AGGAGCCGTCACCTCTCATTACCGCCGAGGACGTACAAGAAGCTAAGTGCGCAGCCGATGAGGCTAAGGA  
GGTGCGTGAAGCCGAGGAGTTGCGCGCAGCTCTACCACCTTTGGCAGCTGATGTTGAGGAGCCCACTCTG  
GAAGCCGATGTAGACTTGATGTTACAAGAGGCTGGGGCCGGCTCAGTGGAGACACCTCGTGGCTTGATAA  
AGGTTACCAGCTACGATGGCGAGGACAAGATCGGCTCTTACGCTGTGCTTTCTCCGAGGCTGTACTCAA  
GAGTGAAAAATTATCTTGCATCCACCCTCTCGCTGAACAAGTCATAGTGATAACACACTCTGGCCGAAAA  
GGGCGTTATGCCGTGGAACCATAACCATGGTAAAGTAGTGGTGCCAGAGGGACATGCAATACCCGTCCAGG  
ACTTTCAAGCTCTGAGTGAAAGTGCCACCATTGTGTACAACGAACGTGAGTTCGTAAACAGGTACCTGCA  
CCATATTGCCACACATGGAGGAGCGCTGAACACTGATGAAGAATATTACAAAACCTGTCAAGCCCAGCGAG  
CACGACGGCGAATACCTGTACGACATCGACAGGAAACAGTGCGTCAAGAAAGAACTAGTCACTGGGCTAG  
GGCTCACAGGCGAGCTGGTGGATCCTCCCTTCCATGAATTGCGCTACGAGAGTCTGAGAACACGACCAGC  
CGCTCCTTACCAAGTACCAACCATAGGGGTGTATGGCGTGCCAGGATCAGGCAAGTCTGGCATCATTAAA  
AGCGCAGTCACCAAAAAAGATCTAGTGGTGAGCGCCAAGAAAGAAAACTGTGCAGAAATTATAAGGGACG  
TCAAGAAAAATGAAAGGGCTGGACGTCAATGCCAGAACTGTGGACTCAGTGCTCTTGAATGGATGCAACA  
CCCCGTAGAGACCCTGTATATTGACGAAGCTTTTGCTTGTCTATGCAGGTACTCTCAGAGCGCTCATAGCC

FIG. 4A

ATTATAAGACCTAAAAAGGCAGTGCTCTGCGGGGATCCCAAACAGTGCGGTTTTTTTAACATGATGTGCC  
TGAAAGTGCATTTTAACCACGAGATTGACACACAAGTCTTCCACAAAAGCATCTCTCGCCGTTGCACTAA  
ATCTGTGACTTCGGTCGTCTCAACCTTGTTTTACGACAAAAAATGAGAACGACGAATCCGAAAGAGACT  
AAGATTGTGATTGACACTACCGGCAGTACCAAACCTAAGCAGGACGATCTCATTCTCACTTGTTTCAGAG  
GGTGGGTGAAGCAGTTGCAAATAGATTACAAAGGCAACGAAATAATGACGGCAGCTGCCTCTCAAGGGCT  
GACCCGTAAAGGTGTGTATGCCGTTCCGGTACAAGGTGAATGAAAATCCTCTGTACGCACCCACCTCAGAA  
CATGTGAACGTCCTACTGACCCGCACGGAGGACCGCATCGTGTGGAACAACACTAGCCGGCGACCCATGGA  
TAAAAAACTGACTGCCAAGTACCCTGGGAATTTCACTGCCACGATAGAGGAGTGGCAAGCAGAGCATGA  
TGCCATCATGAGGCACATCTTGGAGAGACCGGACCCTACCGACGTCTTCCAGAATAAGGCAAACGTGTGT  
TGGGCCAAGGCTTTAGTGCCGGTGCTGAAGACCGCTGGCATAGACATGACCACTGAACAATGGAACACTG  
TGGATTATTTTGAAACGGACAAAGCTCACTCAGCAGAGATAGTATTGAACCAACTATGCGTGAGGTTCTT  
TGGACTCGATCTGGACTCCGGTCTATTTTCTGCACCCACTGTTCCGTTATCCATTAGGAATAATCACTGG  
GATAACTCCCCGTCGCCTAACATGTACGGGCTGAATAAAGAAGTGGTCCGTCAGCTCTCTCGCAGGTACC  
CACAACCTGCCTCGGGCAGTTGCCACTGGAAGAGTCTATGACATGAACACTGGTACACTGCGCAATTATGA  
TCCGCGCATAAACCTAGTACCTGTAAACAGAAGACTGCCTCATGCTTTAGTCTCCACCATAATGAACAC  
CCACAGAGTGACTTTTCTTCATTTCGTAGCAAAATGAAGGGCAGAACTGTCTGGTGGTCCGGGAAAAGT  
TGTCGGTCCAGGCAAATGGTTGACTGGTTGTGAGACCGGCCTGAGGCTACCTTCAGAGCTCGGCTGGA  
TTTAGGCATCCCAGGTGATGTGCCCAAATATGACATAATATTTGTTAATGTGAGGACCCCATATAAATAC  
CATCACTATCAGCAGTGTGAAGACCATGCCATTAAGCTTAGCATGTTGACCAAGAAAAGCTTGTCTGCATC  
TGAATCCCGGCGGAACCTGTGTGAGCATAGGTTATGGTTACGCTGACAGGGCCAGCGAAAGCATCATTTGG  
TGCTATAGCGCGGCAGTTCAAGTTTTCCCGGGTATGCAAACCGAAATCCTCACTTGAAGAGACGGAAGTT  
CTGTTTGTATTTCATTGGGTACGATCGCAAGGCCCCGTACGCACAATCCTTACAAGCTTTCATCAACCTTGA  
CCAACATTTATACAGGTTCCAGACTCCACGAAGCCGGATGTGCACCCTCATATCATGTGGTGCAGGGGGA  
TATTGCCACGGCCACCGAAGGAGTGATTATAAATGCTGCTAACAGCAAAGGACAACCTGGCGGAGGGGTG  
TGCGGAGCGCTGTATAAGAAATTCCCGGAAAGCTTCGATTTACAGCCGATCGAAGTAGGAAAAGCGCGAC  
TGGTCAAAGGTGCAGCTAAACATATCATTTCATGCCGTAGGACCAAACCTTCAACAAAGTTTCGGAGGTTGA  
AGGTGACAAACAGTTGGCAGAGGCTTATGAGTCCATCGCTAAGATTGTCAACGATAACAATTACAAGTCA  
GTAGCGATTCCACTGTTGTCCACCGGCATCTTTTCCGGGAACAAAGATCGACTAACCCAATCATTGAACC  
ATTTGCTGACAGCTTTAGACACCACTGATGCAGATGTAGCCATATACTGCAGGGACAAGAAATGGGAAAT  
GACTCTCAAGGAAGCAGTGGCTAGGAGAGAAGCAGTGGAGGAGATATGCATATCCGACGACTCTTCAGTG  
ACAGAACCTGATGCAGAGCTGGTGAGGGTGCATCCGAAGAGTTCTTTGGCTGGAAGGAAGGGCTACAGCA  
CAAGCGATGGCAAAACTTTCTCATATTTGGAAGGGACCAAGTTTACCAGGCGGCCAAGGATATAGCAGA  
AATTAATGCCATGTGGCCCCGTGCAACGGAGGCCAATGAGCAGGTATGCATGTATATCCTCGGAGAAAAGC  
ATGAGCAGTATTAGGTGAAATGCCCCGTGCAAGAGTGGGAAGCCTCCACACCACCTAGCACGCTGCCTT  
GCTTGTGCATCCATGCCATGACTCCAGAAAGAGTACAGCGCCTAAAAGCCTCACGTCCAGAACAAATTAC

FIG. 4B

TGTGTGCTCATCCTTTCCATTGCCGAAGTATAGAATCACTGGTGTGCAGAAGATCCAATGCTCCCAGCCT  
ATATTGTTCTCACCGAAAGTGCCTGCGTATATTCATCCAAGGAAGTATCTCGTGGAACACCACCGGTAG  
ACGAGACTCCGGAGCCATCGGCAGAGAACCAATCCACAGAGGGGACACCTGAACAACCACCACCTTATAAC  
CGAGGATGAGACCAGGACTAGAACGCCTGAGCCGATCATCATCGAAGAGGAAGAAGAGGATAGCATAAGT  
TTGCTGTCAGATGGCCCGACCCACCAGGTGCTGCAAGTCGAGGCAGACATTCACGGGCGGCCCTCTGTAT  
CTAGCTCATCCTGGTCCATTCTCATGCATCCGACTTTGATGTGGACAGTTTATCCATACTTGACACCCT  
GGAGGGAGCTAGCGTGACCAGCGGGGCAACGTCAGCCGAGACTAACTCTTACTTCGCAAAGAGTATGGAG  
TTTCTGGCGCGACCGGTGCCTGCGCCTCGAACAGTATTCAGGAACCCTCCACATCCCGCTCCGCGCACAA  
GAACACCGTCACTTGCACCCAGCAGGGCCTGCTCGAGAACCAGCCTAGTTTCCACCCCGCCAGGCGTGAA  
TAGGGTGATCACTAGAGAGGAGCTCGAGGCGCTTACCCCGTCACGCACTCCTAGCAGGTGGTCTCGAGA  
ACCAGCCTGGTCTCCAACCCGCCAGGCGTAAATAGGGTGATTACAAGAGAGGAGTTTGAGGCGTTCGTAG  
CACAACAACAATGACGGTTTGATGCGGGTGATACATCTTTTCCCTCCGACACCGGTCAAGGGCATTTACA  
ACAAAAATCAGTAAGGCAAACGGTGCTATCCGAAGTGGTGTGGAGAGGACCGAATTGGAGATTTCTGTAT  
GCCCCGCGCCTCGACCAAGAAAAGAAGATTACTACGCAAGAAATTACAGTTAAATCCACACCTGCTA  
ACAGAAGCAGATACCAGTCCAGGAAGGTGGAGAACATGAAAGCCATAACAGCTAGACGTATTCTGCAAGG  
CCTAGGGCATTATTTGAAGGCAGAAGGAAAAGTGGAGTGCTACCGAACCTGCATCCTGTTCTTTGTAT  
TCATCTAGTGTGAACCGTGCCTTTTCAAGCCCCAAGGTCGCAGTGGAAGCCTGTAACGCCATGTTGAAAG  
AGAACTTTCCGACTGTGGCTTCTTACTGTATTATTCCAGAGTACGATGCCTATTTGGACATGGTTGACGG  
AGCTTCATGCTGCTTAGACACTGCCAGTTTTTGCCCTGCAAAGCTGCGCAGCTTTCCAAAGAAACACTCC  
TATTTGGAACCCACAATACGATCGGCAGTGCCCTTCAGCGATCCAGAACACGCTCCAGAACGTCTGGCAG  
CTGCCACAAAAGAAATTGCAATGTCACGCAAATGAGAGAATTGCCCGTATTGGATTGCGCGGCCCTTTAA  
TGTGGAATGCTTCAAGAAATATGCGTGTAATAATGAATATTGGGAAACGTTTAAAGAAAACCCCATCAGG  
CTTACTGAAGAAAACGTGGTAAATTACATTACCAAATTAAGAGGACCAAAGCTGCTGCTCTTTTTGCGA  
AGACACATAATTTGAATATGTTGCAGGACATACCAATGGACAGGTTTGTAATGGACTTAAAGAGAGACGT  
GAAAGTGACTCCAGGAACAAAACATACTGAAGAACGGCCCCAAGGTACAGGTGATCCAGGCTGCCGATCCG  
CTAGCAACAGCGTATCTGTGCGGAATCCACCGAGAGCTGGTTAGGAGATTAAATGCGGTCTGCTTCCGA  
ACATTCATACACTGTTTGATATGTCGGCTGAAGACTTTGACGCTATTATAGCCGAGCACTTCCAGCCTGG  
GGATTGTGTTCTGGAAACTGACATCGCGTCGTTTGATAAAAGTGAGGACGACGCCATGGCTCTGACCGCG  
TTAATGATTCTGGAAGACTTAGGTGTGGACGCAGAGCTGTTGACGCTGATTGAGGCGGCTTTCCGCGAAA  
TTTCATCAATACATTTGCCCACTAAACTAAATTTAAATTCGGAGCCATGATGAAATCTGGAATGTTCTT  
CACACTGTTTGTGAACACAGTCATTAACATTGTAATCGCAAGCAGAGTGTTGAGAGAACGGCTAACCGGA  
TCACCATGTGCAGCATTCAATTGGAGATGACAATATCGTGAAAGGAGTCAAATCGGACAAATTAATGGCAG  
ACAGGTGCGCCACCTGGTTGAATATGGAAGTCAAGATTATAGATGCTGTGGTGGGCGAGAAAGCGCCTTA  
TTTCTGTGGAGGGTTTATTTTGTGTGACTCCGTGACCGGCACAGCGTGCCGTGTGGCAGACCCCTAAAA  
AGGCTGTTTAAGCTTGGCAAACCTCTGGCAGCAGACGATGAACATGATGATGACAGGAGAAGGGCATTGC

FIG. 4C



ATGAAGAGTCAACACGCTGGAACCGAGTGGGTATTCTTTCAGAGCTGTGCAAGGCAGTAGAATCAAGGTA  
TGAAACCGTAGGAACTTCCATCATAGTTATGGCCATGACTACTCTAGCTAGCAGTGTTAAATCATTTCAGC  
TACCTGAGAGGGGGCCCTATAACTCTCTACGGCTAACCTGAATGGACTACGACATAGTCTAGTCGACGCC  
ACCATGGAAGTGTGATCCTGAAGGCCAACGCCATCACCACCATCCTGACCGCCGTGACCTTCTGCTTCG  
CCAGCGGCCAGAACATCACCAGGAATTCTACCAGAGCACCTGCAGCGCCGTGAGCAAGGGCTACCTGAG  
CGCCCTGCGGACCGGCTGGTACACCAGCGTGATCACCATCGAGCTGTCCAACATCAAAGAAAACAAGTGC  
AACGGCACCGACGCCAAGGTGAACTGATCAAGCAGGAACTGGACAAGTACAAGAACGCCGTGACCGAGC  
TGCAGCTGCTGATGCAGAGCACCCCGCCACCAACAACCGGGCCAGAAGAGAGCTGCCCCGGTTCATGAA  
CTACACCCTGAACAACGCCAAGAAAACCAACGTGACCCTGAGCAAGAAGCGGAAGCGGCGGTTCCTGGGC  
TTCCTGCTGGGCGTGGGCAGCGCCATCGCCAGCGGGGTGGCCGTGTCCAAGGTGCTGCACCTGGAAGGCG  
AGGTGAACAAGATCAAGTCCGCCCTGCTGTCCACCAACAAGGCCGTGGTGTCCCTGAGCAACGGCGTGAG  
CGTGCTGACCAGCAAGGTGCTGGATCTGAAGAACTACATCGACAAGCAGCTGCTGCCCATCGTGAACAAG  
CAGAGCTGCAGCATCAGCAACATCGAGACCGTGATCGAGTTCAGCAGAAGAACAACCGGCTGCTGGAAA  
TCACCCGGGAGTTCAGCGTGAACGCCGGCGTGACCACCCCGTGAGCACCTACATGCTGACCAACAGCGA  
GCTGCTGTCCCTGATCAATGACATGCCCATCACCAACGACCAGAAAAAGCTGATGAGCAACAACGTGCAG  
ATCGTGCGGCAGCAGAGCTACTCCATCATGAGCATCATCAAAGAAGAGGTGCTGGCCTACGTGGTGCAGC  
TGCCCCCTGTACGGCGTGATCGACACCCCTGCTGGAAGCTGCACACCAGCCCCCTGTGCACCACCAACAC  
CAAAGAGGGCAGCAACATCTGCCTGACCCGGACCGACCGGGGCTGGTACTGCGACAACGCCGGCAGCGTG  
AGCTTCTTCCCCAAGCCGAGACCTGCAAGGTGCAGAGCAACCGGGTGTCTGCGACACCATGAACAGCC  
TGACCCTGCCCTCCGAGGTGAACCTGTGCAACGTGGACATCTTCAACCCCAAGTACGACTGCAAGATCAT  
GACCTCCAAGACCGACGTGAGCAGCTCCGTGATCACCTCCCTGGGCGCCATCGTGAGCTGCTACGGCAAG  
ACCAAGTGCACCGCCAGCAACAAGAACCGGGGCATCATCAAGACCTTCAGCAACGGCTGCGACTACGTGA  
GCAACAAGGGCGTGGACACCGTGAGCGTGGGCAACACACTGTACTACGTGAATAAGCAGGAAGGCAAGAG  
CCTGTACGTGAAGGGCGAGCCCATCATCAACTTCTACGACCCCTGGTGTTCCTCCAGCGACGAGTTCGAC  
GCCAGCATCAGCCAGGTCAACGAGAAGATCAACCAGAGCCTGGCCTTCATCCGGAAGAGCGACGAGCTGC  
TGCACAATGTGAATGCCGGCAAGAGCACCACCAATATCATGATCACCACAATCATCATCGTGATCATTGT  
GATCCTGCTGTCTCTGATTGCCGTGGGCTGCTGCTGTACTGCAAGGCCCGCAGCACCCCTGTGACCCTG  
TCCAAGGACCAGCTGTCCGGCATCAACAATATCGCCTTCTCCAAGTGAAGTCTAGACGGCGCGCCACCC  
AGCGGCCGCATACAGCAGCAATTGGCAAGCTGCTTACATAGAAGTTCGCGCGATTGGCATGCCGCCTTAA  
AATTTTATTTTATTTTCTTTTCTTTTCCGAATCGGATTTTGTTTTAAATTTTCAAAAAAAAAAAAAA  
AAAAAAAAAAAAAAAAAAGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCGA  
AGGAGGACGCACGTCCACTCGGATGGCTAAGGGAGAGCCACGTTTAAACCAGCTCCAATTGCCCTATAG  
TGAGTCGTATTACGCGCGCTCACTGGCCGTGTTTTTACAACGTGCTGACTGGGAAAACCTGGCGTTACC  
CAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATC  
GCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGC

FIG. 4D

GGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTC  
TTCCCTTCCTTTCTCGCCACGTTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGT  
TCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCC  
ATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTTC  
CAAACCTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTTCGG  
CCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTAC  
AATTTAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAA  
ATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAG  
TATTCAACATTTCCGTGTGCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCCTTCCTGTTTTTGCTCACCCA  
GAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATCGAACTGGATC  
TCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGT  
TCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCGCATACACTAT  
TCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAG  
AATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGG  
ACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGGGAACCG  
GAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGC  
GCAAACCTATTAACCTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGA  
TAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCC  
GGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTA  
TCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACT  
GATTAAGCATTGGTAACTGTGACACCAAGTTTACTCATATATACTTTAGATTGATTTAAACTTTCATTTT  
TAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAATCCCTTAACGTGAGTTTT  
CGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGT  
AATCTGCTGCTTGCAAACAAAAAACCACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCA  
ACTCTTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGAGCCGT  
AGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT  
GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG  
CAGCGGTGCGGCTGAACGGGGGGTTCGTGCACACAGCCAGCTTGAGCGAACGACCTACACCGAACTGA  
GATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGT  
AAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGAAACGCCTGGTATCTTTATAGT  
CCTGTGCGGTTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTAT  
GGAAAAACGCCAGCAACGCGGCCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTT  
TCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGC  
AGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTC  
TCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGA

FIG. 4E

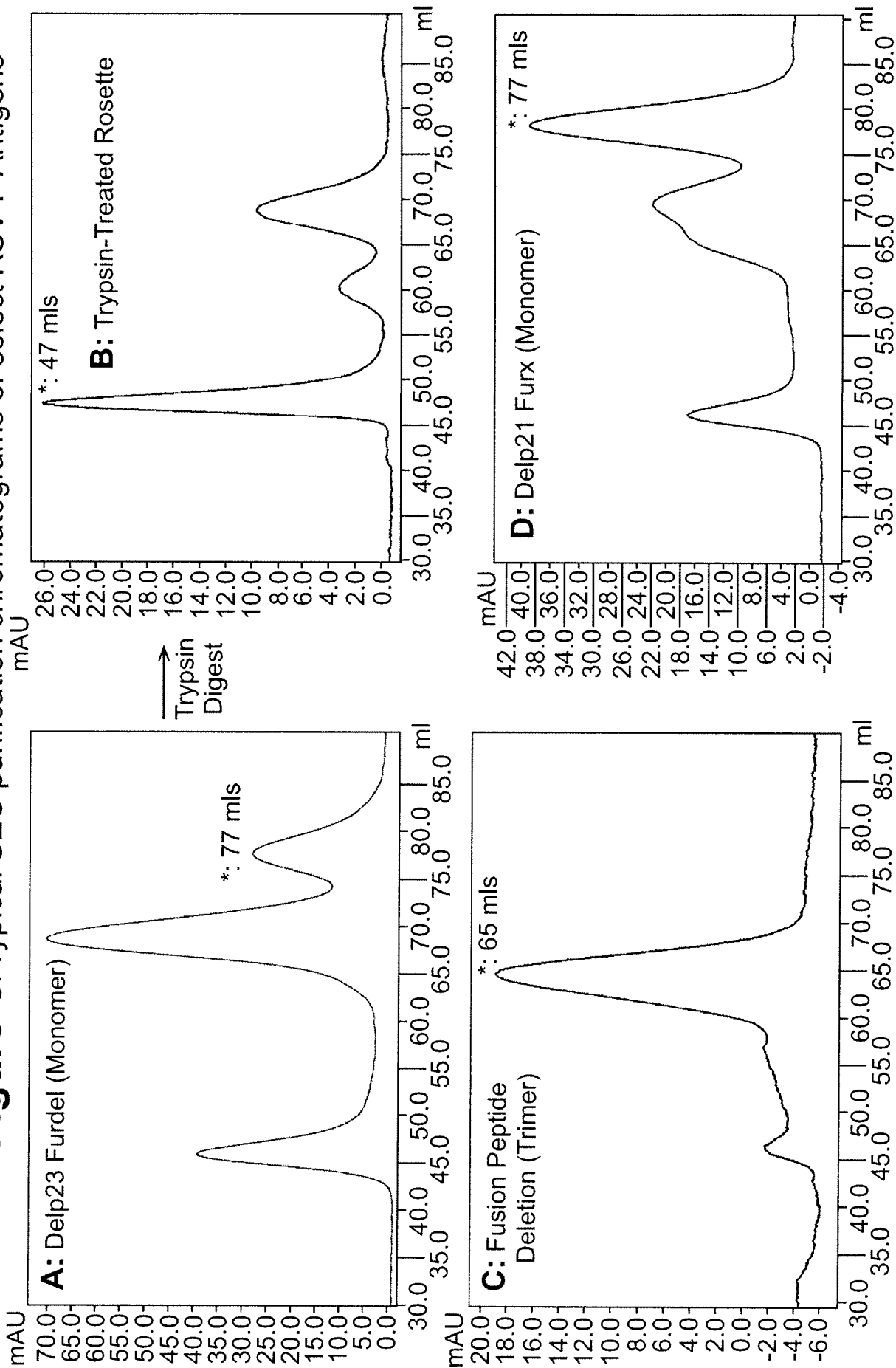
GCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCAGGCTTTACACTTTATGCTCCCGGCT  
CGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTACACAGGAAACAGCTATGACCATGATTACGCCA  
AGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGGTACCGGGCCCACGCGTAATACGACTCACTA  
TAG

FIG. 4F

1	1	10	20	30	40	50	60	70	80	90	100	110	120	130																																																																																															
CP52	MELL	IARLSAIFL	TLAINAL	YL	TSSQN	ITEEF	YQST	CSAY	SRGY	SAL	RTG	HYT	SV	ITIEL	SNIKET	KNG	TOT	KVKL	IKQEL	DKY	KNAV	TEL	QL	LHONT	PRANN	RARE	APQ	YNNY	TINT	TKNL	NVSIS																																																																														
B	MELL	IARLSAIFL	TLAINAL	YL	TSSQN	ITEEF	YQST	CSAY	SRGY	SAL	RTG	HYT	SV	ITIEL	SNIKET	KNG	TOT	KVKL	IKQEL	DKY	KNAV	TEL	QL	LHONT	PRANN	RARE	APQ	YNNY	TINT	TKNL	NVSIS																																																																														
18537strain	MELL	IHRSSAIFL	TLAVNAL	YL	TSSQN	ITEEF	YQST	CSAY	SRGY	SAL	RTG	HYT	SV	ITIEL	SNIKET	KNG	TOT	KVKL	IKQEL	DKY	KNAV	TEL	QL	LHONT	PRANN	RARE	APQ	YNNY	TINT	TKNL	NVSIS																																																																														
A2	MELL	ILKANAI	ITIL	TRVY	CFR	SGQ	NIITEF	YQST	CSAY	SKGYL	SAL	RTG	HYT	SV	ITIEL	SNIKEN	KNG	TOK	AVKL	IKQEL	DKY	KNAV	TEL	QL	LHQST	TPAT	NNR	ARREL	PRF	NNY	TLNNA	KKT	NVTLS																																																																												
Long	MELPIL	KANAI	ITIL	ARVY	CFR	SSQN	ITEEF	YQST	CSAY	SKGYL	SAL	RTG	HYT	SV	ITIEL	SNIKEN	KNG	TOK	AVKL	IKQEL	DKY	KNAV	TEL	QL	LHQST	TARAN	RARE	REL	PRF	NNY	TLNNT	KKT	NVTLS																																																																												
Consensus	MELII	IkanA	ITet	I	Laav	t	f	c	f	a	s	QN	ITEEF	YQST	CSAY	SKGYL	SAL	RTG	HYT	SV	ITIEL	SNIKEN	KNG	TOK	AVKL	IKQEL	DKY	KNAV	TEL	QL	LHQ	s	I	Paan	RARE	EL	P	Y	NNY	T	IN	NT	K	K	et	NV	t	LS																																																													
CP52	KKRR	RFL	GL	FL	GV	SAT	AS	GI	AV	SKVL	HL	EGE	VNK	IK	NAL	ST	NK	AV	VS	L	SN	GV	SV	L	TS	KV	LD	L	KNY	I	NQ	LL	P	IV	NQ	S	CR	IS	NI	G	TV	IE	F	Q	Q	K	N	S	R	L	L	E	T	REF	S	V	N	A	G	V	T	T	P	L	S	T	Y	M	L	T	N	S	E	L	L	S																																	
B	KKRR	RFL	GL	FL	GV	SAT	AS	GI	AV	SKVL	HL	EGE	VNK	IK	NAL	ST	NK	AV	VS	L	SN	GV	SV	L	TS	KV	LD	L	KNY	I	NQ	LL	P	IV	NQ	S	CR	IS	NI	G	TV	IE	F	Q	Q	K	N	S	R	L	L	E	T	REF	S	V	N	A	G	V	T	T	P	L	S	T	Y	M	L	T	N	S	E	L	L	S																																	
18537strain	KKRR	RFL	GL	FL	GV	SAT	AS	GI	AV	SKVL	HL	EGE	VNK	IK	NAL	ST	NK	AV	VS	L	SN	GV	SV	L	TS	KV	LD	L	KNY	I	NQ	LL	P	IV	NQ	S	CR	IS	NI	G	TV	IE	F	Q	Q	K	N	S	R	L	L	E	T	REF	S	V	N	A	G	V	T	T	P	L	S	T	Y	M	L	T	N	S	E	L	L	S																																	
A2	KKRR	RFL	GL	FL	GV	SAT	AS	GI	AV	SKVL	HL	EGE	VNK	IK	S	ALL	ST	NK	AV	VS	L	SN	GV	SV	L	TS	KV	LD	L	KNY	I	D	K	Q	L	L	P	IV	NK	S	CS	IS	NI	A	T	IE	F	Q	Q	K	N	R	L	L	E	T	REF	S	V	N	A	G	V	T	T	P	S	T	Y	M	L	T	N	S	E	L	L	S																															
Long	KKRR	RFL	GL	FL	GV	SAT	AS	GI	AV	SKVL	HL	EGE	VNK	IK	S	ALL	ST	NK	AV	VS	L	SN	GV	SV	L	TS	KV	LD	L	KNY	I	D	K	Q	L	L	P	IV	NK	S	CS	IS	NI	A	T	IE	F	Q	Q	K	N	R	L	L	E	T	REF	S	V	N	A	G	V	T	T	P	S	T	Y	M	L	T	N	S	E	L	L	S																															
Consensus	KKRR	RFL	GL	FL	GV	SAT	AS	G	I	AV	S	KV	L	H	E	G	V	N	K	I	S	A	L	L	S	T	N	K	A	V	V	S	L	T	S	K	V	L	D	L	K	N	Y	I	#	k	q	L	L	P	IV	N	K	S	C	R	I	S	N	I	e	T	V	I	E	F	Q	Q	K	N	R	L	L	E	T	REF	S	V	N	A	G	V	T	T	P	S	T	Y	M	L	T	N	S	E	L	L	S												
CP52	INDMP	IT	NDQ	KKL	HSS	NV	Q	IV	RQ	S	YS	INS	I	K	EE	V	AY	V	Y	Q	L	P	I	G	V	ID	T	P	CH	KL	HT	S	PL	CT	T	N	I	K	E	S	N	I	C	L	T	R	D	R	G	U	Y	C	ON	A	G	S	V	S	F	F	P	Q	A	D	T	C	K	Y	Q	S	N	R	V	F	C	OT	H	N	S	L	T	L	P	S	E	V	S	L	C	N	T	D	I	F	N	S	K												
B	INDMP	IT	NDQ	KKL	HSS	NV	Q	IV	RQ	S	YS	INS	I	K	EE	V	AY	V	Y	Q	L	P	I	G	V	ID	T	P	CH	KL	HT	S	PL	CT	T	N	I	K	E	S	N	I	C	L	T	R	D	R	G	U	Y	C	ON	A	G	S	V	S	F	F	P	Q	A	D	T	C	K	Y	Q	S	N	R	V	F	C	OT	H	N	S	L	T	L	P	S	E	V	S	L	C	N	T	D	I	F	N	S	K												
18537strain	INDMP	IT	NDQ	KKL	HSS	NV	Q	IV	RQ	S	YS	INS	I	K	EE	V	AY	V	Y	Q	L	P	I	G	V	ID	T	P	CH	KL	HT	S	PL	CT	T	N	I	K	E	S	N	I	C	L	T	R	D	R	G	U	Y	C	ON	A	G	S	V	S	F	F	P	Q	A	D	T	C	K	Y	Q	S	N	R	V	F	C	OT	H	N	S	L	T	L	P	S	E	V	S	L	C	N	T	D	I	F	N	S	K												
A2	INDMP	IT	NDQ	KKL	HSS	NV	Q	IV	RQ	S	YS	INS	I	K	EE	V	AY	V	Y	Q	L	P	I	G	V	ID	T	P	CH	KL	HT	S	PL	CT	T	N	I	K	E	S	N	I	C	L	T	R	D	R	G	U	Y	C	ON	A	G	S	V	S	F	F	P	Q	A	E	T	C	K	Y	Q	S	N	R	V	F	C	OT	H	N	S	L	T	L	P	S	E	V	N	L	C	N	V	D	I	F	N	P	K												
Long	INDMP	IT	NDQ	KKL	HSS	NV	Q	IV	RQ	S	YS	INS	I	K	EE	V	AY	V	Y	Q	L	P	I	G	V	ID	T	P	CH	KL	HT	S	PL	CT	T	N	I	K	E	S	N	I	C	L	T	R	D	R	G	U	Y	C	ON	A	G	S	V	S	F	F	P	Q	A	E	T	C	K	Y	Q	S	N	R	V	F	C	OT	H	N	S	L	T	L	P	S	E	V	N	L	C	N	V	D	I	F	N	P	K												
Consensus	INDMP	IT	NDQ	KKL	HS	n	NV	Q	IV	RQ	S	YS	INS	I	K	EE	V	AY	V	Y	Q	L	P	I	G	V	ID	T	P	CH	KL	HT	S	PL	CT	T	N	I	K	E	S	N	I	C	L	T	R	D	R	G	U	Y	C	ON	A	G	S	V	S	F	F	P	Q	A	#	T	C	K	Y	Q	S	N	R	V	F	C	OT	H	N	S	L	T	L	P	S	E	V	N	L	C	N	V	D	I	F	N	P	K											
CP52	YDCK	INT	SK	TD	SS	V	IT	SL	G	A	I	V	S	C	Y	G	K	T	C	T	A	S	N	K	R	G	I	K	T	F	S	N	G	C	D	Y	V	S	N	K	G	V	D	T	S	V	G	N	T	L	Y	Y	N	K	L	E	G	N	L	Y	Y	K	G	E	P	I	I	N	Y	O	P	L	V	F	P	S	D	E	F	D	A	S	I	S	Q	V	N	E	K	I	N	Q	S	L	A	F	I	R	S	D	E	L	L	H	N	V	T	G	K
B	YDCK	INT	SK	TD	SS	V	IT	SL	G	A	I	V	S	C	Y	G	K	T	C	T	A	S	N	K	R	G	I	K	T	F	S	N	G	C	D	Y	V	S	N	K	G	V	D	T	S	V	G	N	T	L	Y	Y	N	K	L	E	G	N	L	Y	Y	K	G	E	P	I	I	N	Y	O	P	L	V	F	P	S	D	E	F	D	A	S	I	S	Q	V	N	E	K	I	N	Q	S	L	A	F	I	R	S	D	E	L	L	H	N	V	T	G	K
18537strain	YDCK	INT	SK	TD	SS	V	IT	SL	G	A	I	V	S	C	Y	G	K	T	C	T	A	S	N	K	R	G	I	K	T	F	S	N	G	C	D	Y	V	S	N	K	G	V	D	T	S	V	G	N	T	L	Y	Y	N	K	L	E	G	N	L	Y	Y	K	G	E	P	I	I	N	Y	O	P	L	V	F	P	S	D	E	F	D	A	S	I	S	Q	V	N	E	K	I	N	Q	S	L	A	F	I	R	S	D	E	L	L	H	N	V	T	G	K
A2	YDCK	INT	SK	TD	SS	V	IT	SL	G	A	I	V	S	C	Y	G	K	T	C	T	A	S	N	K	R	G	I	K	T	F	S	N	G	C	D	Y	V	S	N	K	G	V	D	T	S	V	G	N	T	L	Y	Y	N	K	L	E	G	N	L	Y	Y	K	G	E	P	I	I	N	Y	O	P	L	V	F	P	S	D	E	F	D	A	S	I	S	Q	V	N	E	K	I	N	Q	S	L	A	F	I	R	S	D	E	L	L	H	N	V	T	G	K
Long	YDCK	INT	SK	TD	SS	V	IT	SL	G	A	I	V	S	C	Y	G	K	T	C	T	A	S	N	K	R	G	I	K	T	F	S	N	G	C	D	Y	V	S	N	K	G	V	D	T	S	V	G	N	T	L	Y	Y	N	K	L	E	G	N	L	Y	Y	K	G	E	P	I	I	N	Y	O	P	L	V	F	P	S	D	E	F	D	A	S	I	S	Q	V	N	E	K	I	N	Q	S	L	A	F	I	R	S	D	E	L	L	H	N	V	T	G	K
Consensus	YDCK	INT	SK	TD	SS	V	IT	SL	G	A	I	V	S	C	Y	G	K	T	C	T	A	S	N	K	R	G	I	K	T	F	S	N	G	C	D	Y	V	S	N	K	G	V	D	T	S	V	G	N	T	L	Y	Y	N	K	L	E	G	N	L	Y	Y	K	G	E	P	I	I	N	Y	O	P	L	V	F	P	S	D	E	F	D	A	S	I	S	Q	V	N	E	K	I	N	Q	S	L	A	F	I	R	S	D	E	L	L	H	N	V	T	G	K
CP52	ST	N	D	M	I	T	I	I	I	V	I	V	L	S	L	I	A	I	G	L	L	Y	C	K	A	K	N	T	P	V	T	L	S	K	O	Q	L	S	G	I	N	N	I	A	F	S	K																																																														
B	ST	N	D	M	I	T	I	I	V	I	V	L	S	L	I	A	I	G	L	L	Y	C	K	A	K	N	T	P	V	T	L	S	K	O	Q	L	S	G	I	N	N	I	A	F	S	K																																																															
18537strain	ST	N	D	M	I	T	I	I	V	I	V	L	S	L	I	A	I	G	L	L	Y	C	K	A	K	N	T	P	V	T	L	S	K	O	Q	L	S	G	I	N	N	I	A	F	S	K																																																															
A2	ST	N	D	M	I	T	I	I	V	I	V	L	S	L	I	A	I	G	L	L	Y	C	K	A	K	N	T	P	V	T	L	S	K	O	Q	L	S	G	I	N	N	I	A	F	S	K																																																															
Long	ST	N	D	M	I	T	I	I	V	I	V	L	S	L	I	A	I	G	L	L	Y	C	K	A	K	N	T	P	V	T	L	S	K	O	Q	L	S	G	I	N	N	I	A	F	S	K																																																															
Consensus	ST	N	D	M	I	T	I	I	V	I	V	L	S	L	I	A	I	G	L	L	Y	C	K	A	K	N	T	P	V	T	L	S	K	O	Q	L	S	G	I	N	N	I	A	F	S	K																																																															

## Figure 5

**Figure 6:** Typical SEC purification chromatograms of select RSV F Antigens



**Figure 7:** EM and SDS-PAGE analysis of RSV F antigen

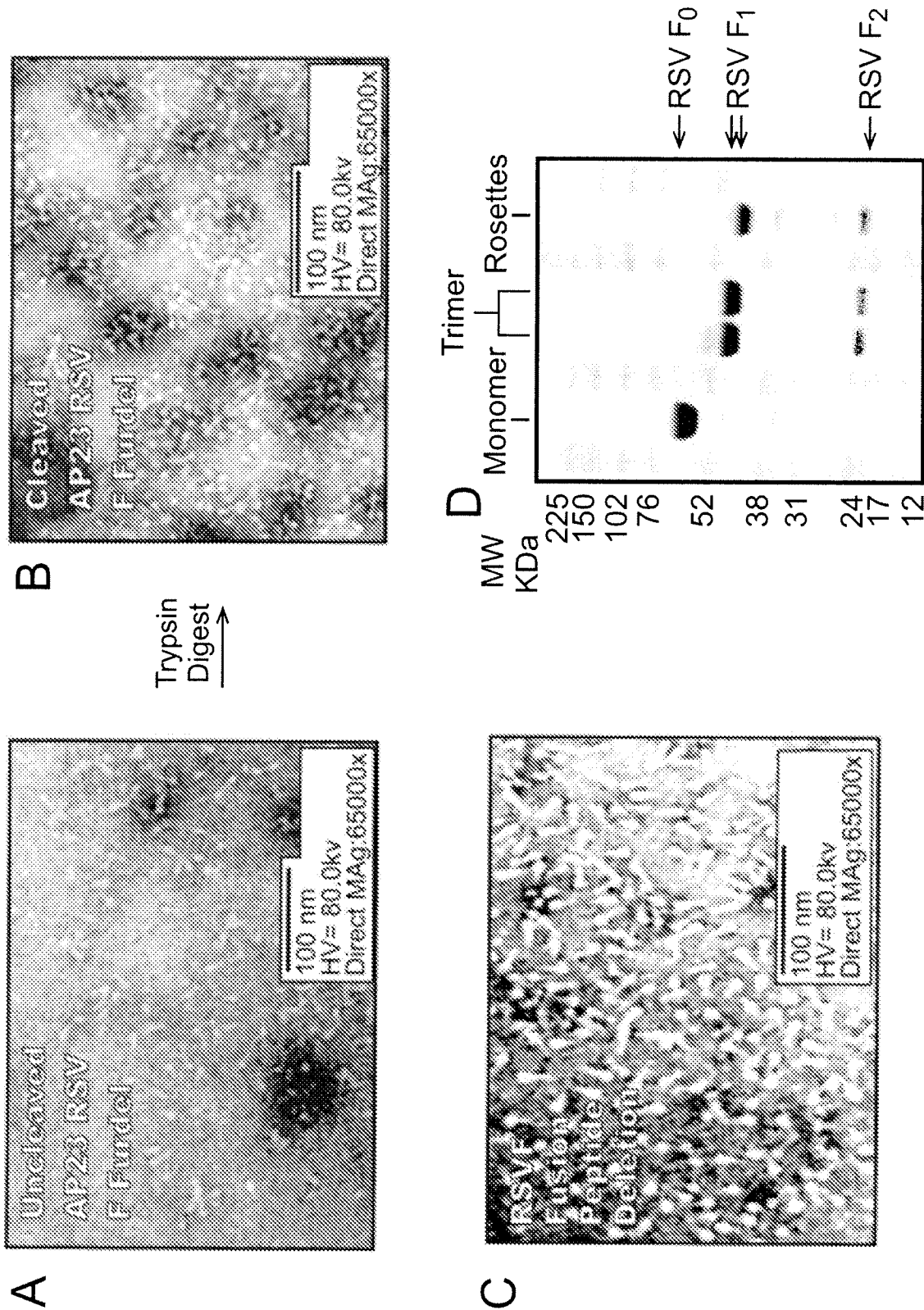


Figure 8: RSV-F subunit immunogenicity in cotton rats

