Cell culture process for producing RSV F protein

Field of the invention

The invention relates to methods for producing an RSV F protein trimer in a fed batch cell culture.

Background

Respiratory syncytial virus, or RSV, is a respiratory virus that infects the lungs and breathing passages. RSV is the leading cause of serious viral lower respiratory tract illness in infants worldwide and an important cause of respiratory illness in the elderly. However, no vaccines have been approved for preventing RSV infection.

RSV is a member of the Paramyxoviridae family. Its genome consists of a single-stranded, negative-sense RNA molecule that encodes 11 proteins, including nine structural proteins (three glycoproteins and six internal proteins) and two non-structural proteins. The structural proteins include three transmembrane surface glycoproteins: the attachment protein G, fusion protein F, and the small hydrophobic SH protein. There are two subtypes of RSV, A and B. They differ primarily in the G glycoprotein, while the sequence of the F glycoprotein is more conserved between the two subtypes.

The mature F glycoprotein has three general domains: ectodomain (ED), transmembrane domain (TM), and a cytoplasmic tail (CT). CT contains a single palmitoylated cysteine residue.

The F glycoprotein of human RSV is initially translated from the mRNA as a single 574-amino acid polypeptide precursor (referred to “F0” or “F0 precursor”), which contains a signal peptide sequence (amino acids 1-25) at the N-terminus. Upon translation the signal peptide is removed by a signal peptidase in the endoplasmic reticulum. The remaining portion of the F0 precursor (i.e., residues 26-574) may be further cleaved at two polybasic sites (a.a. 109/110 and 136/137) by cellular proteases (in particular furin), removing a 27-amino acid intervening sequence designated pep27 (amino acids 110-136) and generating two linked fragments designated F1 (C-terminal portion; amino acids 137-574) and F2 (N-terminal portion; amino acids 26-109). F1 contains a hydrophobic fusion peptide at its N-terminus and two heptad-repeat regions (HRA and HRB). HRA is near the fusion peptide, and HRB is near the TM domain. The F1 and F2 fragments are linked together through two disulfide bonds. Either the uncleaved F0 protein without the signal peptide sequence or a F1-F2 heterodimer can form a RSV F protomer. Three such protomers assemble to form the final RSV F protein complex, which is a homotrimer of the three protomers.

The F proteins of subtypes A and B are about 90 percent identical in amino acid sequence. An example sequence of the F0 precursor polypeptide for the A subtype is provided in SEQ ID NO: 1 (A2 strain; GenBank GI: 138251; Swiss Prot P03420), and for the B subtype is provided in SEQ ID NO: 2 (18537 strain; GenBank GI: 138250; Swiss Prot P13843). SEQ ID NO: 1 and SEQ ID NO:2 are both 574 amino acid sequences. The signal peptide sequence for SEQ ID NO: 1 and SEQ ID NO:2 has also been reported as amino acids 1-25 (GenBank and UniProt). In both sequences the TM domain is from approximately amino acids 530 to 550, but has alternatively been reported as 525-548. The cytoplasmic tail begins at either amino acid 548 or 550 and ends at amino acid 574, with the palmitoylated cysteine residue located at amino acid 550.

One of the primary antigens explored for RSV subunit vaccines is the F protein. The RSV F protein trimer mediates fusion between the virion membrane and the host cellular membrane and also promotes the formation of syncytia. In the virion prior to fusion with the membrane of the host cell, the largest population of F molecules forms a lollipop-shaped structure, with the TM domain anchored in the viral envelope [Dormitzer, P.R., Grandi, G., Rappuoli, R., Nature Reviews Microbiol, 10, 807, 2012.]. This conformation is referred to as the pre-fusion conformation. Pre-fusion RSV F is recognized by monoclonal antibodies (mAbs) D25, AM22, and MPE8, without discrimination between oligomeric states. Pre-fusion F trimers are specifically recognized by mAb AM14 [Gilman MS, Moin SM, Mas V et al. Characterization of a prefusion-specific antibody that recognizes a quaternary, cleavage-dependent epitope on the RSV fusion glycoprotein. PLoS Pathogens,11(7), 2015]. During RSV entry into cells, the F protein rearranges from the pre-fusion state (which may be referred to herein as “pre-F”), through an intermediate extended structure, to a post-fusion state (“post-F”). During this rearrangement, the C-terminal coiled-coil of the pre-fusion molecule dissociates into its three constituent strands, which then wrap around the globular head and join three additional helices to form the post-fusion six helix bundle. If a pre-fusion RSV F trimer is subjected to increasingly harsh chemical or physical conditions, such as elevated temperature, it undergoes structural changes. Initially, there is loss of trimeric structure (at least locally within the molecule), and then rearrangement to the post-fusion form, and then denaturation of the domains.

To prevent viral entry, F-specific neutralizing antibodies presumably must bind the pre-fusion conformation of F on the virion, or potentially the extended intermediate, before the viral envelope fuses with a cellular membrane. Thus, the pre-fusion form of the F protein is considered the preferred conformation as the desired vaccine antigen [Ngwuta, J.O., Chen, M., Modjarrad, K., Joyce, M.G., Kanekiyo, M., Kumar, A., Yassine, H.M., Moin, S.M., Killikelly, A.M., Chuang, G.Y., Druz, A., Georgiev, I.S., Rundlet, E.J., Sastry, M., Stewart-Jones, G.B., Yang. Y., Zhang, B., Nason, M.C., Capella, C., Peeples, M., Ledgerwood, J. E., Mclellan, J.S., Kwong, P.D., Graham, B.S., Science Translat. Med., 14, 7, 309 (2015)]. Upon extraction from

a membrane with surfactants such as Triton X-100, Triton X-114, NP-40, Brij-35, Brij-58, Tween 20, Tween 80, Octyl glucoside, Octyl thioglucoside, SDS, CHAPS, CHAPSO, or expression as an ectodomain, physical or chemical stress, or storage, the F glycoprotein readily converts to the post-fusion form [McLellan JS, Chen M, Leung S et al. Structure of RSV fusion glycoprotein trimer bound to a pre-fusion-specific neutralizing antibody. Science 340, 1113–1117 (2013); Chaiwatpongsakorn, S., Epand, R.F., Collins, P.L., Epand R.M., Peeples, M.E., J Virol. 85(8):3968-77 (2011); Yunus, A.S., Jackson T.P., Crisafi, K., Burimski, I., Kilgore, N.R., Zoumplis, D., Allaway, G.P., Wild, C.T., Salzwedel, K. Virology. 2010 Jan 20;396(2):226-37]. Therefore, the preparation of pre-fusion F as a vaccine antigen has remained a challenge. Since the neutralizing and protective antibodies function by interfering with virus entry, it is postulated that an F antigen that elicits only post-fusion specific antibodies is not expected to be as effective as an F antigen that elicits pre-fusion specific antibodies. Therefore, it is considered more desirable to utilize an F protein vaccine that contains a F protein immunogen in the pre-fusion form (or potentially the extended intermediate form). Mutants of the RSV F protein have been provided to increase the stability of the pre fusion form of the protein (see for example PCT application No WO2017/109629) and are promising vaccine candidate. Therefore, there is a need for a process to produce these antigens in the desired trimer conformation and with a suitable titer. Such process should also be sufficiently robust to be used at large scale. In addition, the amount of host cell proteins (HCP) or other impurities should be minimized in order to facilitate the downstream processing of the produced trimers.

Summary of the invention

The invention relates to a method for producing an RSV F protein trimer in a fed batch cell culture, said method comprising the steps of:

(i) providing mammalian cells that contain a gene encoding an RSV F protein in a cell culture medium to start a cell culture, and,

(ii) culturing the cells at a temperature between about 33.0°C and 35.0°C, and

(iii) providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value.

In some embodiments the method comprises a temperature shift where the temperature is shifted to a lower temperature between about 30.0 and about 32.0°C, preferably about 31.0°C.

Brief description of drawings

Figure 1A shows the effect of the growth temperature on the percentage of HMMS, LMMS and RSV F protein of subtype A trimer as measured by size exclusion chromatography.

Figure 1B shows the effect of the growth temperature on the percentage of HMMS, LMMS and RSV F protein of subtype B trimer as measured by size exclusion chromatography

Figure 2A shows the effect of the growth temperature on the titer of RSV F protein of subtype A as measured by RP-HPLC.

Figure 2B shows the effect of the growth temperature on the titer of RSV F protein of subtype B as measured by RP-HPLC.

Figure 3A shows the effect of the growth temperature on the amount of host cell protein (HCP) as measured by enzyme-linked immunoassay in material harvested from production of RSV F protein of subtype A.

Figure 3B shows the effect of the growth temperature on the amount of host cell protein (HCP) as measured by enzyme-linked immunoassay in material harvested from production of RSV F protein of subtype B.

Figure 4A shows the effect of the growth temperature on the amount of triter in material harvested from production of RSV F protein of subtype A.

Figure 4B shows the effect of the growth temperature on the amount of triter in material harvested from production of RSV F protein of subtype B.

Figure 5A shows the effect of the production temperature on the percentage of HMMS, LMMS and RSV F protein of subtype A trimer as measured by size exclusion chromatography.

Figure 5B shows the effect of the production temperature on the percentage of HMMS, LMMS and RSV F protein of subtype B trimer as measured by size exclusion chromatography

Figure 6A shows the effect of the production temperature on the titer of RSV F protein of subtype A as measured by RP-HPLC.

Figure 6B shows the effect of the production temperature on the titer of RSV F protein of subtype B as measured by RP-HPLC.

Figure 7A shows the effect of the production temperature on the amount of host cell protein (HCP) as measured by enzyme-linked immunoassay in material harvested from production of RSV F protein of subtype A.

Figure 7B shows the effect of the production temperature on the amount of host cell protein (HCP) as measured by enzyme-linked immunoassay in material harvested from production of RSV F protein of subtype B.

Figure 8A shows the effect of the production temperature on the amount of triter in material harvested from production of RSV F protein of subtype A.

Figure 8B shows the effect of the production temperature on the amount of triter in material harvested from production of RSV F protein of subtype B.

Figure 9A shows the effect of the timing of a temperature shift on the percentage of HMMS, LMMS and RSV F protein of subtype A trimer as measured by size exclusion chromatography.

Figure 9B shows the effect of the timing of a temperature shift on the percentage of HMMS, LMMS and RSV F protein of subtype B trimer as measured by size exclusion chromatography.

Figure 10A shows the effect of the timing of a temperature shift on the titer of RSV F protein of subtype A as measured by RP-HPLC.

Figure 10B shows the effect of the timing of a temperature shift on the titer of RSV F protein of subtype B as measured by RP-HPLC.

Figure 11A shows the effect of the timing of a temperature shift on the amount of host cell protein (HCP) as measured by enzyme-linked immunoassay in material harvested from production of RSV F protein of subtype A.

Figure 11B shows the effect of the timing of a temperature shift on the amount of host cell protein (HCP) as measured by enzyme-linked immunoassay in material harvested from production of RSV F protein of subtype B.

Figure 12A shows the effect of the timing of a temperature shift on the amount of triter in material harvested from production of RSV F protein of subtype A.

Figure 12B shows the effect the timing of a temperature shift on the amount of triter in material harvested from production of RSV F protein of subtype B.

Figure 13A shows the effect of the presence of a temperature shift on the percentage of HMMS, LMMS and RSV F protein of subtype A trimer as measured by size exclusion chromatography.

Figure 13B shows the effect of the presence of a temperature shift on the percentage of HMMS, LMMS and RSV F protein of subtype B trimer as measured by size exclusion chromatography.

Figure 14A shows the effect of the presence of a temperature shift on the titer of RSV F protein of subtype A as measured by RP-HPLC.

Figure 14B shows the effect of the presence of a temperature shift on the titer of RSV F protein of subtype B as measured by RP-HPLC.

Figure 15A shows the effect of the presence of a temperature shift on the amount of host cell protein (HCP) as measured by enzyme-linked immunoassay in material harvested from production of RSV F protein of subtype A.

Figure 15B shows the effect of the presence of a temperature shift on the amount of host cell protein (HCP) as measured by enzyme-linked immunoassay in material harvested from production of RSV F protein of subtype B.

Figure 16 shows a western blot of the material harvested from production run from 9 bioreactors with various culture conditions after a hydrophobic interaction chromatography (HIC) on material harvested from production of RSV F protein of subtype A.

Figure 17 shows a western blot of the material harvested from production run from 9 bioreactors with various culture conditions after a hydrophobic interaction chromatography (HIC) on material harvested from production of RSV F protein of subtype B.

Detailed description of the invention

The invention relates to a method for producing an RSV F protein trimer in a fed batch cell culture, said method comprising the steps of:

(i) providing mammalian cells that contain a gene encoding an RSV F protein in a cell culture medium to start a cell culture, and,

(ii) culturing the cells at a temperature between about 33.0°C and about 35.0°C, and

(iii) providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value.

The method of the invention is particularly useful for producing RSV F protein trimers to be used as antigens in immunogenic compositions. The method of the invention can be used for manufacturing RSV F protein trimers at large scale, for example in cell culture medium volume of at least 500L or even at least 3000L. The method of the invention provides high titers and high percentages of RSV protein F in the form of trimers while also minimizing the amount of HCP or other impurities thereby facilitating further downstream processing. In addition, specific conditions optimizing the processing of the protein have been identified and can be used in the method of the invention.

In some embodiments, the RSV F protein is an RSV F protein of subtype A. In some embodiments, the RSV F protein is an RSV F protein of subtype B. In some embodiments, the RSV F protein is a mutant of wild type RSV F protein. In some embodiments, the RSV F protein is a mutant of wild type RSV F protein of subtype A. In some embodiments, the RSV F protein is a mutant of wild type RSV F protein of subtype B. In some embodiments, the mutants display introduced mutations in the amino acid sequence relative to the amino acid sequence of the corresponding wild-type RSV F protein and are immunogenic against the wild-type RSV F protein or against a virus comprising the wild-type F protein. The amino acid mutations in the mutants include amino acid substitutions, deletions, or additions relative to a wild-type RSV F protein.

In some embodiments, the RSV F protein produced by the method of the invention is an RSV protein mutant as disclosed in WO2017/109629 which is incorporated herein by reference.

In some embodiments, the RSV F protein is a mutant of a wild-type RSV F protein, wherein the introduced amino acid mutations are mutation of a pair of amino acid residues in a wild-type RSV F protein to a pair of cysteines ("engineered disulfide mutation"). The introduced pair of cysteine residues allows for formation of a disulfide bond between the cysteine residues that stabilize the protein's conformation or oligomeric state, such as the pre-fusion conformation. Examples of specific pairs of such mutations include: 55C and 188C; 155C and 290C; 103C and 148C; and 142C and 371C, such as S55C and L188C; S155C and S290C; T103C and I148C; and L142C and N371C.

In still other embodiments, the RSV F protein mutants comprise amino acid mutations that are one or more cavity filling mutations. Examples of amino acids that may be replaced with the goal of cavity filling include small aliphatic (e.g. Gly, Ala, and Val) or small polar amino acids (e.g. Ser and Thr) and amino acids that are buried in the pre-fusion conformation, but exposed to solvent in the post-fusion conformation. Examples of the replacement amino acids include large aliphatic amino acids (Ile, Leu and Met) or large aromatic amino acids (His, Phe, Tyr and Trp). In some specific embodiments, the RSV F protein mutant comprises a cavity filling mutation selected from the group consisting of:

(1) substitution of S at positions 55, 62, 155, 190, or 290 with I, Y, L, H, or M;

(2) substitution of T at position 54, 58, 189, 219, or 397 with I, Y, L, H, or M;

(3) substitution of G at position 151 with A or H;

(4) substitution of A at position 147 or 298 with I, L, H, or M;

(5) substitution of V at position 164, 187, 192, 207, 220, 296, 300, or 495 with I, Y, H; and

(6) substitution of R at position 106 with W.

In some particular embodiments, the RSV F protein mutant comprises at least one cavity filling mutation selected from the group consisting of: T54H, S190I, and V296I.

In still other embodiments, the RSV F protein mutants comprise electrostatic mutations, which decrease ionic repulsion or increase ionic attraction between resides in a protein that are proximate to each other in the folded structure. In several embodiments, the RSV F protein mutant includes an electrostatic substitution that reduces repulsive ionic interactions or increases attractive ionic interactions with acidic residues of Glu487 and Asp489 from another protomer of RSV F trimer. In some specific embodiments, the RSV F protein mutant comprises an electrostatic mutation selected from the group consisting of:

(1) substitution of E at position 82, 92, or 487 by D, F, Q, T, S, L, or H;

(2) substitution of K at position 315, 394, or 399 by F, M, R, S, L, I, Q, or T;

(3) substitution of D at position 392, 486, or 489 by H, S, N, T, or P; and

(4) substitution of R at position 106 or 339 by F, Q, N, or W.

In still other embodiments, the RSV F protein mutants comprise a combination of two or more different types of mutations selected from engineered disulfide mutations, cavity filling mutations, and electrostatic mutations. In some particular embodiments, the RSV F protein mutants comprise a combination of mutations relative to the corresponding wild-type RSV F protein, wherein the combination of mutations is selected from the group consisting of:

(1) combination of T103C, I148C, S190I, and D486S;

(2) combination of T54H S55C L188C D486S;

(3) combination of T54H, T103C, I148C, S190I, V296I, and D486S;

(4) combination of T54H, S55C, L142C, L188C, V296I, and N371C;

(5) combination of S55C, L188C, and D486S;

(6) combination of T54H, S55C, L188C, and S190I;

(7) combination of S55C, L188C, S190I, and D486S;

(8) combination of T54H, S55C, L188C, S190I, and D486S;

(9) combination of S155C, S190I, S290C, and D486S;

(10) combination of T54H, S55C, L142C, L188C, V296I, N371C, D486S, E487Q, and D489S; and

(11) combination of T54H, S155C, S190I, S290C, and V296I.

In some embodiments, the RSV F protein is of subtype A and comprises the mutations T103C, I148C, S190I, and D486S.

In some embodiments, the RSV F protein is of subtype B and comprises the mutations T103C, I148C, S190I, and D486S.

In view of the substantial conservation of RSV F sequences, a person of ordinary skill in the art can easily compare amino acid positions between different native RSV F sequences to identify corresponding RSV F amino acid positions between different RSV strains and subtypes. For example, across nearly all identified native RSV F0 precursor proteins, the furin cleavage sites fall in the same amino acid positions. Thus, the conservation of native RSV F protein sequences across strains and subtypes allows use of a reference RSV F sequence for comparison of amino acids at particular positions in the RSV F protein. For the purposes of this disclosure (unless context indicates otherwise), the RSV F protein amino acid positions are given with reference to the amino acid sequence of the full length native F precursor polypeptide of the RSV A2 strain; corresponding to GenInfo Identifier GI 138251 and Swiss Prot identifier P03420.

In some embodiments, the RSV F protein produced by the method of the invention is an RSV protein mutant as disclosed WO2009/079796, WO2010/149745, WO2011/008974, WO2014/160463, WO2014/174018, WO2014/202570, WO2015/013551, WO2015/177312, WO2017/005848 and WO2018/109220. The RSV F proteins disclosed in these references are incorporated herein by reference.

The term "fed-batch culture" as used herein refers to a method of culturing cells in which additional components are provided to the culture at a time or times subsequent to the beginning of the culture process. In some embodiments, these additional components are provided together in a feed medium. Such provided components typically comprise nutritional components for the cells which have been depleted during the culturing process. A fed-batch culture is typically stopped at some point and the cells and/or components in the medium are harvested and optionally purified. In some embodiments, the fed-batch culture comprises a basal medium supplemented with a feed medium.

In some embodiments, the cells are cultured at a temperature of 33.0°C, 33.1°C, 33.2°C, 33.3°C, 33.4°C, 33.5°C, 33.6°C, 33.7°C, 33.8°C, 33.9°C, 34.0°C, 34.1°C, 34.2°C, 34.3°C, 34.4°C, 34.5°C, 34.6°C, 34.7°C, 34.8°C, 34.9°C or 35.0°C. In a preferred embodiment, the cells are cultured at a temperature between 34.0°C and 35.0°C. In a preferred embodiment, the cells are cultured at a temperature of 34.5°C.

The method of the invention comprises a step of providing glucose in a restricted manner to the cells wherein glucose is fed to the cells in response to a rise of pH above a predetermined pH value. Such method of feeding glucose depending on pH variations is also referred to as HiPDOG and is disclosed for example in WO2004/104186 and in Gagnon et al ((2011) (Biotechnology and bioengineering 108: 1328-1337), which are both incorporated herein by reference.

In some embodiments, a pH sensor is used to monitor pH of the cell culture.

In some embodiments, the predetermined pH value of the method of the invention corresponds to an increase of 0.01 to 0.10 such as for example 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 or 0.10 above the pH set point of the culture. In some embodiments, the predetermined pH value corresponds to an increase of 0.05 above the pH set point of the culture.

In some embodiments, the pH set point of the cell culture is between 6.70 and 7.30. In some embodiments, the pH set point of the cell culture is between 6.90 and 7.20. In some embodiments, the pH set point of the cell culture is between 7.00 and 7.10. In a preferred embodiment, the pH set point of the cell culture is 7.05.

In a preferred embodiment, the pH set point of the cell culture is 7.05 and the predetermined pH value corresponds to an increase of 0.05 above said set point.

In some embodiments, during the phase of the cell culture where glucose is provided in a restricted manner, the pH of the cell culture is between 6.70 and 7.30. In some embodiments, during the phase of the cell culture where glucose is provided in a restricted manner, the pH of the cell culture is between 6.90 and 7.20. In some embodiments, during the phase of the cell culture where glucose is provided in a restricted manner, the pH set point is 6.95. In some embodiments, during the phase of the cell culture where glucose is provided in a restricted manner, the pH set point is 7.07. In some embodiments, during the phase of the cell culture where glucose is provided in a restricted manner, the pH set point is 7.01. In some embodiments, during the phase of the cell culture where glucose is provided in a restricted manner, the pH set point is 7.20.

In some embodiments, after the phase of the cell culture where glucose is provided in a restricted manner, the pH set point is 7.20. In some embodiments, after the phase of the cell culture where glucose is provided in a restricted manner, the pH set point is 7.20 and the pH operating range is 7.05 to 7.35. In some embodiments, after the phase of the cell culture where glucose is provided in a restricted manner, the pH set point is 6.90. In some embodiments, after the phase of the cell culture where glucose is provided in a restricted manner, the pH set point is 6.90 and the pH operating range is 6.75 to 7.05.

In some embodiments, feeding glucose to the cell culture in response to rise of pH above a predetermined pH value comprises feeding glucose until the pH decreases to reach the pH set point of the culture.

In some embodiments, glucose is provided in a restricted manner to the cell culture during the growth phase of the culture. In some embodiments, glucose is provided in a restricted manner to the cell culture for 1 to 6 days, preferably 3, 4 or 5 days, more preferably for 4 or 5 days.

In some embodiments, the step of providing glucose in a restricted manner to the cell culture starts on day 0, day 1 or day 2.

In some embodiments, when glucose is provided in a restricted manner, it is provided as an independent feed i.e not comprising other components of the feed medium.

In some embodiments, when glucose is provided in a restricted manner, it is provided as part of the feed medium.

In some embodiments of the method disclosed herein, the temperature is shifted to a lower temperature between about 30.0°C and about 32.0°C, preferably about 31.0°C. In some embodiments, the temperature is shifted to a lower temperature between day 3 and day 7 (i.e between the third day of culture and the seventh day of culture). In a preferred embodiment, the temperature is shifted to a lower temperature on day 5 or on day 6. In a preferred embodiment the temperature is shifted to a lower temperature after the provision of glucose in a restricted manner is stopped.

In some embodiments, the method of the invention results in an improved titer as compared to other methods such as for example methods conducted at a temperature higher or lower than the temperature or temperature ranges defined herein and/or methods without temperature shift and/or methods using a medium comprising glucocorticoids and/or methods not comprising a step of providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value. Titer can be determined by any method known in the art. In one embodiment, titer is measured by reverse phase high-performance liquid chromatography (RP-HPLC).

In some embodiments, the method of the invention results in an increased percentage of trimer and a reduced percentage high molecular mass species (HMMS) and/or low molecular mass species (LMMS) as compared to other methods such as for example methods conducted at a temperature higher or lower than the temperature or temperature ranges defined herein and/or methods without temperature shift and/or methods using a medium comprising glucocorticoids and/or methods not comprising a step of providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value. Percentage of trimer, HMMS and LMMS can be determined by any method known in the art. In some embodiments, percentage of trimer, HMMS and LMMS are measured by size exclusion chromatography (SEC-HPLC).

In some embodiments, the method of the invention results in an increased triter as compared to other methods such as for example methods conducted at a temperature higher or lower

than the temperature or temperature ranges defined herein and/or methods without temperature shift and/or methods using a medium comprising glucocorticoids and/or methods not comprising a step of providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value. Triter values are calculated by multiplying percentage of trimer, preferably as obtained by SEC-HPLC, by titer, preferably obtained by RP-HPLC. Triter provides an estimate of how much protein is produced in the trimeric form.

In some embodiments, the method of the invention results in a reduced amount of Host Cell Protein as compared to other methods such as for example methods conducted at a temperature higher or lower than the temperature or temperature ranges defined herein and/or methods without temperature shift and/or methods using a medium comprising glucocorticoids and/or methods not comprising a step of providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value. HCP can be measured by any method known in the art. In some embodiments, HCP was measured by enzyme-linked immunoassay (ELISA).

In some embodiments, the method of the invention results in an improved amount of processed RSV F (A) or RSV F (B) in a form suitable for forming trimers that can be used as antigens in immunogenic compositions as compared to other methods such as for example methods conducted at a temperature higher or lower than the temperature or temperature ranges defined herein and/or methods without temperature shift and/or methods using a medium comprising glucocorticoids and/or methods not comprising a step of providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value. Amount of processed RSV F (A) or RSV F (B) in a suitable form can be determined by any method known in the art. In one embodiment, such amount is measured by western blot, for example as shown in example 3.

In some embodiments, the method of the invention results in an improved titer and/or an increased percentage of trimer and a reduced percentage high molecular mass species (HMMS) and/or low molecular mass species (LMMS) and/or a reduced amount of Host Cell Protein as compared to other methods such as for example methods conducted at a temperature higher or lower than the temperature or temperature ranges defined herein and/or methods without temperature shift and/or methods using a medium comprising glucocorticoids and/or methods not comprising a step of providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value.

The terms “medium”, “cell culture medium” and “culture medium” as used herein refer to a solution containing nutrients which nourish growing mammalian cells. Typically, such solutions provide essential and non-essential amino acids, vitamins, energy sources, lipids, and trace elements required by the cell for minimal growth and/or survival. In one embodiment, the medium may comprise Ala, Arg, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val and Cystine and/or Cys.

Such a solution may also contain supplementary components that enhance growth and/or survival above the minimal rate, including, but not limited to, hormones and/or other growth factors, particular ions (such as sodium, chloride, calcium, magnesium, and phosphate), buffers, vitamins, nucleosides or nucleotides, trace elements (inorganic compounds usually present at very low final concentrations), inorganic compounds present at high final concentrations (e.g., iron), amino acids, lipids, and/or glucose or other energy source. In some embodiments, a medium is advantageously formulated to a pH and salt concentration optimal for cell survival and proliferation. For example, the medium may be formulated to a pH between around 7.1 and 7.3 and a final osmolality between around 1000 and 1300mOsm.

Example of known basal and/or feed cell culture media which can be used in the method of the invention include those disclosed in WO2006/026445, WO2008/109410, WO2008/063892, EP2243827, WO2002/066603, WO2015/140708 and WO2006/050050.

In a preferred embodiment, the feed medium used in the method of the invention comprises 4 to 10mM Ala, 30 to 60mM Arg, 50 to 90mM Asn, 10 to 30mM Asp, 2 to 40mM Glu, 2 to 15mM Gly, 8 to 20mM His, 25 to 32mM Ile, 35 to 60mM Leu, 28 to 60mM Lys, 9 to 25mM Met, 10 to 30mM Phe, 15 to 40mM Pro, 44 to 80mM Ser, 20 to 45mM Thr, 2 to 10mM Trp and 20 to 50mM Val.

In some embodiments, the medium is a chemically defined medium, wherein the components of the medium are both known and controlled. In some embodiments, the medium is a complex medium, in which not all components of the medium are known and/or controlled.

Chemically defined growth media for mammalian cell culture have been extensively developed and published over the last several decades. All components of defined media are well characterized, and so defined media do not contain complex additives such as serum or hydrolysates. Early media formulations were developed to permit cell growth and maintenance of viability with little or no concern for protein production. More recently, media formulations have been developed with the express purpose of supporting highly productive recombinant protein producing cell cultures. Such media are preferred for use in the method of the invention.

Such media generally comprises high amounts of nutrients and in particular of amino acids to support the growth and/or the maintenance of cells at high density. If necessary, these media can be modified by the skilled person for use in the method of the invention.

Not all components of complex media are well characterized, and so complex media may contain additives such as simple and/or complex carbon sources, simple and/or complex nitrogen sources, and serum, among other things. In some embodiments, complex media suitable for the present invention contains additives such as hydrolysates in addition to other components of defined medium as described herein.

In some embodiments, defined media typically includes roughly fifty chemical entities at known concentrations in water. Some of them also contain one or more well-characterized proteins such as insulin, IGF-1, transferrin or BSA, but others require no protein components and so are referred to as protein-free defined media. Typical chemical components of the media fall into five broad categories: amino acids, vitamins, inorganic salts, trace elements, and a miscellaneous category that defies neat categorization.

Cell culture medium may be optionally supplemented with supplementary components. The term “supplementary components” as used herein refers to components that enhance growth and/or survival above the minimal rate, including, but not limited to, hormones and/or other growth factors, particular ions (such as sodium, chloride, calcium, magnesium, and phosphate), buffers, vitamins, nucleosides or nucleotides, trace elements (inorganic compounds usually present at very low final concentrations), amino acids, lipids, and/or glucose or other energy source. In some embodiments, supplementary components may be added to the initial cell culture. In some embodiments, supplementary components may be added after the beginning of the cell culture.

Typically, trace elements refer to a variety of inorganic salts included at micromolar or lower levels. For example, commonly included trace elements are zinc, selenium, copper, and others. In some embodiments, iron (ferrous or ferric salts) can be included as a trace element in the initial cell culture medium at micromolar concentrations. Manganese is also frequently included among the trace elements as a divalent cation ($MnCl_2$ or $MnSO_4$) in a range of nanomolar to micromolar concentrations. Numerous less common trace elements are usually added at nanomolar concentrations.

In some embodiments, the cell culture medium used in the method of the invention does not comprise glucocorticoid compounds.

Glucocorticoid compounds are known to modulate various cellular functions such as cell proliferation, metabolism, glycosylation, and secretion of many proteins and are therefore often included in cell culture media, in particular for use in large scale manufacturing process.

Examples of glucocorticoid compounds used as cell culture media components include, but are not limited to hydrocortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone and fludrocortisone acetate.

As shown in below example 3, the presence of a glucocorticoid such as hydrocortisone in the cell culture medium has a detrimental effect on the amount of RSV F protein in the correct form. Without being bound by any theory, this effect may be due to an interference of the glucocorticoid compounds with the processing of the RSV F protein resulting in an increased amount of unprocessed RSV protein in the harvested material.

In some embodiments, the cell culture medium used in the methods of the invention does not comprise glucocorticoid compounds. In some embodiments, the basal medium used in the methods of the invention does not comprise glucocorticoid compounds. In some embodiments, the feed medium used in the methods of the invention does not comprise glucocorticoid compound. In some embodiments, the basal medium and the feed medium used in the methods of the invention do not comprise glucocorticoid compounds.

In some embodiments, the cell culture medium used in the methods of the invention does not comprise any of hydrocortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone and fludrocortisone acetate. In some embodiments, the basal medium used in the methods of the invention does not comprise any of hydrocortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone and fludrocortisone acetate. In some embodiments, the feed medium used in the methods of the invention does not comprise any of hydrocortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone and fludrocortisone acetate. In some embodiments, the basal medium and the feed medium used in the methods of the invention do not comprise any of hydrocortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone and fludrocortisone acetate.

In some embodiments, the cell culture medium used in the methods of the invention does not comprise any of hydrocortisone, prednisolone, betamethasone and dexamethasone. In some embodiments, the basal medium used in the methods of the invention does not comprise any of hydrocortisone, prednisolone, betamethasone and dexamethasone. In some embodiments, the feed medium used in the methods of the invention does not comprise any of hydrocortisone, prednisolone, betamethasone and dexamethasone. In some embodiments,

the basal medium and the feed medium used in the methods of the invention do not comprise any of hydrocortisone, prednisolone, betamethasone and dexamethasone.

In some embodiments, the cell culture medium used in the methods of the invention does not comprise hydrocortisone. In some embodiments, the basal medium used in the methods of the invention does not comprise hydrocortisone. In some embodiments, the feed medium used in the methods of the invention does not comprise hydrocortisone. In some embodiments, the basal medium and the feed medium used in the methods of the invention do not comprise hydrocortisone.

In some embodiments, the medium used in the method of the invention is a medium suitable for supporting high viable cell density, such as for example 1 x 10^6cells/mL, 5 x 10^6cells/mL, 1 x 10^7cells /mL, 5 x 10^7 cells/mL, 1X10^8 cells/mL or 5X10^8 cells/mL, in a cell culture. In some embodiments, the cell culture is a CHO cell fed-batch culture. In some embodiments, the cells are grown to a viable cell density greater than 1 x 10^6cells/mL, 5 x 10^6cells/mL, 1 x 10^7cells /mL, 5 x 10^7 cells/mL, 1X10^8 cells/mL or 5X10^8 cells/mL.

The term “viable cell density” as used herein refers to the number of cells present in a given volume of medium. Viable cell density can be measured by any method known to the skilled person. Preferably, viable cell density is measured using an automated cell counter such as Bioprofile Flex®. The term maximum cell density as used herein refers to the maximum cell density achieved during the cell culture. The term “cell viability” as used herein refers to the ability of cells in culture to survive under a given set of culture conditions or experimental variations. Those of ordinary skill in the art will appreciate that one of many methods for determining cell viability are encompassed in this invention. For example, one may use a dye (e.g., trypan blue) that does not pass through the membrane of a living cell, but can pass through the disrupted membrane of a dead or dying cell in order to determine cell viability.

Cell culture methods

The terms “culture” and “cell culture” as used herein refer to a cell population that is suspended in a medium under conditions suitable to survival and/or growth of the cell population. As will be clear to those of ordinary skill in the art, in some embodiments, these terms as used herein refer to the combination comprising the cell population and the medium in which the population is suspended.

The term “fed-batch culture” or “fed-batch cell culture” as used herein refers to a method of culturing cells in which additional components are provided to the culture at a time or times

subsequent to the beginning of the culture process. Such provided components typically comprise nutritional components for the cells which have been depleted during the culturing process. A fed-batch culture is typically stopped at some point and the cells and/or components in the medium are harvested and optionally purified. In some embodiments, the fed-batch culture comprises a basal medium supplemented with feed media.

Cells may be grown in any convenient volume chosen by the practitioner. For example, cells may be grown in small scale reaction vessels ranging in volume from a few milliliters to several liters. Alternatively, cells may be grown in large scale commercial bioreactors ranging in volume from at least 500, 1000, 2500, 5000, 8000, 10,000, 12,000, 15000, 20000 or 25000 liters or more, or any volume in between. In some embodiments, the volume of the cell culture is at least 500L. In some embodiments, the volume of the cell culture is at least 3000L.

In some embodiments, the cells may be grown during the initial growth phase (or growth phase) for a greater or lesser amount of time, depending on the needs of the practitioner and the requirement of the cells themselves. In some embodiments, the cells are grown for a period of time sufficient to achieve a predefined cell density. In some embodiments, the cells are grown for a period of time sufficient to achieve a predefined cell density of about 1 x 10^6cells/mL, about 5 x 10^6cells/mL, about 1 x 10^7cells /mL, about 5 x 10^7 cells/mL, about 1X10^8 cells/mL or about 5X10^8 cells/mL. In some embodiments, the cells are grown for a period of time sufficient to achieve a cell density that is a given percentage of the maximal cell density that the cells would eventually reach if allowed to grow undisturbed. For example, the cells may be grown for a period of time sufficient to achieve a desired viable cell density of 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 percent of maximal cell density. In some embodiments, the cells are grown until the cell density does not increase by more than 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% per day of culture. In some embodiments, the cells are grown until the cell density does not increase by more than 5% per day of culture.

In some embodiments the cells are allowed to grow for a defined period of time. For example, depending on the starting concentration of the cell culture and the intrinsic growth rate of the cells, the cells may be grown for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more days, preferably for 4 to 10 days. The practitioner of the present invention will be able to choose the duration of the initial growth phase depending on protein production requirements and the needs of the cells themselves.

The cell culture may be agitated or shaken during the initial culture phase in order to increase oxygenation and dispersion of nutrients to the cells. In accordance with the present invention, one of ordinary skill in the art will understand that it can be beneficial to control or regulate certain internal conditions of the bioreactor during the initial growth phase, including but not limited to pH, temperature, oxygenation, etc.

In accordance with the present invention, one of ordinary skill in the art will understand that the temperature at which the cells are cultured is a temperature set point and is controlled during the cell culture to limit the variation of temperature around the set point.

A temperature shift to a lower temperature can be used in the method of the invention. In such case, one of ordinary skill in the art will understand that a lower temperature set point is defined and that once the temperature has reached the lower set point, it is controlled to limit the variation of temperature around said lower set point. When shifting the temperature of the culture, the temperature shift may be relatively gradual. For example, it may take several hours or days to complete the temperature change. Alternatively, the temperature shift may be relatively abrupt. For example, the temperature change may be complete in less than several hours. Given the appropriate production and control equipment, such as is standard in the commercial large-scale production of polypeptides or proteins, the temperature change may even be complete within less than an hour.

In some embodiments, once the conditions of the cell culture have been shifted as discussed above, the cell culture is maintained for a subsequent production phase under conditions conducive to the survival and viability of the cell culture and appropriate for expression of the desired polypeptide or protein at commercially adequate levels. In some embodiments, the cells may be maintained in the subsequent production phase until a desired cell density or production titer is reached. In some embodiments, the duration of the production phase is comprised between 2 and 10 days, i.e 2, 3 ,4, 5, 6, 7, 8, 9 or 10 days, preferably between 4 to 8 days, preferably 6 days.

In some embodiment the duration of the growth phase is about 6 days and the duration of the production phase is about 6 days.

The cell culture may be agitated or shaken during the subsequent production phase in order to increase oxygenation and dispersion of nutrients to the cells. In accordance with the present invention, one of ordinary skill in the art will understand that it can be beneficial to control or

regulate certain internal conditions of the bioreactor during the subsequent growth phase, including but not limited to pH, temperature, oxygenation, etc.

Cells

Any mammalian cell susceptible to cell culture may be utilized in accordance with the present invention. Non-limiting examples of mammalian cells that may be used in accordance with the present invention include BALB/c mouse myeloma line (NSO/l, ECACC No: 85110503); human retinoblasts (PER.C6, CruCell, Leiden, The Netherlands); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59,1977); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells +/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216, 1980); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1 587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68, 1982); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2). In some preferred embodiments, the cells are CHO cells. In some preferred embodiments, the cells are GS-CHO cells.

Expression of Proteins

As noted above, in many instances the cells will be selected or engineered to produce high levels of desired products. Often, cells will be manipulated by the hand of man to produce high levels of recombinant protein, for example by introduction of a gene encoding the protein of interest and/or by introduction of genetic control elements that regulate expression of that gene (whether endogenous or introduced).

Even amongst a population of cells of one particular type engineered to express a specific protein, variability within the cellular population exists such that certain individual cells will grow better, produce more protein of interest. In certain embodiments, a cell line is empirically selected by the practitioner for robust growth under the particular conditions chosen for culturing the cells. In some embodiments, individual cells engineered to express a particular protein are chosen for large-scale production based on cell growth, final cell density, percent cell viability, titer of the expressed protein or any combination of these or any other conditions deemed important by the practitioner.

The term “host cell” as used herein refers to a cell that is manipulated to produce a protein of interest as described herein. A protein may be expressed from a gene that is

endogenous to the cell, or from a heterologous gene that is introduced into the cell. A protein may be one that occurs in nature, or may alternatively have a sequence that was engineered or selected by the hand of man.

Isolation of the Expressed Protein

In general, it will typically be desirable to isolate and/or purify proteins expressed according to the present invention. In certain embodiments, the expressed protein is secreted into the medium and thus cells and other solids may be removed, as by centrifugation or filtering for example, as a first step in the purification process.

The expressed protein may be isolated and purified by standard methods including, but not limited to, chromatography (e.g., ion exchange, affinity, size exclusion, and hydroxyapatite chromatography), gel filtration, centrifugation, or differential solubility, ethanol precipitation and/or by any other available technique for the purification of proteins (See, e.g., Scopes, Protein Purification Principles and Practice 2nd Edition, Springer-Verlag, New York, 1987; Higgins, S.J. and Hames, B.D. (eds.), Protein Expression : A Practical Approach, Oxford Univ Press, 1999; and Deutscher, M.P., Simon, M.I., Abelson, J.N. (eds.), Guide to Protein Purification : Methods in Enzymology (Methods in Enzymology Series, Vol. 182), Academic Press, 1997, each of which is incorporated herein by reference). For immunoaffinity chromatography in particular, the protein may be isolated by binding it to an affinity column comprising antibodies that were raised against that protein and were affixed to a stationary support. Alternatively, affinity tags such as an influenza coat sequence, poly-histidine, or glutathione-S-transferase can be attached to the protein by standard recombinant techniques to allow for easy purification by passage over the appropriate affinity column. Protease inhibitors such as phenyl methyl sulfonyl fluoride (PMSF), leupeptin, pepstatin or aprotinin may be added at any or all stages in order to reduce or eliminate degradation of the protein during the purification process. Protease inhibitors are particularly advantageous when cells must be lysed in order to isolate and purify the expressed protein.

One of ordinary skill in the art will appreciate that the exact purification technique will vary depending on the character of the protein to be purified, the character of the cells from which the protein is expressed, and/or the composition of the medium in which the cells were grown.

Introduction of genes for the expression of proteins into host cells

Generally, a nucleic acid molecule introduced into the cell encodes the protein desired to be expressed according to the present disclosure.

Methods suitable for introducing nucleic acids sufficient to achieve expression of a protein of interest into mammalian host cells are known in the art. See, for example, Gething *et al.*, *Nature*, 293:620-625, 1981; Mantei *et al.*, *Nature*, 281:40-46, 1979; Levinson *et al.* EP

117,060; and EP 117,058, each of which is incorporated herein by reference. For mammalian cells, common methods of introducing genetic material into mammalian cells include the calcium phosphate precipitation method of Graham and van der Erb (*Virology*, 52:456-457, 1978) or the lipofectamine™ (Gibco BRL) Method of Hawley-Nelson (*Focus* 15:73, 1993). General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued Aug. 16, 1983. For various techniques for introducing genetic material into mammalian cells, see Keown *et al.*, *Methods in Enzymology*, 1989, Keown *et al., Methods in Enzymology*, 185:527-537, 1990, and Mansour *et al.*, *Nature*, 336:348-352, 1988. In some embodiments, a nucleic acid to be introduced is in the form of a naked nucleic acid molecule. For example, the nucleic acid molecule introduced into a cell may consist only of the nucleic acid encoding the protein and the necessary genetic control elements. Alternatively, a nucleic acid encoding the protein (including the necessary regulatory elements) may be contained within a plasmid vector. Non-limiting representative examples of suitable vectors for expression of proteins in mammalian cells include pCDNA1; pCD, see Okayama, *et al.* Mol. Cell Biol. 5:1136-1142, 1985; pMClneo Poly-A, see Thomas, *et al.* Cell 51:503-512, 1987; a baculovirus vector such as pAC 373 or pAC 610; CDM8 , see Seed, B. Nature 329:840, 1987; and pMT2PC, see Kaufman, *et al.* EMBO J. 6:187-195, 1987, each of which is incorporated herein by reference in its entirety. In some embodiments, a nucleic acid molecule to be introduced into a cell is contained within a viral vector. For example, a nucleic acid encoding the protein may be inserted into the viral genome (or a partial viral genome). Regulatory elements directing the expression of the protein may be included with the nucleic acid inserted into the viral genome (i.e., linked to the gene inserted into the viral genome) or can be provided by the viral genome itself.

Naked DNA can be introduced into cells by forming a precipitate containing the DNA and calcium phosphate. Alternatively, naked DNA can also be introduced into cells by forming a mixture of the DNA and DEAE-dextran and incubating the mixture with the cells or by incubating the cells and the DNA together in an appropriate buffer and subjecting the cells to a high-voltage electric pulse (e.g., by electroporation). A further method for introducing naked DNA cells is by mixing the DNA with a liposome suspension containing cationic lipids. The DNA/liposome complex is then incubated with cells. Naked DNA can also be directly injected into cells by, for example, microinjection.

Alternatively, naked DNA can also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. J. Biol. Chem. 263:14621, 1988; Wilson *et al.* J. Biol. Chem. 267:963-967, 1992; and U.S. Patent No. 5,166,320, each of which is hereby incorporated by reference in its entirety). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis.

Use of viral vectors containing particular nucleic acid sequences, e.g., a cDNA encoding a protein, is a common approach for introducing nucleic acid sequences into a cell. Infection of cells with a viral vector has the advantage that a large proportion of cells receive the nucleic acid, which can obviate the need for selection of cells which have received the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are generally expressed efficiently in cells that have taken up viral vector nucleic acid.

Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. Blood 76:271, 1990). A recombinant retrovirus can be constructed having a nucleic acid encoding a protein of interest inserted into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. Such a replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques.

The genome of an adenovirus can be manipulated such that it encodes and expresses a protein of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner *et al.* BioTechniques 6:616, 1988; Rosenfeld et al. Science 252:431-434, 1991; and Rosenfeld *et al.* Cell 68:143-155, 1992. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld *et al.,* 1992, cited supra), endothelial cells (Lemarchand *et al.,* Proc. Natl. Acad. Sci. USA 89:6482-6486, 1992), hepatocytes (Herz and Gerard, Proc. Natl. Acad. Sci. USA 90:2812-2816, 1993) and muscle cells (Quantin *et al.,* Proc. Natl. Acad. Sci. USA 89:2581-2584, 1992). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner *et al.* cited supra; Haj-Ahmand and Graham, J. Virol. 57:267, 1986). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material.

Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka *et al.* Curr. Topics in Micro. and Immunol., 158:97-129, 1992). It is also one of the few viruses that may integrate its DNA into

non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte *et al.*, Am. J. Respir. Cell. Mol. Biol. 7:349-356, 1992; Samulski *et al.*, J. Virol. 63:3822-3828, 1989; and McLaughlin *et al.,* J. Virol. 62:1963-1973, 1989). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (Mol. Cell. Biol. 5:3251-3260, 1985) can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.,* Proc. Natl. Acad. Sci. USA 81:6466-6470, 1984; Tratschin *et al.*, Mol. Cell. Biol. 4:2072-2081, 1985; Wondisford *et al.,* Mol. Endocrinol. 2:32-39, 1988; Tratschin *et al.,* J. Virol. 51:611-619, 1984; and Flotte *et al.,* J. Biol. Chem. 268:3781-3790, 1993).

When the method used to introduce nucleic acid molecules into a population of cells results in modification of a large proportion of the cells and efficient expression of the protein by the cells, the modified population of cells may be used without further isolation or subcloning of individual cells within the population. That is, there may be sufficient production of the protein by the population of cells such that no further cell isolation is needed and the population can be immediately be used to seed a cell culture for the production of the protein. Alternatively, it may be desirable to isolate and expand a homogenous population of cells from a few cells or a single cell that efficiently produce(s) the protein.

A gene encoding a protein of interest may optionally be linked to one or more regulatory genetic control elements. In certain embodiments, a genetic control element directs constitutive expression of the protein. In certain embodiments, a genetic control element that provides inducible expression of a gene encoding the protein of interest can be used. The use of an inducible genetic control element (e.g., an inducible promoter) allows for modulation of the production of the protein in the cell. Non-limiting examples of potentially useful inducible genetic control elements for use in eukaryotic cells include hormone- regulated elements (e.g., see Mader, S. and White, J.H., *Proc. Natl. Acad. Sci. USA* 90:5603-5607, 1993), synthetic ligand-regulated elements (see, e.g. Spencer, D.M. *et al., Science* 262:1019-1024, 1993) and ionizing radiation-regulated elements (e.g., see Manome, Y. *et al., Biochemistry* 32:10607-10613, 1993; Datta, R. *et al., Proc. Natl. Acad. Sci. USA* 89:10149-10153, 1992). Additional cell-specific or other regulatory systems known in the art may be used in accordance with the invention.

One of ordinary skill in the art will be able to choose and, optionally, to appropriately modify the method of introducing genes that cause the cell to express the protein of interest in accordance with the teachings of the present invention.

Immunogenic compositions

The RSV F proteins of subtype A and B produced by the methods disclosed herein can be included in immunogenic compositions for use as vaccines.

In addition to the immunogenic component, the vaccine may further comprise an immunomodulatory agent, such as an adjuvant. Examples of suitable adjuvants include aluminum salts such as aluminum hydroxide and/or aluminum phosphate; oil-emulsion compositions (or oil-in-water compositions), including squalene-water emulsions, such as MF59 (see e.g., WO 90/14837); saponin formulations, such as, for example, QS21 and Immunostimulating Complexes (ISCOMS) (see e.g., U.S. Pat. No. 5,057,540; WO 90/03184, WO 96/11711, WO 2004/004762, WO 2005/002620); bacterial or microbial derivatives, examples of which are monophosphoryl lipid A (MPL), 3-O-deacylated MPL (3dMPL), CpG-motif containing oligonucleotides, ADP-ribosylating bacterial toxins or mutants thereof, such as E. coli heat labile enterotoxin LT, cholera toxin CT, and the like. It is also possible to use vector-encoded adjuvant, e.g., by using heterologous nucleic acid that encodes a fusion of the oligomerization domain of C4-binding protein (C4 bp) to the antigen of interest (e.g., Solabomi et al., 2008, Infect Immun 76: 3817-23). In certain embodiments the compositions hereof comprise aluminum as an adjuvant, e.g., in the form of aluminum hydroxide, aluminum phosphate, aluminum potassium phosphate, or combinations thereof, in concentrations of 0.05-5 mg, e.g., from 0.075-1.0 mg, of aluminum content per dose.

Examples

GS-CHO clones recombinantly expressing RSV F protein of subtype A (hereafter RSV F (A)) or of subtype B (hereafter RSV F (B)) were maintained at 36.5ºC and 5% CO_2 in a 120 or 140 rpm shaking incubator. Cultures were seeded at 0.35 x 10^6 cells/mL or 0.20 x 10^6 cells/mL for 3 or 4 day passages during seed expansion, respectively. The N-1 seed cultures for all experiments were run in 2L Applikon® bioreactors with 1L working volume and passaged at 0.70 x 10^6 cells/mL for 4 days in a medium with high nutrient content.

Production experiments were performed in 2 L Applikon® bioreactors with BioNet® controllers using a glucose restricted fed-batch process, hereafter referred to as a HiPDOG process (Gagnon et al (2011) Biotechnology and bioengineering 108: 1328-1337). Specific methods and parameters are listed in the subsequent experiment sections.

On the day of harvest, the cell culture broth is clarified by centrifugation and depth filtration. Downstream processing includes ultrafiltration and diafiltration 1 (UF/DF1), to concentrate and buffer exchange material prior to the capture chromatography step, an anion exchange

chromatography (AEX) column, operated in bind and elute mode. The polishing columns include a ceramic hydroxyapatite chromatography (CHA) in flow through mode and hydrophobic interaction chromatography (HIC) column in bind and elute mode. The downstream process concludes with a virus retaining filtration step, an ultrafiltration and diafiltration 2 (UF/DF2), and a final filtration step.

In the following experiments, titer, trimer, high molecular mass species (HMMS), low molecular mass species (LMMS) and host cell protein (HCP) are reported.

Titer can be determined by any method known in the art. In the following experiment, titer was measured by reverse phase high-performance liquid chromatography (RP-HPLC). Reversed phase chromatography separates molecules based on polarity. Relatively non-polar molecules, including RSV F protein of subtype A or B, bind to the column, while polar molecules flow through the column without binding. The bound molecules are eluted from the column through the application of a mobile phase gradient that passes from polar to less polar conditions. Molecules are eluted in order of decreasing polarity. Detection is performed using ultraviolet (UV) absorption at 220 nm. Titer determination is accomplished through comparison of sample peak area to that of a calibration standard.

The following conditions were used in the following experiments disclosed herein:

Condition	Setting
Column Type	Agilent Zorbax, 300SB-C3, 150 x 3.0 mm, 3.5 µm
Mobile Phase A (MPA)	0.1 % TFA (v/v) in water
Mobile Phase B (MPB)	0.1 % TFA (v/v) in 90 % acetonitrile
Column Temperature	55 ± 5 °C
Flow Rate & Run Time	0.75 mL/minute for 20 minutes
Autosampler Temperature	5 ± 3 °C
Injection Volume	5 – 100 µL (15 µg target load)
Detector Wavelength	UV at 220 nm

Gradient Conditions

Time (minutes)	Flow Rate (mL/min)	% MPA	% MPB
0	0.75	90	10
2	0.75	90	10
2.1	0.75	65	35
12	0.75	27	73
12.1	0.75	5	95
16	0.75	5	95
16.1	0.75	90	10
20	0.75	90	10

Trimer, HMMS and LMMS were measured by size exclusion chromatography (SEC-HPLC). SEC-HPLC is an analytical method known to the skilled person and used to determine the relative content of high molecular mass species (HMMS), trimer and low molecular mass species (LMMS) in the RSV F protein of subtype A or B samples obtained by the methods of the invention. SEC-HPLC separates molecules by their hydrodynamic volume. When the analyte is applied to the head of the column bed, molecules that are smaller than the pores of the packing material can diffuse into and out of the pores, whereas those that are larger do not enter the pores. As a result, the larger molecules pass through the column more quickly and smaller molecules more slowly. Once the species elute, they are detected by UV absorption at 280 nm. Low Molecular Mass species (LMMS) is the term used for all species of apparent molecular mass less than the trimer as measured by SEC-HPLC. They elute after the trimer peak. High Molecular Mass species (HMMS) is the term used for all peaks of apparent molecular mass greater than the trimer as measured by SEC-HPLC. They elute before the trimer peak and may include aggregates.

HCP was measured by enzyme-linked immunoassay (ELISA), a quantitative assay which measures residual Chinese Hamster Ovary (CHO) Host Cell Proteins (HCPs), using a sandwich-type ELISA analysis. The major steps in the HCP assay are outlined below.

A set of standard samples are prepared from highly enriched CHO HCP material. The standard samples range in concentration from 2 ng/mL to 256 ng/mL of CHO HCPs. Test samples are diluted to four RSV protein F of subtype A or B concentrations. Lastly, a control sample is tested on each assay plate. The assay plate is coated with polyclonal antibodies raised against the highly enriched preparation of the CHO HCPs (anti-CHO HCPP pAbs). After the coating is completed, the plate is blocked to minimize non-specific binding of analytes and reagents. After blocking, the standards, the test samples, and the control sample are added to the assay plate and incubated to allow the HCPs in these samples to be captured by the anti-CHO HCP antibodies. The plate is then washed to remove any unbound proteins and leave the HCP-antibody complex. To quantify the amount of bound HCPs in each well, a preparation of the anti-CHO HCP antibody conjugated to biotin is added to the assay plate and allowed to bind to the captured HCPs. The plate is washed to remove any unbound biotinylated antibody and a streptavidin-horseradish peroxidase (HRP) conjugate is added which binds to the biotin-anti-CHO HCP conjugate. The plate is washed to remove any unbound streptavidin-HRP and a solution of 3,3',5,5'-tetramethyl benzidine (TMB) is added to the assay plate. TMB is a substrate which generates a blue color in the presence of HRP. The assay plates are incubated with the TMB reagent for a period of time to generate an appropriate signal in each of the wells and the peroxidase reaction is quenched by the addition of sulfuric acid. Lastly the absorbance in each well is measured and recorded at 450 nm using a suitable plate reader. The generated

signal is proportional to the amount of HCPs captured on the assay plate. The signal in the standard sample wells is plotted against the standard HCP concentration. The plot is fit to a four-parameter logistic (4PL) fit to generate an HCP standard curve. The signal in the test samples and the external control sample is then used to determine the HCP content in these samples by interpolation of the absorbance signal against the pseudo linear portion of the standard 4PL function.

From an overall productivity and downstream filterability perspective, the process is most optimal when titer and trimer are maximized and HMMS, LMMS and HCP are minimized. RP-HPLC titer measures the total amount of RSV protein present in the sample, including aggregate and RSV protein that is not in the trimeric form. Trimer, as measured by SEC, provides an estimate of approximately how much RSV molecule in the trimeric form is present as a percentage of the total amount of protein present (including some process impurities). The manipulation of process parameters, such as growth temperature, may increase trimer while negatively impacting titer (or vice versa). To demonstrate the overall impact to both titer and trimer, "triter" is reported, which is calculated by multiplying trimer by titer. Triter provides an estimate of how much protein is produced in the trimeric form.

Example 1 – effect of temperature on RSV F protein production in CHO cells

This set of experiments was designed to assess the effect of the temperature pre and post shift as well as the timing of the shift on titer and trimer formation during the production of RSV F proteins of subtype A and B by CHO cells.

Production experiments were performed in 2 L Applikon® bioreactors with BioNet® controllers using conditions detailed in Table 1. All conditions were run in a fedbatch process comprising a phase where the amount of glucose provided to the cells is restricted (HipDOG from day 0 to day 5 for RSV F (A) and day 0 to day 4 for RSV F (B)) and using a cell culture medium without hydrocortisone.

Table 1. Bioreactor Production Process Parameters

Inoculation Density	3.0 x 10^6 cells/mL
Process	Fed batch with HiPDOG
pH set point during HiPDOG	7.075 +/- 0.025
pH set point post HiPDOG	7.05 +/- 0.15
DO set point	40%
Agitation	80 W/m^3

Impellers	Rushton (1)
Sparger	100 µm sintered steel
Sparge	Pure O_2
Headspace	Air/7% CO_2 mix @ 100 sccm
Feed Rate Post HiPDOG	RSV F subtype A: 21 mL/L/day RSV F subtype B: 25.5 mL/L/day
Glucose Feed	500 g/L glucose, target 2 g/L
Titrant	0.94 M sodium carbonate + 0.06 M potassium carbonate
Antifoam	EX-CELL® as needed
Process Duration	12 days
Vessel Size	2 L Applikon®
Working Volume	1 L

1.1 Effect of growth temperature

In this experiment, the cells were grown at a temperature of 33°C, 34.5°C or 36°C to assess the effect of the growth temperature on titer, percentage of trimer, HMMS, LMMS, triter and the amount of Host Cell Protein (HCP). The results are shown in Table 2 and in Figures 1A, 1B, 2A, 2B, 3A, 3B, 4A and 4B.

Table 2 – Effect of growth temperature

Cell line	Growth Temp. (°C)	Production Temp. (°C)	Temp. Shift (hours)	HCP (µg/mL)	RP-HPLC (g/L)	SEC HMMS (%)	SEC LMMS (%)	SEC Trimer (%)	Triter (g/L)
RSV F (A)	33	31	144	242	0.62	32	24	44	0.27
	34.5	31	144	267	0.73	39	23	37	0.27
	36	31	144	307	0.63	45	25	30	0.19
RSV F (B)	33	31	144	131	1.44	33	12	55	0.78
	34.5	31	144	130	1.91	38	19	43	0.82
	36	31	144	243	1.74	42	21	37	0.64

For both antigens, the growth temperature negatively correlated with percentage of trimer and positively correlated with percentage of HMMS and LMMS (see figures 1A and AB). The highest titer was consistently obtained with the temperature of 34.5°C (see figures 2A and 2B).

A growth temperature between 34°C and 35°C, and preferably 34.5°C is suitable for maximizing trimer, titer and minimizing impurities. For both antigens, HCP levels positively correlated with temperature (see figures 3A and 3B). For subtype B, the highest triter was obtained with the temperature of 34.5°C and for subtype A the triter at 33°C and 34.5°C was higher than at 36°C (see figures 4A and 4B).

1.2 Effect of production temperature

In this experiment, the growth temperature was 34.5°C and the production temperature was varied (28.5°C, 31°C or 34°C) to assess the effect of the production temperature on titer, percentage of trimer, HMMS, LMMS, triter, and the amount of HCP. The results are shown in Table 3 and in Figures 5A, 5B, 6A, 6B, 7A, 7B, 8A and 8B.

Table 3 – Effect of production temperature

Cell line	Growth Temp. (°C)	Prod Temp. (°C)	Temp. Shift (hrs)	HCP (µg/mL)	RP-HPLC (g/L)	SEC HMMS (%)	SEC LMMS (%)	SEC Trimer (%)	Triter (g/L)
RSV F (A)	34.5	28.5	144	314	0.60	37	23	39	0.24
	34.5	31	144	267	0.73	39	23	37	0.27
	34.5	34	144	355	0.71	45	26	29	0.21
RSV F (B)	34.5	28.5	144	198	1.47	36	15	49	0.72
	34.5	31	144	130	1.91	38	19	43	0.82
	34.5	34	144	327	2.08	41	22	38	0.79

The production temperature (post temperature shift) had a negative linear correlation with trimer and a positive linear correlation with LMMS, and HMMS for both antigens (see figures 5A and 5B). The lowest HCP levels and the highest triter levels were obtained for the 31°C production temperature (see figures 7A,7B and 8A and 8B).

1.3 Effect of timing of temperature shift

In this experiment, the timing of the temperature shift was varied to assess its effect on titer, percentage of trimer, HMMS, LMMS triter, and the amount HCP. The results are shown in Table 4 and in Figures 9A, 9B, 10A, 10B, 11A, 11B, 12A and 12B.

Table 4 – Effect of temperature shift timing

Cell line	Growth Temp. (°C)	Prod. Temp. (°C)	Temp. Shift (hrs)	HCP (µg/mL)	RP-HPLC (g/L)	SEC HMMS (%)	SEC LMMS (%)	SEC Trimer (%)	Triter (g/L)
RSV F (A)	34.5	31	114	273	0.61	46	28	26	0.16
	34.5	31	144	267	0.73	39	23	37	0.27
	34.5	31	185.5	375	0.44	42	29	28	0.12
RSV F (B)	34.5	31	114	167	1.79	37	17	46	0.83
	34.5	31	144	130	1.91	38	19	43	0.82
	34.5	31	185.5	194	1.82	33	18	49	0.90

A shift of the temperature at 144 hours after the start of the culture improved the amount of trimer, titer and level of HCP as compared to a shift at a different culture duration. This is true for both antigens and all attributes apart from trimer for RSV F (B) which was highest with a temperature shift at 185.5 hours after the start of the culture. The highest triter was obtained at a temperature shift of 144 hours for RSV F (A). Triter levels for RSV F (B) were similar at 144 and 114 hours, both lower than at 185.5 hours.

Example 2 – effect of temperature shift on RSV F protein production in CHO cells

This experiment was designed to assess the effect of the presence of a temperature shift on process performance, titer and trimer formation during the production of RSV F proteins of subtype A and B by CHO cells.

Production experiments were performed in 2 L Applikon® bioreactors with BioNet® controllers using conditions detailed in Table 5. All conditions were run in a fedbatch process comprising a phase where the amount of glucose provided to the cells is restricted (HipDOG from day 0 to day 5 for RSV F (A) and day 0 to day 4 for RSV F (B)) and using a cell culture medium without hydrocortisone.

Table 5. Bioreactor Production Process Parameters

Inoculation Density	2.5 x 10^6 cells/mL
Process	Fed batch with HiPDOG
pH set point during HiPDOG	7.025 +/- 0.025
pH set point post HiPDOG	7.05 +/- 0.15
DO set point	40%
Agitation	80 W/m^3
Impellers	Rushton (1)
Sparger	100 µm sintered steel
Sparge	Pure O_2
Headspace	Air/7% CO_2 mix @ 100 sccm
Feed Rate Post HiPDOG	847A: 21 mL/L/day 847B: 25.5 mL/L/day
Glucose Feed	500 g/L glucose, target 1.5 g/L
Titrant	0.94 M Na_2CO_3 + 0.06 M K_2CO_3
Antifoam	EX-CELL® as needed
Process Duration	12 days
Vessel Size	2 L Applikon®
Working Volume	1 L

Results are shown in Tables 6 and 7 and Figures 13A, 13B, 14A, 14B, 15A and 15C.

Table 6. Results with and without a temperature shift (averages).

	Average								
	Growth Temp. (°C)	Prod. Temp (°C)	Temp. Shift Day	RP-HPLC Titer (g/L)	HMMS (%)	Trimer (%)	LMMS (%)	HCP (µg/ml)	Triter (g/L)
RSV F (A)	34.5	31	6	0.89	38	39	22	273	0.35
	34.5	34.5	N/A	0.86	45	32	23	381	0.27
RSV F (A)	34.5	31	6	1.81	47	37	16	267	0.67
	34.5	34.5	N/A	1.55	40	35	25	470	0.54

Table 7. Results with and without a temperature shift (standard deviations).

Standard Deviation									
Antigen	Growth Temp. (°C)	Prod. Temp. (°C)	Temp. Shift Day	RP-HPLC Titer (g/L)	HMMS (%)	Trimer (%)	LMMS (%)	HCP (µg/ml)	Triter (g/L)
RSV F (A)	34.5	31	6	0.09	5	4	2	65	0.05
	34.5	34.5	N/A	0.07	5	4	2	124	0.03
RSV F (A)	34.5	31	6	0.08	7	7	3	88	0.13
	34.5	34.5	N/A	0.06	3	3	2	105	0.06

The presence of a temperature shift increased trimer levels, decreased HCP, and increased titer for both antigens (see Figures 13A, 13B, 14A, 14B, 15A, and 15B).

Example 3 – effect of glucocorticoid compounds on RSV F protein production in CHO cells

This experiment was designed to understand the effect of glucocorticoid compounds such as hydrocortisone on titer and product quality of RSV F protein of subtype A and B produced in CHO cells.

Production experiments were performed in 2 L Applikon® bioreactors with BioNet® controllers using the process detailed in Table 8 in cell culture media with or without hydrocortisone.

All bioreactors were run at 34.5°C and a temperature shift to 31°C was performed with bioreactor (B08) on day 6. Glucose was provided in a restricted manner (Hipdog) from day 0 to day 4 for RSV F (B) and day 0 to day 5 for RSV F (A).

Table 8. Bioreactor Production Process Parameters

Inoculation Density	2.5 x 10^6 cells/mL
Process	Fed batch with HiPDOG
HiPDOG End	Day 4 for RSV F (B); Day 5 for RSV F (A)
pH set point during HiPDOG	7.025 +/- 0.025
pH set point post HiPDOG	7.05 +/- 0.15
DO set point	40%
Temperature	34.5°C
Agitation	80 W/m^3

Impellers	Rushton (1)
Sparger	100 µm sintered steel
Sparge	Pure O_2
Headspace	Air/7% CO_2 mix @ 100 sccm
Production Medium	+/- 0.54 mg/L hydrocortisone
Feed Medium	+/- 1.08 mg/L hydrocortisone
Feed Rate Post HiPDOG	RSV F (A): 21 mL/L/day RSV F (B): 25.5 mL/L/day
Glucose Feed	500 g/L glucose with 7.5 g/L cysteine, target 1.5 g/L
Titrant	0.94 M sodium carbonate + 0.06 M potassium carbonate
Antifoam	EX-CELL® as needed
Process Duration	12 days
Vessel Size	2 L Applikon
Working Volume	1 L

Hydrocortisone had a negative effect on furin processing of RSV F protein as indicated by the Western blot results shown in figures 16 and 17. The Western blot allows monitoring of processed RSV F (A) or RSV F (B) monomers and related species. Pre-fusion F trimers are specifically recognized by mAb AM14 (Gilman MS et al, PLoS Pathogens,11(7), 2015). The term "AM14" refers to an antibody described in WO 2008/147196 A2, which has a heavy chain variable domain comprising an amino acid sequence of SEQ ID NO:3 and a light chain variable domain comprising an amino acid sequence of SEQ ID NO:4. Results are collected to monitor the process capabilities and levels of processed RSV F (A) or RSV F (B) monomer, partially processed or unprocessed F+p27 or other size variants. The lanes for those conditions which contained hydrocortisone (B-07, B-04, B-03 and A-01 in Figure 16 and A-04, A-05, B-03, B-07) present a smear directly above the RSV band (approximately 60kDa) as identified by binding of the AM-14 antibody. The presence of a smear is an indication of partially processed RSV variants.

Therefore, it is advantageous not to include hydrocortisone or other related glucocorticoid compound in the cell culture medium to be used in the method of the invention in order to improve the amount of processed material suitable for being used in vaccine composition in particular in the form of trimer.

Example 4 - effect of HiPDOG on RSV F production in CHO cells

Stabilization of the prefusion conformation is important for the RSV protein as the postfusion conformation is energetically favored and less immunogenic, with the transition from prefusion to postfusion being irreversible. RSV F protein of subtype A and B can be engineered to stabilize the protein in the prefusion conformation and disulfide bonds contribute to this stability. Consequentially, disulfide bond integrity could impact the stability of the desired conformation. An inter-subunit disulfide bond in RSV was found to be unpaired to a small extent in the initial fed batch process. The two corresponding unpaired cysteines were found modified with cysteinyl moieties. This modification is measured and reported as "cysteinylation" which is measured by amino acid analysis coupled to a QDa mass detector.

This experiment was designed to understand the effect of HiPDOG on the level of cysteinylation on the RSV F of subtype A and B produced in CHO cells. Bioreactor parameters are listed in Table 9.

Table 9. Bioreactor Production Process Parameters

Inoculation Density	3.0 x 10^6 cells/mL
Process	Fed batch with or without HiPDOG
HiPDOG End	Day 4 for RSV F (B); Day 5 for RSV F (A)
pH set point during HiPDOG	7.075 +/- 0.025
pH set point post HiPDOG	7.05 +/- 0.15
DO set point	40%
Temperature	34.5°C throughout
Agitation	80 W/m^3
Impellers	Rushton (1)
Sparger	100 µm sintered steel
Sparge	Pure O_2
Headspace	Air/7% CO_2 mix @ 100 sccm
Feed Rate Post HiPDOG	RSV F subtype A: 21 mL/L/day RSV F subtype B: 25.5 mL/L/day
Glucose Feed	500 g/L glucose, target 2 g/L
Titrant	0.94 M sodium carbonate + 0.06 M potassium carbonate
Antifoam	EX-CELL® as needed
Process Duration	12 days
Vessel Size	2 L Applikon
Working Volume	1 L

The level of cysteinylation was reduced for both the RSV F (A) and RSV F (B) antigens when HiPDOG control was employed (Table 10).

Table 10. Cysteinylation Results

Cell line	Condition	Cysteinylation (%)
RSV F (A)	Without HiPDOG	7.63
RSV F (A)	With HiPDOG	3.84
RSV F (B)	Without HiPDOG	2.08
RSV F (B)	With HiPDOG	1.38

In addition, titer was improved for both the RSV F (A) and RSV F (B) antigens when HiPDOG was employed (Table 11). The titer measurements reported are after the first purification step (ultrafiltration).

Table 11. Titer Results

Cell line	Condition	Ultrafiltration Pool Titer (g/L)
RSV F (A)	Without HiPDOG	0.70
RSV F (A)	With HiPDOG	1.80
RSV F (B)	Without HiPDOG	1.87
RSV F (B)	With HiPDOG	3.43

Example 5 – Large scale manufacturing processs

The suitability of the method of the invention for use at large scale was tested. CHO cells expressing RSV protein F of subtype A or subtype B were cultured in a 12 day fed batch process using HiPDOG, a growth temperature of 34.5°C and a production temperature of 31°C with a temperature shift on day 6. As shown in below Table 12, the method of the invention provided advantageous triter values even when performed in 2500 or 12500L bioreactors.

Table 12 – Results from large scale experiments

Cell line	Scale (L)	HCP (ug/mL)	RP-HPLC (g/L)	HMMS (%)	Trimer (%)	LMMS (%)	Triter (g/L)
RSV F (A)	2500	303	0.86	38	38	24	0.33
	12500	300	0.84	39	36	24	0.30
RSV F (B)	2500	268	1.49	40	41	19	0.61
	12500	211	1.79	39	38	23	0.68

Listing of Raw Sequences

SEQ ID NO: 1. Amino Acid Sequence of the Full Length F0 of Native RSV A2 (GenBank GI: 138251; Swiss Prot P03420)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKENKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPPTNNRARRELPRFMNYTLNNAKKTNVTLSKKRKRRF
LGFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPIVNKQSCSISNIE
TVIEFQQKNNRLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVL
AYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSL
TLPSEINLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGMDTVSV
GNTLYYVNKQEGKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVI
IVILLS LIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

SEQ ID NO: 2. Amino Acid Sequence of the Full Length F0 of Native RSV B (18537 strain; GenBank GI: 138250; Swiss Prot P13843)

MELLIHRSSAIFLTLAVNALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
LSNIKETKCNGTDTKVKLIKQELDKYKNAVTELQLLMQNTPAANNRARREAPQYMNYTINTTKNLNVSISKKRKRRF
LGFLLGVGSAIASGIAVSKVLHLEGEVNKIKNALLSTNKAVVSLSNGVSVLTSKVLDLKNYINNRLLPIVNQQSCRISNIE
TVIEFQQMNSRLLEITREFSVN
AGVTTPLSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYV
VQLPIYGVIDTPCWKLHTSPLCTTNIKEGSNICLTRTDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMNSLTLPS
EVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLY
YVNKLEGKNLYVKGEPIINYYDPLVFPSDEFDASISQVNEKINQSLAFIRRSDELLHNVNTGKSTTNIMITTIIIVIIVVLLS
LIAIGLLLYCKAKNTPVTLSKDQLSGINNIAFSK

SEQ ID NO:3: Amino Acid Sequence of Heavy Chain Variable Domain of Antibody AM14:

EVQLVESGGGVVQPGRSLRLSCAASGFSFSHYAMHWVRQAPGKGLEWVAVISYDGENTYYADSVKGRFSISRDNS
KNTVSLQMNSLRPEDTALYYCARDRIVDDYYYYGMDVWGQGATVTVSS

SEQ ID NO:4: Amino Acid Sequence of Light Chain Variable Domain of Antibody AM14:

DIQMTQSPSSLSASVGDRVTITCQASQDIKKYLNWYHQKPGKVPELLMHDASNLETGVPSRFSGRGSGTDFTLTISS
LQPEDIGTYYCQQYDNLPPLTFGGGTKVEIKRTV

Claims

1. A method for producing an RSV F protein trimer in a fed batch cell culture, said method comprising the steps of:
(i) providing mammalian cells that contain a gene encoding an RSV F protein in a cell culture medium to start a cell culture, and,
(ii) culturing the cells at a temperature between about 33.0°C and 35.0°C, and
(iii) providing glucose in a restricted manner to the cell culture by feeding glucose to the cell culture in response to rise of pH above a predetermined pH value.

2. The method according to claim 1, wherein the temperature is about 34.5°C.

3. The method according to claim 1 or 2 wherein the temperature is shifted to a lower temperature, preferably between about 30.0°C and about 32.0°C.

4. The method according to claim 3 wherein the lower temperature is about 31.0°C

5. The method according to claim 3 or 4 wherein the temperature is shifted to a lower temperature between day 3 and day 7.

6. The method according to any one of claims 3 to 5 wherein the temperature is shifted to a lower temperature on day 5 or on day 6.

7. The method according to any one of the preceding claims, wherein a pH sensor is used to monitor pH of the cell culture.

8. The method according to any one of the preceding claims, wherein the predetermined pH value corresponds to an increase of 0.01 to 0.10 above the pH set point of the culture.

9. The method according to any one of the preceding claims, wherein the predetermined pH value corresponds to an increase of 0.05 above the pH set point of the culture.

10. The method according to any one of the preceding claims wherein the pH set point of the cell culture is between 6.70 and 7.30.

11. The method according to any one of the preceding claims wherein the pH set point of the cell culture is between 6.90 and 7.20.

12. The method according to any one of the preceding claims wherein the pH set point of the cell culture is between 7.00 and 7.10.

13. The method according to any one of the preceding claims wherein the pH set point of the cell culture is 7.05.

14. The method according to any one of claims 1 to 9 wherein:
- the pH set point is 6.95, 7.01, 7.05, 7.07 or 7.20 during the phase of the cell culture where glucose is provided in a restricted manner and,
- the pH set point is 6.90 or 7.20 after the phase of the cell culture where glucose is provided in a restricted manner.

15. The method according to any one of the preceding claims wherein feeding glucose to the cell culture in response to rise of pH above a predetermined pH value comprises feeding glucose until the pH decreases to reach the pH set point of the culture.

16. The method according to any one of the preceding claims wherein glucose is provided in a restricted manner from day 1 to day 6.

17. The method according to any one of the preceding claims, wherein the cell culture medium does not comprise glucocorticoid compounds.

18. The method according to any one of the preceding claims, wherein the cell culture medium does not comprise any of hydrocortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone and fludrocortisone acetate.

19. The method according to any one of the preceding claims, wherein the cell culture medium does not comprise hydrocortisone.

20. The method according to any one of the preceding claims, wherein the cell culture medium is serum free.

21. The method according to any one of the preceding claims, wherein the cell culture medium is protein free.

22. The method according to any one of the preceding claims, wherein the cell culture medium is defined.

23. The method according to any one of the preceding claims, wherein the cell culture is further provided with a feed medium.

24. The method according to claim 23, wherein the feed medium is provided continuously or at multiple intervals.

25. The method according to claim 23 or 24, wherein the feed medium does not comprise glucocorticoid compounds.

26. The method according to any of claims 23 to 25, wherein the feed medium does not comprise any of hydrocortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone and fludrocortisone acetate.

27. The method according to any of claims 23 to 26, wherein the cell culture medium does not comprise hydrocortisone.

28. The method according to any of claims 23 to 27, wherein the feed medium is serum free.

29. The method according to any of claims 23 to 28, wherein the feed medium is protein free.

30. The method according to any of claims 23 to 29, wherein the feed medium is defined.

31. The method of any one of the preceding claims wherein the maximum viable cell density during the cell culture is above 1 x 10^6 cells/mL.

32. The method of any one of the preceding claims wherein the maximum viable cell density is above 5 x 10^6cells/mL, 1 x 10^7cells /mL, 5 x 10^7 cells/mL, 1X10^8 cells/mL or 5X10^8 cells/mL.

33. The method of any one of the preceding claims wherein the volume of the cell culture medium is at least 500L.

34. The method of claim 33, wherein the volume of the cell culture medium is at least 3000L.

35. The method according to any one of the preceding claims wherein the mammalian cells are selected from BALB/c mouse myeloma line, human retinoblasts (PER.C6), monkey kidney cells, human embryonic kidney line (293), baby hamster kidney cells (BHK), Chinese hamster ovary cells (CHO), mouse sertoli cells, African green monkey kidney cells (VERO-76), human cervical carcinoma cells (HeLa), canine kidney cells, buffalo rat liver cells, human lung cells, human liver cells, mouse mammary tumor cells, TRI cells, MRC 5 cells, FS4 cells, or human hepatoma line (Hep G2).

36. The method according to any one of the preceding claims wherein the mammalian cells are Chinese hamster ovary cells (CHO).

37. The method according to any one of the preceding claims wherein the mammalian cells are GS-CHO cells.

38. The method according to any one of the preceding claims, wherein the RSV F protein is of subtype A.

39. The method according to any one of the preceding claims, wherein the RSV F protein is of subtype B.

40. The method according to any one of the preceding claims wherein the RSV F protein comprises mutations stabilizing the trimer in the pre-fusion conformation.

41. The method according to any one of the preceding claims wherein the RSV F protein comprise a combination of mutations selected from the group consisting of:

(1) combination of T103C, I148C, S190I, and D486S;

(2) combination of T54H S55C L188C D486S;

(3) combination of T54H, T103C, I148C, S190I, V296I, and D486S;

(4) combination of T54H, S55C, L142C, L188C, V296I, and N371C;

(5) combination of S55C, L188C, and D486S;

(6) combination of T54H, S55C, L188C, and S190I;

(7) combination of S55C, L188C, S190I, and D486S;

(8) combination of T54H, S55C, L188C, S190I, and D486S;

(9) combination of S155C, S190I, S290C, and D486S;

(10) combination of T54H, S55C, L142C, L188C, V296I, N371C, D486S, E487Q, and D489S; and

(11) combination of T54H, S155C, S190I, S290C, and V296I.

42. The method of any one of claims 1 to 41, further comprising obtaining the RSV F protein trimer produced by the cells.

43. The method of claim 41, further comprising purifying the RSV F protein trimer.

44. A pharmaceutical composition comprising a purified RSV F protein trimer obtained by the method according to claim 42 in combination with a pharmaceutically acceptable carrier.