

## PRODUCTION OF POXVIRUSES FROM QUAIL CELL CULTURES

### FIELD OF THE INVENTION

The present invention relates to methods of optimizing production of poxvirus viral vector-based vaccine products from avian cell cultures. The poxvirus viral vector can be a Modified vaccinia virus Ankara (“MVA”) or recombinant MVA that encodes heterologous antigens. The viruses from the cell cultures can be used to produce vaccines.

### BACKGROUND OF THE INVENTION

Poxviruses have a long history of providing vaccines for immune protection against infection and disease. One example is Modified Vaccinia Virus Ankara (“MVA”), a highly attenuated strain of vaccinia virus (genus *Orthopoxvirus*). MVA-BN® virus developed by Bavarian Nordic® A/S has been used as a vaccine against smallpox and monkeypox marketed under the brand names IMVAMUNE®, IMVANEX®, and JYNNEOS®. Moreover, recombinant MVA-BN® virus encoding various heterologous antigens has also been used as a vaccine. For example, a recombinant MVA-BN® virus encoding antigens from four different filoviruses (Ebola virus, Sudan virus, Tai Forest virus, and Marburg virus) provides an improved vaccine against Ebola virus and is disclosed, for example, in WO 2016/034678. This MVABEA® vaccine, used in combination with Zabdeno® vaccine as part of a 2-component vaccine regimen, has been approved for use in the prevention of Ebola virus disease.

The cells approved for production of MVA-BN® virus were primary chicken embryonic fibroblast cells (“CEF” cells). CEF cells have been widely used to study the interactions between cells and viruses and in the production of vaccines. However, there are several drawbacks of primary CEF cells in this context, for example, the time, cost, and labor involved in preparing these cells (see, *e.g.*, Farzaneh *et al.* (2017) *British Poultry Science* 58: 681-686), variability of the cell substrate in each batch, and the preparation procedure being prone to contamination.

One alternative to CEF cells may be the duck embryo-derived EB66® cell line (see, *e.g.*, Leon *et al.* (2016) *Vaccine* 34: 5878-85). Continuous avian cell lines have also been developed from the Muscovy duck (Jordan *et al.* (2016) *Avian Pathology* 45: 137-155) and the peacock (Wang *et al.* (2022) *Poultry Sci.* 101: 102147). Another alternative to CEF cells may be

continuous quail cell lines, which have been produced by various means (see, *e.g.*, Kraus *et al.* (2011) *BMC Proceedings* 5 (Suppl. 8): P52; Lee *et al.* (2008) *J. Virol. Meth.* 153: 22-8). Quail cells lack most of the endogenous retroviral (ERV) sequences detectable in chicken cells. For example, sequences from the subgroup of the endogenous avian retrovirus family termed EAV-HP were found in chickens but are completely absent in quails (see, *e.g.*, Smith *et al.* ((1999) *J. Gen. Virol.* 80: 261-268). A comparative mapping of quail and chicken genomes revealed that only 393 intact ERV were identified in quail, versus 1212 in chicken (Morris *et al.* ((2020) *BMC Biol.* 18: 14).

However, manufacturing costs for vaccines remain high, in part because cell lines often fail to meet the desired yields (see, *e.g.*, Hoeksema *et al.* (2018) *Vaccine* 36: 2093-2103), or because the resulting product has unacceptably high levels of impurities such as total protein and host cell DNA. Regulatory authorities require very low levels of host cell DNA in the final vaccine product, for example, less than 100 nanograms per vaccine dose. Therefore, there remains a need for safe, efficient, and commercially viable methods for processing virus-infected cell lines to produce vaccines, including vaccines comprising recombinant poxviruses.

### **BRIEF SUMMARY OF THE INVENTION**

The present invention relates to methods of cultivating and processing avian cells infected with poxviruses to produce viral vector-based vaccine products. In some embodiments, the viral vector is Modified Vaccinia Virus Ankara ("MVA"), such as MVA-BN® virus. In some embodiments, the viral vector is a recombinant MVA encoding one or more heterologous antigens and the avian cells are used to produce a vaccine comprising the recombinant MVA and/or the encoded antigens. In some embodiments, the recombinant MVA encodes one or more antigens of Respiratory Syncytial Virus (RSV) and the avian cells are used to produce a vaccine protecting against disease caused by RSV. In some embodiments, the recombinant MVA encodes a tumor-associated antigen and the avian cells are used to produce a vaccine that stimulates an immune response to the antigen.

In some embodiments, methods of cultivating avian cells to increase viral titers are also provided. In some embodiments, the avian cells are quail cells. Higher viral titers in infected cell lines can be helpful in producing a superior vaccine product with lower levels of contaminants such as host cell DNA. Thus, in some embodiments, the methods of the invention

include an “upstream process” designed to maximize the viral titer produced by the cultured cells and also include a “downstream process” for isolating and purifying the virus for use in a vaccine. Vaccines comprising MVAs and recombinant MVAs are produced by the methods of the invention and thus are also provided by the invention.

### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** shows the effects of Denerase® enzyme addition on the Host Cell DNA (“HCD”) present in collected material (*i.e.*, material collected from a poxvirus-infected cell culture) and the reduction of HCD levels at different steps in the downstream process depicted on the X-axis (see Example 1). HCD content per vaccine dose is shown on the Y axis; note log scale. Each line connects data points from a particular run of the downstream process. Abbreviations on horizontal axis represent stages of the downstream process as follows: PI = post infection with poxvirus (Day 3); PT = post treatment with trypsin; PN = post addition of NaCl; PL = post cell lysis; PF = post depth filtration (5 µM); TF1 = sample following TFF filtration; PD = sample following second treatment with Denerase® enzyme; BDS = sample of final product (“Bulk Drug Substance”). The data demonstrate that Denerase® enzyme acts to decrease HCD even in the presence of high salt (1M NaCl).

**Figure 2** shows the effect of NaCl addition on Host Cell DNA (“HCD”) reduction during later steps of the downstream purification process (see Example 1; note log scale). Abbreviations on horizontal axis represent stages of the downstream process as follows: PI = post infection with poxvirus; PT = post treatment with trypsin; PN = post addition of NaCl; PL = post cell lysis; PF = post depth filtration (5 µM); TF = sample following TFF filtration; PD = post treatment with Denerase® enzyme; TF2 UF – sample following second TFF ultrafiltration step; TF2 DF = sample following second TFF diafiltration step; BDS = sample of final product (“Bulk Drug Substance”). These data show the surprising result that NaCl addition produces a lower HCD level in later steps of the process, despite showing an initial increase in earlier steps.

**Figure 3** shows virus yields from quail suspension cells cultivated in various media (see Example 3). Quail cells (CCX.E10 cells (Nuvonis, Vienna, Austria)) were seeded in stirrer flasks in SCGM medium without addition of anti-clumping (“ac”) agent (“SCGM-ac”). After three days of cultivation, cells were collected and re-seeded in different media, either SCGM-ac with CaCl<sub>2</sub>, OptiPRO™ medium (with Pluronic F68 additive and a final concentration of 4mM

GlutaMAX<sup>TM</sup> supplement), or a 50:50 mix of SCGM-ac and OptiPRO<sup>TM</sup> medium (with Pluronic F68 additive), at a cell density between  $8 \times 10^5 - 1.1 \times 10^6$  cells/mL. Cells were transferred to either 30°C or 32°C for 1 hour before infection with MVA-BN-RSV at a MOI (Multiplicity of Infection) of 0.1 (=Day 0). For analysis of viral yields, cell culture samples were collected at Days 1-4 post infection, sonicated, frozen at -20°C, and subsequently titrated (vortexed  $\geq 1$  min) by the standard TCID<sub>50</sub> assay. Figure 3 shows viral yields as geometric means (GM) with geometric standard deviation (geoSD) of TCID<sub>50</sub>/mL. Data points represent results from nine independent experiments. Fold-differences shown below the graph are in comparison to yields with SCGM-ac +CaCl<sub>2</sub> at the corresponding time points (Day 1 (“d1”) through Day 4 (“d4”)).

**Figures 4A and 4B** show viral yields from quail suspension cells cultivated with media comprising different percentages of conditioned SCGM and OptiPRO<sup>TM</sup> medium (see Example 4). Quail cells (CCX.E10) were seeded at Day minus 3 in a stirrer flask in SCGM medium without addition of anti-clumping (ac) agent (“SCGM-ac medium”), eventually resulting in conditioned medium (“cSCGM-ac medium”). At Day 0 (the day of viral infection), aliquots of cell culture in this conditioned medium were transferred to shaker flasks and mixed with: an equal volume of OptiPRO<sup>TM</sup> medium (resulting in a 50:50 mixture); cSCGM-ac premixed with OptiPRO<sup>TM</sup> medium (resulting in proportions ranging from 60:40 to 90:10 of cSCGM-ac and OptiPRO<sup>TM</sup> medium); or cSCGM with CaCl<sub>2</sub> (no OptiPRO<sup>TM</sup> medium). All media were supplemented with a final concentration of 2 mM GlutaMAX<sup>TM</sup> supplement and Pluronic F68 additive. Cells were transferred to 30°C for 1 hour before infection with MVA-BN-RSV that also encoded mRFP (monomeric Red Fluorescent Protein) to allow for analysis of viral infection and spread (see Figure 4B). Infection was at a MOI (Multiplicity of Infection) of 0.1, and cells were subsequently cultured at 30°C. For analysis of viral yields, duplicate samples were collected at Days 1-4 post infection, sonicated, and frozen at -20°C. Samples from Day 1/ Day 2 (d1, d2) and Day 3/ Day 4 (d3, d4) were titrated in parallel (vortexed  $\geq 1$  min) by the standard TCID<sub>50</sub> assay. Figure 2A shows viral yields as geometric means (GM) of TCID<sub>50</sub>/mL with geometric standard deviation (geoSD). For analysis of viral infection and spread, mRFP expression was analyzed at Days 2-4 post infection by flow cytometry (Figure 4B) (GMFI, geometric mean fluorescence intensity). For both Figure 4A and Figure 4B, for each day represented in the bar graphs, data is shown from left to right for the following media: 50% cSCGM-ac/ 50% OptiPRO<sup>TM</sup> medium (“50:50”); 60% cSCGM-ac/ 40% OptiPRO<sup>TM</sup> medium

("60:40"); 70% cSCGM-ac/ 30% OptiPRO™ medium ("70:30"); 80% cSCGM-ac/ 20% OptiPRO™ medium ("80:20"); 90% cSCGM-ac/ 10% OptiPRO™ medium ("90:10"); cSCGM + CaCl<sub>2</sub>.

**Figure 5** shows viral yields from quail suspension cells cultivated with different media (see Example 5). Quail suspension cells (CCX.E10) were initially grown in SCGM medium without addition of anti-clumping (ac) medium (SCGM-ac). At Day 0, aliquots of the cell culture were transferred to different shaker flasks and mixed with OptiPRO™ medium, DMEM-F12 medium, and/or cSCGM media to obtain a final concentration of 50% or 30% of OptiPRO™ medium or DMEM-F12 medium ("F12") in 40 mL of culture volume with  $1.1 \times 10^6$  cells/mL. All media were supplemented with Pluronic F68 additive and final 2mM GlutaMAX™ supplement. Cells were transferred to 30°C for 1 h before infection with MVA-BN-RSV (co-encoding mRFP) at a MOI (Multiplicity of Infection) of 0.1 and further cultured at 30°C. Samples were collected at Days 1–4 post infection and titrated with the standard TCID<sub>50</sub> assay. Viral yields are shown in Figure 5 as geometric means of TCID<sub>50</sub>/mL with geometric standard deviation (geoSD).

**Figure 6** shows data from a comparison of MVA-BN® virus yields obtained from culturing Chicken Embryonic Fibroblast cells (CEF cells) and from quail suspension cells (CCX.E10 cells (Nuvonis, Vienna, Austria); see Example 6). CCX.E10 cells were seeded at Day -3 in SCGM medium without addition of anti-clumping (ac) agent (SCGM-ac) in S-125 shaker flasks. At Day 0, one hour before infection, cells were counted and mixed with an equal volume of OptiPRO™ medium supplemented with Pluronic F68 additive and final 4 mM GlutaMAX™ supplement, resulting in a 50:50 mix of SCGM-ac and the OptiPRO™ medium. CEF cells were seeded at Day -1 in VP-SFM medium (Thermo Fisher Scientific) in 6-well plates and counted at the day of infection. CCX.E10 and CEF cells were infected in parallel at a MOI (Multiplicity of Infection) of 0.1 with MVA-BN® virus (IMVAMUNE® vaccine) and subsequently cultured at 32°C. For analysis of viral yields, triplicate samples of CEF and CCX.E10 cells were collected daily at Day 1-4 post infection, sonicated, frozen at -20°C, and titrated in parallel by the standard TCID<sub>50</sub> assay (vortexed  $\geq 1$  min). Data shown in Figure 6 are viral yields as geometric means (GM) of TCID<sub>50</sub>/mL with geometric standard deviation (geoSD); numbers above bars for Day 4 (d4) indicate cell-specific peak viral yields (TCID<sub>50</sub>/cell).

**Figure 7** shows a schematic of the hypothesized effect of the first enzymatic treatment step with trypsin enzymatic activity and nuclease (*e.g.*, TrypLE and Denarase® enzyme) on the harvested cells, amplified virus, and impurities that result from lysis of the cells during the virus purification process (showing MVA-BN-RSV as an exemplary virus). While the invention is not bound by any particular mechanism of operation, it is thought that addition of these enzymatic activities prior to cell disruption facilitates the removal of impurities by decreasing cell clumping so that enzymes are better able to access their substrates, providing the improved virus yield seen from this process. This anti-clumping effect can also be seen in the Transmission Electron micrographs in Figure 7, showing cell material without added trypsin (TrypLE) (left micrograph) and with added trypsin (TrypLE) (right micrograph). These micrographs demonstrate that the added trypsin decreases the formation of large clumps of cells that are likely to be difficult for enzymes to penetrate. Figure 7 also demonstrates that the protease having trypsin activity functions in this process to de-aggregate these cells that have been grown in suspension, in contrast to the more common use of trypsin to detach cells grown as an adherent cell culture (*e.g.*, cells growing in a monolayer in a cell culture dish)).

## DETAILED DESCRIPTION OF THE INVENTION

The invention relates to methods of producing viral vector-based vaccine products from avian cell cultures. In some embodiments, the viral vector is Modified Vaccinia Virus Ankara (“MVA”), such as MVA-BN® virus. In some embodiments, the viral vector is a recombinant MVA encoding one or more heterologous antigens and the methods are used to produce a vaccine comprising the recombinant MVA. In some embodiments, the recombinant MVA encodes, for example, one or more antigens of Respiratory Syncytial Virus (RSV) and the methods are used to produce a vaccine comprising the recombinant MVA that protects against RSV-induced lower respiratory tract disease. In some embodiments, the recombinant MVA encodes a Tumor-Associated Antigen and can stimulate an immune response against the antigen. In some embodiments, the avian cells are quail cells from a suspension cell line and are processed according to the methods of the invention to provide a vaccine comprising MVA or recombinant MVA. In some embodiments, the quail cells are CCX.E10 cells (Nuvonis (Vienna, Austria)).

The invention provides methods of processing avian cell lines infected with MVA and recombinant MVA to produce vaccines. In some embodiments, the methods include an “upstream process” designed to maximize the viral titer produced by the cultured cells and also include a “downstream process” for isolating and purifying the virus for use in a vaccine. Vaccines comprising MVAs (including MVA-BN® virus) and recombinant MVAs can be produced using the methods of the invention and therefore are also provided by the invention.

The use of vaccinia viruses to protect humans against smallpox has a long history, and includes the use of the chorioallantois vaccinia virus Ankara (CVA) that was maintained in the Vaccination Institute in Ankara, Turkey, for many years. However, there were often severe post-vaccine complications associated with these viruses, so efforts were made to generate a more attenuated, safer smallpox vaccine. The attenuated CVA-derived virus Modified Vaccinia Virus Ankara (“MVA”) was obtained by serial propagation of more than 570 passages of CVA on primary chicken embryo fibroblasts (“CEF” cells; for review see Mayr *et al.* (1975) *Infection* 3: 6-14). As a result of the passaging used to attenuate MVA, there are a number of different strains or isolates of MVA, which are sometimes named according to the passage number in CEF cells. For example, MVA-572 was used in Germany during the smallpox eradication program, and MVA-575 was extensively used as a veterinary vaccine. MVA-575 was deposited on Dec. 7, 2000, at the European Collection of Animal Cell Cultures (ECACC) as deposit number V00120707.

### **MVAs and Recombinant MVAs**

Strains of MVA having enhanced safety profiles for the development of safer products, such as vaccines or pharmaceuticals, have been developed by Bavarian Nordic® A/S. For example, MVA was further passaged by Bavarian Nordic® A/S and is designated MVA-BN® virus. MVA as well as MVA-BN® virus lacks approximately 15% of the genome compared with the ancestral CVA virus; specifically, 31 kb from six regions. These deletions affect a number of virulence and host range genes, as well as the gene for Type A inclusion bodies. A sample of MVA-BN® virus corresponding to passage 583 was deposited on August 30, 2000 at the European Collection of Cell Cultures (ECACC) under deposit number V00083008.

MVA-BN® virus can attach to and enter human cells, where virally-encoded genes are expressed very efficiently. However, assembly and release of progeny virus does not occur, and

no infectious virus is produced. Although MVA-BN® virus does not replicate in human cells, it is strongly adapted to primary chicken embryo fibroblast (CEF) cells. MVA-BN® virus is classified as a Biosafety Level 1 organism according to the Centers for Disease Control and Prevention in the United States. Preparations of MVA-BN® virus and derivatives have been administered to many types of animals and to more than 2000 human subjects, including immune-deficient individuals. All vaccinations have proven to be generally safe and well tolerated. Despite its high attenuation and reduced virulence, in preclinical studies, MVA-BN® virus has been shown to elicit both humoral and cellular immune responses to vaccinia and to heterologous gene products encoded by genes cloned into the MVA genome (see Harrer *et al.* (2005) *Antivir. Ther.* 10(2): 285-300; Cosma *et al.* (2003) *Vaccine* 22(1): 21-9; Di Nicola *et al.* (2003) *Hum. Gene Ther.* 14(14): 1347-1360; and Di Nicola *et al.* (2004) *Clin. Cancer Res.* 10(16): 5381-5390).

The virus MVA-BN® is licensed under the tradename IMVANEX® in the European Union (EU) for the prevention of smallpox infection, and under IMVAMUNE® in Canada for the prevention of smallpox, monkeypox and other Orthopoxvirus infections. In the United States, MVA-BN® virus is licensed under the trade name JYNNEOS™ for active immunization against smallpox and monkeypox in adults considered at high risk for these diseases. Thus, MVA-BN® virus itself (*i.e.*, not expressing any additional heterologous antigens) is also a vaccine that can be produced using the methods of the invention.

“Derivatives” or “variants” of MVAs are viruses exhibiting essentially the same replication characteristics as MVA as described herein but have differences in one or more parts of their genomes. MVA-BN® virus as well as a derivative or variant thereof fails to reproductively replicate *in vivo* in humans and mice, even in severely immune-suppressed mice. More specifically, MVA-BN® virus or a derivative or variant thereof preferably also has the capability of reproductive replication in chicken embryo fibroblasts (CEF), but no capability of reproductive replication in the human keratinocyte cell line HaCat (Boukamp *et al.* (1988) *J. Cell Biol.* 106: 761-771), the human bone osteosarcoma cell line 143B (ECACC No. 91112502), the human embryo kidney cell line 293 (ECACC No. 85120602), and the human cervix adenocarcinoma cell line HeLa (ATCC No. CCL-2). Additionally, a derivative or variant of MVA-BN® virus has a virus amplification ratio at least two-fold less and more preferably three-fold less than MVA-575 in HeLa cells and HaCaT cell lines. Tests and assays for these

properties of MVA variants are described in WO 2002/042480 (US 2003/0206926) and WO 2003/048184 (US 2006/0159699). The amplification or replication of a virus is normally expressed as the ratio of virus produced from an infected cell (output) to the amount originally used to infect the cell (input), referred to as the “amplification ratio.” An amplification ratio of “1” defines an amplification status where the amount of virus produced from the infected cells is the same as the amount initially used to infect the cells, meaning that the infected cells are permissive for virus infection and reproduction. In contrast, an amplification ratio of less than 1 (*i.e.*, a decrease in output compared to the input level) indicates a lack of reproductive replication and therefore attenuation of the virus.

The advantages of MVA-based vaccines include their safety profile as well as availability for large scale vaccine production. Preclinical tests have revealed that MVA-BN® virus demonstrates superior attenuation and efficacy compared to other MVA strains (WO 2002/042480). An additional property of MVA-BN® virus strains is the ability to induce substantially the same level of immunity in prime/boost regimes utilizing vaccinia virus prime and vaccinia virus boost when compared to regimes utilizing a DNA prime and vaccinia virus boost. The recombinant MVA-BN® viruses, the most preferred embodiment herein, are considered to be safe because of their distinct replication deficiency in mammalian cells and their well-established avirulence. Also, with MVA-BN® virus, the feasibility of industrial scale manufacturing can be beneficial. Furthermore, MVA-based vaccines can deliver multiple heterologous antigens and allow for simultaneous induction of humoral and cellular immunity.

In addition to recombinant MVAs expressing RSV antigens (referred to herein generally as “MVA-RSV”), discussed in detail below, other recombinant MVAs can be produced using the methods of the invention comprising culture in quail cells. Any recombinant MVA that can reproduce in quail cells can be produced using the methods of the invention. Accordingly, the invention provides methods of preparing vaccines comprising any recombinant MVA that can reproduce in quail cells. Such recombinant MVAs include, for example, those expressing EBV antigens (“MVA-EBV”), Equine Encephalitis Virus antigens, Foot and Mouth Disease Virus antigens, filovirus antigens, as well as other disease, viral, or cancer-related antigens or tumor-associated antigens, or heterologous genes such as monomeric Red Fluorescent Protein (mRFP; see, *e.g.*, Campbell *et al.* (2002) *Proc. Nat’l. Acad. Sci. USA* 99: 7877-82). Thus, it will be

appreciated that recombinant MVAs for use in the methods and compositions of the invention comprise heterologous nucleotide sequences encoding one or more heterologous antigens.

Since the MVAs and recombinant MVA viruses described herein are highly replication restricted and thus highly attenuated, they are ideal candidates for the treatment of a wide range of mammals including humans and even immune-compromised humans. MVAs and recombinant MVAs produced in the methods, cells, cell cultures, and populations of cells of the invention can be isolated and/or purified and used to provide compositions for further use, including pharmaceutical compositions such as vaccines. Suitable techniques and formulations for these purposes are known in the art.

In another aspect, an MVA viral strain suitable for generating the recombinant virus may be strain MVA-572, MVA-575, or any similarly attenuated MVA strain. Also suitable may be a mutant MVA, such as the deleted chorioallantois vaccinia virus Ankara (dCVA). A dCVA comprises del I, del II, del III, del IV, del V, and del VI deletion sites of the MVA genome. The deletion sites are particularly useful for the insertion of multiple heterologous sequences. The dCVA can reproductively replicate (with an amplification ratio of greater than 10) in a human cell line (such as human 293, 143B, and MRC-5 cell lines), which then enables optimization by further mutation and can be useful for a virus-based vaccination strategy (see WO 2011/092029).

### **MVA-RSV**

RSV is a significant respiratory pathogen and is the most clinically important cause of acute lower respiratory tract (LRT) infection, which causes significant morbidity and mortality in infants and children under the age of five years worldwide (see, *e.g.*, Aliyu *et al.* (2010), *Bayero J. Pure Appl. Sci.* 3(1): 147-155). Primary infection with RSV does not induce complete immunity to RSV, so frequent re-infections occur throughout life, with the most severe infections developing in the very young, the very old, and in immune-compromised patients of any age (see, *e.g.*, Murata (2009) *Clin. Lab. Med.* 29(4): 725-39).

RSV is an enveloped RNA virus of the family *Paramyxoviridae*. Each RSV virion contains a non-segmented, negative-sense, single-stranded RNA molecule containing ten genes encoding eleven separate proteins, including eight structural (G, F, SH, M1, N, P, M2-1, and L) and three non-structural proteins (NS1, NS2, and M2.2); M2 contains two open reading frames

(Murata (2009) *Clin. Lab. Med.* 29(4): 725-39). Much is known about the roles and interactions of RSV proteins.

Recombinant MVAs expressing at least one RSV antigen are referred to herein generally as MVA-RSV. Some embodiments of an MVA-RSV comprise MVA-BN® virus and are referred to herein generally as MVA-BN-RSV.

It had been shown that vaccination with a recombinant vaccinia virus Ankara (MVA) expressing at least one antigen of an RSV membrane glycoprotein and at least one antigen of an RSV nucleocapsid protein (*e.g.*, MVA-mBN201B) induced better immune protection than an RSV vaccine comprising only the RSV-F and/or RSV-G antigens (see WO 2014/019718). In addition, such constructs induced almost complete sterile immunity when applied by the intranasal route, and enhanced protection could be obtained by administering candidate RSV vaccines intranasally in comparison to intramuscular or subcutaneous administration (see, *e.g.*, Wyatt *et al.* (2000) *Vaccine* 18: 392-97). Unfortunately, in a phase 3 clinical trial conducted by Bavarian Nordic A/S, this recombinant MVA did not meet all the primary endpoints of preventing lower respiratory tract disease from RSV and commercial development of this vaccine candidate was discontinued (see Bavarian Nordic A/S press release dated July 22, 2023).

### **RSV Nucleotide Sequences and Proteins**

Exemplary RSV vaccines that can be produced using methods of the instant invention are known in the art, for example, as described in WO 2014019718, incorporated specifically in its entirety herein by reference. For use in the methods of the invention, recombinant MVAs encoding RSV genes (herein, “MVA-RSVs”) as mentioned herein refer to the genes, or to a homolog or variant of the genes, encoding the corresponding protein in any RSV strain or isolate, even though the exact sequence and/or genomic location of the gene may differ between strains or isolates. Likewise, the RSV proteins mentioned herein refer to proteins, or to a homolog or variant of the proteins, encoded and expressed by the corresponding gene as defined above. Generally, MVA-RSVs encode RSV proteins that are antigens. In some embodiments, an MVA-RSV is an “MVA-BN-RSV” that comprises MVA-BN® virus such as, for example, MVA-mBN294B.

When referring to the RSV F protein gene, other terms may also be used, such as “F protein gene,” “F glycoprotein gene,” “RSV F glycoprotein gene,” or “F gene,” all of which refer to the gene, or to a homolog or variant of the gene, encoding the transmembrane fusion glycoprotein in any RSV strain or isolate, even though the exact sequence and/or genomic location of the F protein gene may differ between RSV strains or isolates. For example, in the A2 strain of RSV, the F(A2) protein gene comprises nucleotides 5601-7499 (endpoints included) as numbered in GenBank Accession Number M11486. The F(A2) protein gene further comprises a protein coding open reading frame (ORF) spanning nucleotides 5614-7338 (endpoints included) as numbered in GenBank Accession No. M11486. The nucleotide sequence of the F protein gene from RSV A2 (SEQ ID NO:1) is known in the art (see WO 2014019718).

Also interchangeably used herein are the terms “F protein,” “F glycoprotein,” “RSV F protein,” “RSV F glycoprotein,” or “F” which refer to the heavily glycosylated transmembrane fusion glycoprotein, or to a homolog or variant of the protein encoded and expressed by an RSV F protein gene as defined above. The amino acid sequence of the F protein from RSV A2 (SEQ ID NO:2) is known in the art (see WO 2014019718). The RSV(A2) F protein comprises a signal peptide, an extracellular domain, a transmembrane domain, and a cytoplasmic domain (*see, e.g.*, UniProtKB/Swiss-Prot Accession No. P03420). Other proteins and fragments are known in the art and can be used to provide MVA-RSVs, which in turn can be produced using the methods of the instant invention to provide vaccines.

When referring to the RSV G protein G, similarly, other terms such as “G protein gene,” “G glycoprotein gene,” “RSV G protein gene,” “RSV G glycoprotein gene,” and “G gene” are used interchangeably herein. For example, in the A2 strain of RSV, the G(A2) protein gene comprises nucleotides 4626-5543 (endpoints included) as numbered in GenBank Accession Number M11486. The G(A2) protein gene further comprises a protein coding open reading frame (ORF) spanning nucleotides 4641-5537 (endpoints included) as numbered in GenBank Accession No. M11486. The nucleotide sequence of the G protein gene from RSV A2 (SEQ ID NO:3) is known in the art (see WO 2014019718).

The terms “G protein,” “G glycoprotein,” “RSV G protein,” “RSV G glycoprotein,” or “G” refer to the heavily glycosylated transmembrane attachment glycoprotein, or to a homolog or variant of the protein. The amino acid sequence of the G protein from RSV A2 (SEQ ID NO:4) is known in the art (see WO 2014019718). RSV A2 G protein comprises an extracellular

domain, a transmembrane domain, and a cytoplasmic domain (*see, e.g.*, UniProtKB/Swiss-Prot Accession No. P03423). These domains are also known in the art and taught in WO 2014019718; for example, the extracellular domain of RSV A2 G protein consists of amino acids 67-298 of SEQ ID NO:4; the transmembrane domain of RSV A2 G protein consists of amino acids 38-66 of SEQ ID NO:4; and the cytoplasmic domain of RSV A2 G protein consists of amino acids 1-37 of SEQ ID NO:4 of WO 2014019718.

Interchangeably used herein are also the terms “M2-1 protein gene,” “M2.1 protein gene,” “M2-1 transcription elongation factor,” “RSV M2-1 protein gene,” “RSV M2.1 protein gene,” “RSV M2-1 transcription elongation factor gene,” or “M2 gene.” For example, in the A2 strain of RSV, the M2(A2) protein gene comprises nucleotides 7550-8506 (endpoints included) as numbered in GenBank Accession Number M11486. The M2(A2) protein gene further comprises a protein coding open reading frame (ORF) spanning nucleotides 7559-8143 (endpoints included) as numbered in GenBank Accession No. M11486. The nucleotide sequence of the M2 protein gene from RSV A2 (SEQ ID NO:5) and the amino acid sequence of the M2 protein from RSV A2 (SEQ ID NO:6) are known in the art (see WO 2014019718; *see, e.g.*, UniProtKB/Swiss-Prot Accession No. P04545).

When referring to the RSV N protein gene, the terms “N protein gene,” “N nucleocapsid protein gene,” “RSV N nucleocapsid protein gene,” or “N gene” may be used interchangeably herein. For example, in the A2 strain of RSV, the N(A2) protein gene comprises nucleotides 1081-2277 (endpoints included) as numbered in GenBank Accession Number M11486. The N(A2) protein gene further comprises a protein coding open reading frame (ORF) spanning nucleotides 1096-2271 (endpoints included) as numbered in GenBank Accession No. M11486. The nucleotide sequence encoding the N protein gene from RSV A2 (SEQ ID NO:7) is known in the art (see WO 2014019718). The amino acid sequence of the RSV N protein (also referred to as “N protein,” “N nucleocapsid protein,” “RSV N nucleocapsid protein,” or “N”) from RSV strain A2 (SEQ ID NO:8) is known in the art (see WO 2014019718; UniProtKB/Swiss-Prot Accession No. P03418).

#### **Certain Recombinant MVAs Encoding RSV Antigens**

Recombinant MVAs used in the methods and compositions of the invention can encode one or more RSV antigens suitable for use in an RSV vaccine (generally referred to herein as

“MVA-RSV”). For example, recombinant MVAs for use in an RSV vaccine can comprise at least one nucleotide sequence encoding an antigen of an RSV membrane glycoprotein and at least one nucleotide sequence encoding an antigen of an RSV nucleocapsid protein.

In some embodiments, an MVA-RSV encodes antigens of the surface fusion protein (F), two glycoproteins (G) from RSV subtype A and RSV subtype B, and internal proteins which are the nucleoprotein N and the transcription elongation factor M2-1. In some embodiments, the F protein is based on the native surface protein F from the RSV A subtype.

In certain embodiments, an MVA-RSV encodes at least one antigen of an RSV membrane glycoprotein. In certain embodiments, the MVA-RSV encodes an RSV F antigen and/or an RSV G antigen. In certain embodiments, the recombinant MVA encodes an RSV F antigen that is derived from RSV strain A2 and/or an RSV G antigen that is derived from RSV strain A2. In certain embodiments, an MVA-RSV encodes at least two antigens of an RSV membrane glycoprotein that are an RSV F antigen and an RSV G antigen. In certain embodiments, the RSV F antigen and/or the RSV G antigen are derived from RSV strain A2.

In certain embodiments, the MVA-RSV encodes an antigen of an RSV membrane glycoprotein and comprises at least one heterologous nucleotide sequence encoding an antigen of an RSV nucleocapsid protein. In certain embodiments, the recombinant MVA encodes at least one antigen of an RSV F membrane glycoprotein and at least one antigen of an RSV M2 nucleocapsid protein or an RSV N nucleocapsid protein. In certain embodiments, the MVA-RSV encodes at least an antigen of an RSV G membrane glycoprotein and an antigen of an RSV M2 nucleocapsid protein and/or an antigen of an RSV N nucleocapsid protein.

In certain embodiments, the MVA-RSV encodes at least one antigen of each of: an RSV F membrane glycoprotein; an RSV G membrane glycoprotein; and an RSV M2 or N nucleocapsid protein. In certain embodiments, both the RSV F antigen and the RSV G antigen are derived from RSV strain A2. In certain embodiments, the MVA-RSV encodes at least one antigen of each of: an RSV F membrane glycoprotein, an RSV G membrane glycoprotein, an RSV M2 nucleocapsid protein, and an RSV N nucleocapsid protein. In certain embodiments, both the RSV F antigen and the RSV G antigen are derived from RSV strain A2.

In certain embodiments, an MVA-RSV that encodes an antigen of an RSV F membrane glycoprotein encodes a full-length RSV F membrane glycoprotein or alternatively encodes a truncated or partial RSV F membrane glycoprotein, and/or is a variant of the wildtype RSV F

membrane glycoprotein. In certain embodiments, the MVA-RSV encodes a full-length RSV F membrane glycoprotein from strain A2, or a full-length, truncated, or variant RSV F antigen derived from RSV strain A<sub>Long</sub>. In some embodiments, the MVA-RSV encodes a truncated RSV(A<sub>Long</sub>) F antigen and/or RSV (A2) F antigen that lacks the cytoplasmic and transmembrane domains of the native F protein.

In certain embodiments, MVA-RSV encodes an antigen of an RSV G membrane glycoprotein, optionally from RSV strain A2 or B, which is full-length or truncated, or a variant of wildtype RSV G protein. In certain embodiments, the MVA-RSV encodes a truncated RSV G antigen that lacks the cytoplasmic and transmembrane domains of the full-length RSV G protein.

In certain embodiments, the MVA-RSV encodes an antigen of an RSV M2 nucleocapsid protein which is full-length, truncated, or a variant of wildtype RSV M2 protein, and in some embodiments is derived from RSV strain A2. In certain embodiments, the MVA-RSV encodes an antigen of an RSV N nucleocapsid protein which is full-length, truncated, or a variant of wildtype RSV N protein, and in some embodiments is derived from RSV strain A2. In certain embodiments, the MVA-RSV encodes an antigen of RSV N and an antigen of RSV M2 that are encoded by a single open reading frame and separated by a self-cleaving protease domain, for example such as the self-cleaving protease 2A fragment from Foot and Mouth Disease Virus. In certain embodiments, the MVA-RSV comprises a heterologous nucleotide sequence encoding an RSV N antigen and an RSV M2 antigen that comprises the nucleotide sequence set forth in SEQ ID NO:9 and/or that encodes the amino acid sequence of SEQ ID NO:10.

In certain embodiments (referred to herein for convenience as “Embodiment A”), the recombinant Modified Vaccinia Virus Ankara (MVA) comprises: (a) at least one nucleotide sequence encoding an antigen of a respiratory syncytial virus (RSV) membrane glycoprotein, wherein the nucleotide sequence encodes a full-length RSV F membrane glycoprotein; and (b) at least one nucleotide sequence encoding RSV nucleocapsid antigens, wherein the nucleotide sequence encodes both a full-length RSV N nucleocapsid protein and a full-length RSV M2-1 transcription elongation factor protein, which are encoded by a single open reading frame, wherein the single open reading frame comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:12 or comprises the nucleotide sequence of SEQ ID NO:11; and further comprises (c) at least one nucleotide sequence encoding an antigen of an RSV G

membrane glycoprotein, wherein the nucleotide sequence encodes a full-length RSV G membrane glycoprotein.

Embodiment (B) is the recombinant MVA of embodiment (A), wherein the nucleotide sequence encoding the RSV F membrane glycoprotein is from RSV strain A, preferably from A2 and/or A<sub>Long</sub>. Embodiment (C) is the recombinant MVA of embodiment (A) or embodiment (B), wherein the nucleotide sequence encoding the RSV F membrane glycoprotein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:2 or comprises the nucleotide sequence of SEQ ID NO:1. Embodiment (D) is the recombinant MVA of any of Embodiments (A), (B), or (C), wherein the nucleotide sequence encoding the RSV G membrane glycoprotein is from RSV strain A, preferably from strain A2 and/or B. Embodiment (E) is the recombinant MVA of Embodiment (A), (B), (C), or (D), wherein the nucleotide sequence encoding the RSV G membrane glycoprotein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:4 or comprising the nucleotide sequence nucleotide sequence of SEQ ID NO:3.

Embodiment (F) is the recombinant MVA of embodiment (A), (B), (C), (D), or (E), wherein the MVA used for generating the recombinant MVA is MVA-BN® virus deposited at the European Collection of Cell Cultures (ECACC) under number V00083008. Embodiment (G) is the recombinant MVA of embodiment (A), (B), (C), (D), (E), or (F) for use in preventing at least one symptom of RSV infection, or in preventing RSV-caused disease. Embodiment (H) is the recombinant MVA of embodiment (G) for use in preventing at least one symptom of RSV infection or in preventing RSV-caused disease, wherein the recombinant MVA is administered intranasally and/or subcutaneously, preferably intranasally.

In some embodiments, the recombinant MVA (MVA-RSV) for use in the methods and compositions of the invention is an MVA-BN-RSV, for example, MVA-mBN294B (see, *e.g.*, WO 2014019718, specifically incorporated herein by reference). Several clinical trials of MVA-mBN294B were conducted and successfully demonstrated the safety and immunogenicity of this vaccine. These trials include the Phase 1 trial RSV-MVA-001 and the Phase 2 trial RSV-MVA-002, which provided promising safety and immunogenicity results. Unfortunately a Phase 3 clinical trial of this RSV vaccine candidate for adults 60 years of age and older did not meet all the primary endpoints of preventing lower respiratory tract disease (LRTD) caused by RSV, and

the commercial development of this vaccine candidate was discontinued (see Bavarian Nordic A/S press release dated July 22, 2023).

Immunogenicity was assessed by evaluating antibody and T cell responses, and doses of vaccine were administered at  $1 \times 10^8$  Infectious Units (IU) per 0.5 mL or  $5 \times 10^8$  IU per 0.5 mL. The results of the completed Phase 1 and 2 trials showed that a single dose of MVA-BN-RSV induced broad humoral, cellular and mucosal immune responses persisting over at least 6 months. A single dose of MVA-BN-RSV vaccine elicited increases in neutralizing antibodies (identified using PRNT to RSV-A and B subtype) and total antibodies (IgG and IgA ELISA) as well as a broad Th1-biased cellular immune response (IFN- $\gamma$ /IL-4 ELISPOT) to all 5 inserts encoded in the vaccine also confirming results from non-clinical studies in different animal models. Overall, the vaccine-induced antibody responses (Geometric Mean Titers (GMT)) remained above baseline 30 weeks post-vaccination.

### **Vaccines and Pharmaceutical Compositions**

Since the MVAs and recombinant MVA viruses described herein are highly replication restricted and, thus, highly attenuated, they are ideal candidates for the treatment of a wide range of mammals including humans and even immune-compromised humans. Thus, provided herein are methods for producing these recombinant MVAs and compositions comprising them for use as pharmaceutical compositions and vaccines, all intended for inducing an immune response in a living animal body, including a human.

For this, the MVA or recombinant MVA, vaccine, or pharmaceutical composition can be formulated in solution in a concentration range of about  $10^4$  to  $10^9$  IU/mL,  $10^5$  to  $5 \times 10^8$  IU/mL,  $10^6$  to  $10^8$  IU/mL, or  $10^7$  to  $10^8$  IU/mL. A preferred dose for humans comprises between  $10^6$  to  $10^9$  IU/mL, including a dose of at least about:  $10^6$  IU/mL,  $10^7$  IU/mL,  $10^8$  IU/mL or  $5 \times 10^8$  IU/mL. In some embodiments, a pharmaceutical composition that is a vaccine comprising MVA or MVA-RSV comprises  $1 \times 10^8$  IU/0.5 mL or  $5 \times 10^8$  IU/0.5 mL (*i.e.*,  $1 \times 10^8$  IU in a volume of 0.5 mL or  $5 \times 10^8$  IU in a volume of 0.5 mL), or about  $1.58 \times 10^9$  InfU/mL in a final (vaccine) volume of 0.5 mL.

The pharmaceutical compositions produced by the methods of the invention may generally include one or more pharmaceutically acceptable and/or approved buffers, carriers, additives, antibiotics, preservatives, adjuvants, diluents and/or stabilizers. Such auxiliary

substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like. Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, and the like.

For the preparation of vaccines, the MVAs or recombinant MVAs produced by the methods of the invention can be converted into a physiologically acceptable form. This can be done, for example, based on experience in the preparation of poxvirus vaccines used for vaccination against smallpox as described by Stickl *et al.* ((1974) *Dtsch. med. Wschr.* 99: 2386-2392). For example, purified viruses can be stored at -80°C with a titer of  $5 \times 10^8$  IU/mL formulated in about 10 mM Tris, 140 mM NaCl pH 7.7. For the preparation of vaccine doses, *e.g.*,  $10^2$ - $10^8$  or  $10^2$ - $10^9$  particles of the virus can be lyophilized in 100 ml of phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine doses can be produced by stepwise freeze-drying of the virus in a formulation. This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose, or polyvinylpyrrolidone or other aids such as antioxidants or inert gas, stabilizers or recombinant proteins (*e.g.*, human serum albumin) suitable for *in vivo* administration. In some embodiments, the glass ampoule is then sealed and can be stored between 4°C and room temperature for several months. However, the ampoule can also be stored, for example, at temperatures below -20 °C.

For administration to a subject, the lyophilisate can be dissolved in an aqueous solution, preferably physiological saline or Tris buffer, and administered either systemically or locally, *i.e.*, by a route of administration that is parenteral, subcutaneous, intravenous, intramuscular, intranasal, or any other suitable path of administration. The mode of administration, the dose, and the number of administrations can be optimized by those skilled in the art in a known manner. However, most commonly a subject is vaccinated with a second shot about one month to six weeks after the first vaccination shot.

In some embodiments, vaccination with an MVA or recombinant MVA of the invention (*i.e.*, a recombinant MVA of the invention or a recombinant MVA produced by the methods of the invention) produces sterile immunity in a treated subject. However, sterile immunity is not required for a vaccine to provide benefit to a vaccinated subject. The usefulness of recombinant MVAs as vaccines is illustrated, for example, by MVA-BN-RSV (*i.e.*, MVA-mBN294B; see

data provided in working examples). Vaccination of a subject with MVA-BN-RSV optimally protects against RSV-caused disease by stimulating various aspects of the adaptive immune system such as, for example, the production of RSV-specific antibodies and/or T cells. Efficacy can be assessed using an animal model such as the RSV challenge model in BALB/c mice (see, *e.g.*, Waris *et al.* (1996) *J. Virol.* 70: 2852-60). Efficacy of stimulation of the immune response can be evaluated, for example, by decrease in the viral load of vaccinated subjects in the event of exposure to RSV and possible subsequent infection. Efficacy can also be assessed across a population of vaccinated subjects, as a statistical decrease in the occurrence or severity of infections, as is known in the art.

In this manner, the methods and compositions of the invention provide vaccines that are useful in protecting subjects against diseases, for example, Lower Respiratory Tract Disease caused by RSV, or smallpox or monkeypox. Other recombinant MVAs or MVAs (such as MVA-BN® virus) produced using the methods of the invention can similarly be evaluated with corresponding assays known in the art. In some embodiments, an MVA or recombinant MVA produced using the methods of the invention stimulates an immune response in a vaccinated subject that increases the production of specific antibodies and/or T cells above a background level observed prior to vaccination.

### **Definitions**

The term “antigen” as used herein refers to any molecule that stimulates a host's immune system to make an antigen-specific immune response, whether a cellular response and/or a humoral antibody response. Antigens may include proteins, polypeptides, protein fragments, and epitopes that elicit an immune response in a host. Thus, antigens that are proteins, polypeptides, protein fragments, and epitopes are not limited to particular native amino acid sequences but also encompass modifications to the native sequence, such as deletions, additions, insertions, and substitutions that result in variant amino acid sequences. Antigen-encoding sequences may be from another virus or pathogen, or associated with a disease such as cancer or a tumor, or may be another sequence.

By a “day” as used herein is approximately 24 hours, or at least 12 hours but less than 36 hours. By “overnight” as used herein is approximately about 6 or 8 hours, or about 8 to 12 hours, or about 12 to 16 hours.

Preferably, as the term is used herein, “sequence variants” or “variants” have at least about 80% or 85%, or at least about 90%, 91%, 92%, 93%, or 94% or at least about 95%, 96%, 97%, 98% or 99% identity with the referenced nucleic acid or amino acid sequence. The term “variant” also encompasses truncated, deleted, or otherwise modified nucleic acid or protein sequences such as, for example, soluble forms of the RSV-F or RSV-G proteins lacking the signal peptide as well as the transmembrane and/or cytoplasmic domains of the full-length RSV-F or RSV-G proteins and the nucleic acids encoding them, and deleted, truncated, or otherwise mutated versions of the full-length RSV-M2 or RSV-N proteins and the nucleic acids encoding them. A deleted or truncated version of a protein or nucleic acid can differ from the full-length version by deletion or truncation of one or more elements, and/or by deletion or truncation of particular amino acids or nucleic acids, such as fewer than 30, 20, or 10 amino acids or nucleic acids.

Techniques for determining sequence identity between different nucleic acids and between different amino acids are known in the art. Two or more sequences can be compared by determining their “percent identity.” The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. Alternatively, and particularly if it is necessary to introduce gaps in the alignment of sequences to achieve the maximum percent sequence identity, “percent (%) sequence identity” as used herein is the percentage of nucleic acid or amino acid residues in a candidate sequence that are identical with the nucleic acid or amino acid residues in the reference sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for example, using publicly available computer software such as BLAST, ALIGN, or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for producing a sequence alignment and calculating sequence identity, including any algorithms needed to achieve maximum alignment over the full length of the sequences being compared. Details of these programs are known in the art and can be found, for example, at the website for the National Center for Biotechnology Information.

As used herein, a “heterologous” gene, nucleic acid, or protein is understood to have a nucleic acid or amino acid sequence which is not present in the wild-type poxviral genome (*e.g.*,

sequences encoding antigens of another virus, or pathogen, or associated with a disease such as cancer or a tumor, or another heterologous sequence). The skilled person understands that a nucleic acid which is a “heterologous gene,” when present in a recombinant poxvirus such as MVA, is to be incorporated into the poxviral genome in such a way that, following administration of the recombinant poxvirus to a host cell, it is expressed as the corresponding heterologous gene product, *i.e.*, as the “heterologous antigen” and/or “heterologous protein.” Expression is normally achieved by operatively linking the heterologous gene to regulatory elements that allow expression in the poxvirus-infected cell. In some embodiments, the regulatory elements include a natural or synthetic poxviral promoter.

“Sterile immunity” as used herein means protective immunity provided by a vaccine in the absence of detectable pathogen in a subject (for example, absence of RSV genome when sensitive detection methods, such as RT-qPCR, are applied). By “subject” is intended any animal which is being treated, for example, by administration of a vaccine; as used herein, a subject may be a mammal, including farm animal or companion animal, or may be a human subject or patient.

An “increased inflammatory response” or “enhanced inflammation response” as used herein is characterized by one or more of the following: increased production of IL-12 p70, M-CSF, and/or IL-33; increased antigen-specific CD8<sup>+</sup> T cells, increased percentages of CD8<sup>+</sup> T cells expressing IFN-gamma and TNF-alpha; decrease in tumor size and/or growth rate; and improved survival of treated subjects, and the like, which can be detected by assays known in the art. As used herein, “increased inflammatory response” generally refers to an increase in production of a particular cytokine or cell type associated with inflammation, in comparison to baseline levels prior to treatment, for example, with compositions of the invention. In an “increased inflammatory response,” the amount of a cytokine or cell type is increased by at least 10%, 20%, 30%, 50%, 70%, or 100% or more in comparison to baseline levels in a subject prior to a treatment.

Viral titer can be measured in several ways; one of skill in the art is familiar with techniques for determining viral titer and comparing different measures of titer. “TCID<sub>50</sub>” is the abbreviation of “tissue culture infectious dose,” which is the amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated, expressed as TCID<sub>50</sub>/mL. Methods for determining TCID<sub>50</sub> are well known to the person skilled in the art, for example, as

described in Example 2 of WO 03/053463. “IU” as used herein stands for “Infectious Units” and is also a measurement of viral titer, typically expressed as IU/mL.

### **Avian cells and methods**

The methods of the invention involve processing avian cells infected with poxviruses to produce viral vector-based vaccine products and may be performed using avian cells. Thus, the methods of the invention can be performed using avian cells such as, for example, Chicken Embryonic Fibroblasts (“CEF” cells), duck cells, and quail cells.

Continuous quail cell lines, including CCX.E10 (Nuvonis, Vienna, Austria), were obtained from starting material of embryonic quail cells without the introduction of foreign genes, viral sequences, or chemical treatment; the most likely mechanism of immortalization was the occurrence of spontaneous mutations in the embryonic quail cells used as starting material. Briefly, cell lines were generated from quail cells through passaging of adherent cells, followed by adaptation to growth in suspension. Thereafter, single clones were selected and amplified, and adapted to serum-free suspension cell growth medium. For CCX.E10, the final selected clone was amplified in spinner flasks and frozen as a cell bank.

In some embodiments, “conditioned medium” is present during infection of cells; in some embodiments, this refers to the medium the cells have been expanded in before infection for a time (for example, about 12 hours, about 24 hours, about 36 hours, or about 48 hours or more) and can continue to be used to infect those cells, optionally by adding additional fresh medium that is the same as the original medium and/or by adding additional fresh medium that is different from the original medium.

Cells from frozen cell banks can be thawed and used to produce MVA or recombinant MVA for use in vaccines as further described below. In this manner, the invention provides avian cells including quail cells, quail cell lines, and populations of avian cells (such as, for example, quail cells) for use in producing vaccines comprising MVA and recombinant MVA.

### **Methods for cultivation of avian cells**

In some embodiments, cells are grown in different media at different stages of production that can be characterized as a cell growth phase, a virus infection phase, and a virus production phase. That is, in some embodiments, cells are seeded into a container containing an initial

medium and grown or cultured for an initial stage (also referred to herein as “cell growth phase”). This cell growth phase may continue for a period of time or it may continue until or after a particular cell density is reached or until or after a particular cell property is expressed or observed, such as, for example, increased aggregation of cells in culture. Generally, avian cells such as quail cells tend to grow more quickly when they are in suspension as single cells rather than in groups of cells or aggregated cells. Thus, in some embodiments, the medium composition, temperature, and cell density is adjusted so that cells are mostly growing as single cells during the cell growth phase or are substantially growing as single cells during the growth phase. In some embodiments, quail cells are cultivated in a cell growth phase until the cell density is from  $1 \times 10^6$  cells/mL to  $5 \times 10^6$  cells/mL, or preferably from  $2 \times 10^6$  cells/mL to  $4 \times 10^6$  cells/mL, or more preferably about  $3 \times 10^6$  cells/mL. Cells can be assessed by microscopy or FACS analysis or other techniques known in the art.

In some embodiments, the cell growth phase is conducted in a bioreactor capable of perfusion (*i.e.*, gradual removal of older media and introduction of new media while cells continue to be cultured); alternatively, the cell growth phase can be conducted in cell bags (also referred to as “wave bags”), shaker flasks, or spinner flasks. In some embodiments, the cell growth phase is conducted in roller bottles and the cells are suspended in media prior to the viral infection phase.

After the end of this cell growth phase is the virus infection phase, in which the MVA or recombinant MVA to be produced is added to the cell culture and allowed to attach and enter cells in order to reproduce. Generally, viral infection is enhanced by aggregation or clumping of cells in the culture. In preparation for the infection of cells by virus, the medium in which the cells are cultured may have the same composition as in the previous cell growth phase or may be modified or replaced to comprise a different composition of medium. In some embodiments, cells are transferred from an initial growth medium to a second medium; in some embodiments, cells are cultured in continuous cell culture in an initial medium for the cell growth phase and later a different or subsequent medium is added to the cell culture gradually or in aliquots to modify the composition of the medium in preparation for the virus infection phase. In some embodiments, fresh medium of the same composition as the original medium may be added. In some embodiments, said different or subsequent medium is added to a continuous cell culture (*e.g.*, in a bioreactor or perfusion vessel) while older culture medium is removed so as to

essentially replace said initial medium or a previous medium with said different or subsequent medium, and in some embodiments said different or subsequent medium is added to the continuous cell culture until the culture vessel comprises about a 1:1 mix (volume to volume, or v/v) of said previous medium and said different or subsequent medium. In some embodiments, the different or subsequent medium comprises about 1/5, 1/3, 1/2, 2/3, or 3/4 of the total volume, or about an 80:20 mixture v/v of the media mixture in the vessel after it is added. In some embodiments, the total volume of the cell culture (cells plus medium) remains approximately the same before and after the addition of different or subsequent medium, and in some embodiments, the total volume increases with the addition of the different or subsequent medium. In some embodiments, additional medium is later added to the culture, and can comprise either said initial medium and/or said different or subsequent medium.

In some embodiments, quail cells or other avian cells are seeded into flasks for the cell growth phase and cultured in FS/YIE or SCGM-ac medium for several days (*e.g.*, about 2, 3, 4, or 5 days). The medium can be SCGM (Suspension Cell Growth Medium), which as used herein comprises FreeStyle 293<sup>TM</sup> expression medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with: 1 g/L Difco yeast extract (Difco, Becton Dickinson & Co., Franklin Lakes, NJ, USA); 10 µg/L EGF, human recombinant (Gibco, Thermo Fisher Scientific, Waltham, MA, USA); 50 µg/L Long R3 IGF human (recombinant) (Sigma-Aldrich, St. Louis, MO, USA); 20 mg/L L-ornithine monohydrochloride; and 20 mg/L putrescine dihydrochloride (both from Sigma-Aldrich, St. Louis, MO, USA).

In some embodiments, anti-clumping agent (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) is added at 1:100, or in a range between 1:50 and 1:150. Because commercial media often includes anti-clumping agent, if anti-clumping agent is not added, the medium is referred to as “SCGM-ac” herein. Thus, preferably the SCGM does not include anti-clumping agent so is referred to as SCGM-ac. All of the media used to grow cells according to the methods of the invention is serum-free. In some embodiments, L-ornithine and putrescine are omitted from the SCGM; this medium is generally referred to herein as “FS/YIE.” FS/YIE is FreeStyle 293<sup>TM</sup> expression medium supplemented with yeast extract, EGF, and IGF (for example, 1 g/L Difco yeast extract; 10 µg/L EGF; and 50 µg/L Long R3 IGF human (recombinant), and is serum-free.

It is understood by one of skill in the art that some variation in the amounts of additives to cell culture medium can be tolerated and produce the same results; thus, for each additive, amounts +/- 50% of the amounts recited above are considered to be encompassed by this description. That is, generally, SCGM comprises FreeStyle 293<sup>TM</sup> expression medium supplemented with 0.5 – 1.5 g/L Difco yeast extract; 5 - 15 µg/L EGF, human recombinant; 25 - 75 µg/L Long R3 IGF human (recombinant); 10 – 30 mg/L L-ornithine monohydrochloride; and 10 - 30 mg/L putrescine dihydrochloride. As used herein, “SCGM” does not contain any animal serum; that is, it is “serum-free.” In “FS/YIE” medium, FreeStyle 293<sup>TM</sup> expression medium can be supplemented, for example, with 0.5 – 1.5 g/L Difco yeast extract; 5 - 15 µg/L EGF; and 25 - 75 µg/L Long R3 IGF human (recombinant). OptiPRO<sup>TM</sup> medium as used herein is generally supplemented with 1 – 5 mM GlutaMAX<sup>TM</sup> supplement and 0.01% to 0.5% poloxamer 188 (also referred to herein as Pluronic F68 or just “F68”). Optionally, in some embodiments, OptiPRO<sup>TM</sup> medium is used without additional glutamine, L-glutamine, or GlutaMAX<sup>TM</sup> supplement and/or without poloxamer 188 or similar additive. The term “poloxamer 188” or “poloxamer” is used interchangeably herein with the associated brand name, “Pluronic F68,” sometimes also called “F68.” Generally, components of cell culture medium can be substituted with similar compounds; for example, sometimes glutamine or L-glutamine can be substituted for GlutaMAX<sup>TM</sup> supplement with similar results. FS/YIE medium does not contain any animal serum; that is, it is “serum-free.” In some embodiments of the methods of the invention, the media used does not contain any animal serum; that is, it is “serum-free.”

For preparation for the virus infection phase, cells are cultured in a mixture of conditioned SCGM-ac or FS/YIE medium with OptiPRO<sup>TM</sup> medium (in approximately a 1:1 ratio by volume); in some embodiments, this is accomplished by adding an equal volume of OptiPRO<sup>TM</sup> medium to the existing cell culture. In some embodiments, the quail cells are cultivated for a time in FS/YIE or SCGM-ac medium and then the medium is modified by adding OptiPRO<sup>TM</sup> medium prior to infection. In some embodiments, the cells are in continuous culture so that the composition of the medium is modified by infusion of OptiPRO<sup>TM</sup> medium and removal of previous medium until the desired media mixture is achieved. In some embodiments, the cells are initially cultivated in FS/YIE or SCGM-ac medium and the composition of the medium is modified with addition of OptiPRO<sup>TM</sup> medium to comprise a mixture of 40:60 (v/v) or 50:50 (v/v) of FS/YIE or SCGM-ac to OptiPRO<sup>TM</sup> medium.

In some embodiments, this pre-infection cell growth phase continues until cells in culture are mostly aggregated into groups of cells, or growing in “clumps.” Generally, cells are considered to be aggregated when they appear as clusters rather than individual cells when a sample of cell culture is viewed microscopically. By “mostly aggregated” is intended that at least 50% of the cells in culture appear to be clustered together in the suspension culture. In some embodiments, quail cells are cultivated in this pre-infection cell growth phase until the cells are mostly aggregated or about 50% to 70%, or about 60 to 80%, or about 90% of the cells are aggregated. Cells can be assessed by microscopy or other techniques known in the art. In some embodiments, this pre-infection cell growth phase continues for about an hour, or about 2-4 hours, or about 8-12 hours, or overnight. In some embodiments, MVA or recombinant MVA can be added to the cell culture prior to aggregation of the cells so that infection of cells can occur during aggregation.

For the viral infection, MVA or recombinant MVA (for example, MVA-BN-RSV) is added to the culture at an MOI (Multiplicity of Infection) of approximately 0.1, or between about 0.05 and 0.15, to allow the MVA or recombinant MVA to attach to the cells and enter the cells in the culture. By MOI (Multiplicity of Infection) is intended the ratio of a number of infectious virus particles such as MVA or recombinant MVA to the estimated number of cells in the culture. In some embodiments, MVA or recombinant MVA is added to the culture at an MOI of about 0.0005, 0.001, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, or about 0.5 or more. As will be appreciated by one of skill in the art, because of the limitations of technology, each of these values (infectious virus particles of MVA or recombinant MVA and the number of cells in the culture) are subject to measurement error, and thus the actual MOI may vary from the calculated MOI, but generally will be within about 100%, 50%, 25%, 20%, or 10% of the calculated MOI. In some embodiments, prior to the addition of MVA or recombinant MVA to the cells, the cells are transferred to a lower temperature (*e.g.*, about 30°C or 32°C, or between about 30°C and 34°C) and incubated until the culture reaches that temperature.

The cells and/or cell cultures containing the MVA or recombinant MVA then continue to be cultured during a viral production phase, either in the same medium or, for example, in a perfusion bioreactor in which preferably some medium is removed and replaced with fresh medium as the culture continues. After about 2 days, or after about 24 hours to 36 hours, or about 36 hours to 48 hours, or about 48 hours to about 72 hours, or about 72 hours to 96 hours,

or about 2 days to 4 days, preferably about 3 days, the culture is harvested and products such as viruses can be purified. By “harvested” or “harvesting” as used herein is intended that the cell culture is prepared for further processing to isolate the virus from the cell culture. In some embodiments, “harvesting” entails centrifugation to separate cells from the cell culture medium, and in some embodiments, the cell culture including both cells and medium is processed to isolate virus, for example, by adding reagents such as salt, nucleases, proteases, and the like; in some embodiments, the cell culture is placed in a suitable tank or vessel for said processing and this constitutes “harvesting.”

For the virus infection phase and/or virus production phase, in some embodiments, a mixture of medium is used comprising OptiPRO™ medium (with 2 mM GlutaMAX™ supplement and 0.025% Pluronic F68 (poloxamer 188; all from Gibco, Thermo Fisher Scientific, Waltham, MA, USA)) and FS/YIE, which is FreeStyle™ 293 expression medium with yeast extract (Difco, Becton Dickinson & Co., Franklin Lakes, NJ, USA), IGF-1 Long R3 (Sigma-Aldrich, St. Louis, MO, USA) and EGF (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), with the two media being mixed in a ratio of approximately 1:1, or with the final mixture comprising about one part of OptiPRO™ medium to 9 parts FS/YIE medium, or about a 20:80 mixture (v/v) of OptiPRO™ medium to FS/YIE medium; or 30:70, 40:60, preferably 50:50, 60:40, or 70:30 (v/v) mixture. In some embodiments, the final mixture preferably comprises about a 1:1 mixture of OptiPRO™ medium and FS/YIE medium.

In some embodiments, the cell growth phase, virus infection phase, and virus production phase are performed as follows: Quail cells from a suspension cell line are seeded into a shaker or spinner flask containing a medium such as Suspension Cell Growth Medium without anti-clumping agent (SCGM-ac) or FS/YIE medium (see above). In some embodiments, the quail suspension cell line is CCX.E10 (Nuvonis (Vienna, Austria)). In some embodiments, L-ornithine and putrescine are omitted from the SCGM-ac medium. Cells are seeded into this medium at a cell density of approximately  $1.5 \times 10^5$ /mL and incubated at a temperature between 35° - 39° (+/- 2°) Celsius, or at 37° Celsius (+/- 2°). Cells are then propagated and passaged to larger vessels and volumes of medium as appropriate, for example, first in a shaker flask, then a 50L bioreactor, then a 200L bioreactor. Prior to infection of the cells with MVA or recombinant MVA, the medium is changed or adjusted to approximately a 1:1 mix of OptiPRO™ medium (with GlutaMAX™ supplement and poloxamer 188) and FS/YIE or SCGM-ac, and temperature

adjusted to between about 30°C and 34°C, or about 31°C +/- 0.5°C. MVA or recombinant MVA is added to the cell culture at an MOI of 0.05 (+/- 0.010) IU/cell and incubation is continued, for example, at a temperature of to between about 30°C and 34°C, or about 31°C +/- 0.5°C. In some embodiments, cell concentration at the time of infection is between about  $1 \times 10^6$  to  $5 \times 10^6$  cells/mL, or between about  $2 \times 10^6$  to  $4 \times 10^6$  cells/mL, or between about  $3 \times 10^6$  to  $3.5 \times 10^6$  cells/mL. In some embodiments, cells are incubated for between about 24 to 36 hours, or between about 36 to 48 hours or about 60 to 84 hours to allow virus growth at the same temperature or higher.

These methods of culturing quail cells in a mixture of media during virus infection and/or virus production phase were superior to other methods, for example, such as culturing in one of the media alone (see Example 3) or culturing CEF cells on microcarriers, which produced viral titers of about 40-49 IU/cell, versus titers of 121-147 IU/cell obtained using quail cells. This dramatic improvement in specific yield increases the amount of product available for recovery and also reduced the amount of impurities and debris that must be removed to purify product in a downstream process. In this manner, the present invention provides improved methods of cultivating poxviruses in quail cells for production of viral vector-based vaccine products, for example, comprising MVAs or recombinant MVAs.

#### **Downstream process for purification of virus from cell culture**

Briefly, the downstream process comprises collection of product (*e.g.*, MVA or recombinant MVA) from the culture and removal of impurities. In an exemplary embodiment, this process includes steps of: harvesting a cell culture comprising cells infected with MVA or recombinant MVA; treating the cell culture or cells collected therefrom ("material") with a protease (*e.g.*, trypsin); inactivation of the protease with inhibitor; treatment with a nuclease (*e.g.*, Denarase® endonuclease or Benzonase® endonuclease) and incubating the material; lysing cells in the material (*e.g.*, with high pressure homogenization or ultrasonication); removal of cell debris and remaining intact cells in a first filtration step (*e.g.*, with depth filtration to remove components above 5 µm in size)); continuing incubation under conditions suitable for nuclease activity; and concentrating product (*e.g.*, using tangential flow filtration to retain components larger than 100 nm while removing smaller impurities). The solution or buffer comprising the product can then be changed, if desired, using diafiltration.

In some embodiments of this process, the pH and salt concentration of the material can be adjusted before, after, or at the same time as the addition of the nuclease (*e.g.*, Denarase® endonuclease or Benzonase® endonuclease); in some embodiments, the salt concentration of the material is adjusted to 0.5M NaCl or above, or about 1M NaCl after nuclease is added to the material. In some embodiments, the process further includes a second enzymatic treatment with a nuclease; a second concentration of product (*e.g.*, using tangential flow filtration); and optionally an exchange of buffer with diafiltration. In some embodiments, the product is MVA or recombinant MVA which is provided in a pharmaceutically acceptable buffer or other solution and is thus a pharmaceutical composition. The product can then be stored, for example, at very low temperatures (below -20°C). The nuclease steps assist with removal of host cell DNA; in some embodiments, the nuclease is Denarase® nuclease, and in other embodiments, any suitable nuclease may be used, such as Benzonase® endonuclease (Sigma Aldrich, St. Louis, MO, USA) or TurboNuclease™ nuclease (VitaScientific, Beltsville, MD, USA). In some embodiments, the product which is an MVA or recombinant MVA can be separated from impurities and other debris using the sucrose cushion technique and/or ultracentrifugation.

By “material” as used herein is intended the remaining matter from the cell culture that is being processed to recover product such as virus, *etc.*; at various stages, “material” may comprise cells, lysed cells, host cell DNA, residual protein, and the like. The term “product” as used herein refers to the MVA or recombinant MVA that was used to infect the cell culture and can be purified by the methods described herein. In some embodiments, when sufficiently purified, the product is suitable for use as a vaccine. By “purified” as used herein is intended that most of the cellular debris and other contaminants such as host cell DNA have been removed from the cell culture material to provide product essentially comprising MVA or recombinant MVA, with little additional contamination or no significant additional contamination, for example, that would interfere with the therapeutic function of the product if used as a vaccine. In some embodiments, product is sufficiently purified for use as a pharmaceutical composition such as a vaccine. For example, as contemplated herein, a product may be considered sufficiently purified for use as a vaccine if the level of host cell DNA is less than 100 ng per vaccine dose (*e.g.*, per dose of  $1 \times 10^8$  IU/0.5 mL or  $5 \times 10^8$  IU/0.5 mL, or equivalent per dose and volume). That is, a product may be considered sufficiently purified if the level of host cell

DNA is less than 100 ng per 0.5mL volume of vaccine formulation comprising  $1 \times 10^8$  IU or  $5 \times 10^8$  IU of the virus.

By “about” as used in the context of values of parameters of the methods and compositions of the invention is intended that the actual value of said parameter(s) may vary somewhat from the recited value of said parameter and still have the same qualities and provide the benefit or result of the methods and compositions. Thus, as used herein, a parameter that is recited to have “about” a particular value has an actual value within 10% of the recited value (*e.g.*, a solution having a temperature of “about 30°C” has a temperature between 27°C and 33°C), unless the context clearly indicates otherwise. In this manner, “about” encompasses the recited value of a parameter (*e.g.*, a solution having a temperature of “about 30°C” can have a temperature of 30°C).

In an exemplary embodiment(s), the details of the downstream process are as follows: To harvest the cell culture and collect product, material is collected into a mixer tank. A first series of enzymatic steps is then performed (sometimes referred to as “Enzymatic Treatment 1”). The material is treated with a protease; in some embodiments, the protease has trypsin activity (*e.g.*, trypsin or recombinant trypsin such as TrypLE (Gibco, Fisher Scientific, Waltham, MA, USA)). In some embodiments, the material is treated with recombinant trypsin (*e.g.*, at a ratio of 1:5) and incubated to allow time for digestion, for example, for 30 minutes to 1 hour at ambient temperature. Before the addition of nuclease, the material is treated to inactivate the protease (*e.g.*, with trypsin inhibitor (1:5) for 15 minutes at ambient temperature) and also to adjust the pH, salt concentration, and volume of the material as needed; these adjustments can be made in any order and can be made simultaneously or separately. For example, the pH of the material is adjusted to about 7.5, 8.0, 8.5, 8.7, or any appropriate pH, for example, any pH in a range between 8.0 and 8.6, such as about 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, or 8.7. In some embodiments,  $MgCl_2$  is added to a concentration of about 2 mM or in a range of 2 mM to 25 mM, and a nuclease is added (*e.g.*, Denarase® endonuclease (c-LEcta (Leipzig, Germany)), for example at a concentration of 20 U/mL). NaCl or another salt or chaotropic agent is added to a concentration of about 1M, or at least about 0.5M, 0.6M, 0.7M, 0.8M, 0.9M, 1.0M, 1.1M, 1.2M, 1.3M, or at least about 1.4M. Optionally, the material is cooled to about 4°C, or to between 2°C and 8°C, for about 2 hours, about 4 hours, about 12 hours, or between 2 and 24 hours, or between 14 and 24 hours while being stirred or agitated, or material can proceed to the next step

without incubation (*e.g.*, material can be held at 4°C overnight; this step can be referred to as “Hold Up 1”). Optionally, if a higher biomass burden is present (*i.e.*, more cells are being treated), the additional impurities and other debris can be removed with minor modifications to this process by increasing the amount of nuclease, increasing the incubation temperature, and/or increasing incubation time during these steps. Also, in some embodiments, the material is diluted as a result of or in addition to the adjustments of pH and salt described above, for example, the volume of the material is increased by about 40%, 50%, 60%, or about 100% or 200%, which may aid in removal of impurities.

Cells are then lysed in order to collect intracellular viruses or other product. This step, which can be referred to as “Cell Lysis,” is accomplished with any suitable technique such as high pressure homogenization (HPH) or ultrasonication, which releases cell debris, including host cell DNA and host cell protein. Impurities including cell debris and remaining cells are then removed, for example, by depth filtration (*e.g.*, through a 5 µm or 3 µm depth filter), a step which can be referred to as “Clarification.” The material can then be stored at ambient temperature or warmed to about 30°C or in the range between 25-37°C and incubated overnight or for about 1 hour, or about 2 to 6 hours, or about 8 to 12 hours, or about 22 to 30 hours to allow the nuclease (*e.g.*, Denarase® endonuclease) to degrade the host cell DNA (a step that can be referred to as “Hold Up 2”); in some embodiments, the material is stirred or agitated continually during this step. Optionally, the material is held at  $\leq 10^{\circ}\text{C}$ . This step typically removes much of the host cell DNA.

A further filtration is then performed, for example, using Tangential Flow Filtration (TFF); this step can be referred to as “TFF1.” This step can be performed using any suitable filter, for example, it can be performed with a 0.1 µm PESU E-screen to obtain a concentration (*e.g.*, of 5-6x) via ultrafiltration while removing small impurities, followed with a further concentration (*e.g.*, of 1.3 x) using diafiltration (in some embodiments, for a total concentration of at least about 2x, about 4x, about 6x, or about 8x). In some embodiments, for this step, filtration is performed using hollow fiber (HF)-based filtration with a Polysulfone (PS)-based filter with a 0.5 mm diameter and a cutoff of 0.05 µm. In some embodiments, filtration is performed using HF-based filtration with a mixed cellulose ester (“ME”)-based filter with a 0.63 mm diameter and a cutoff of 0.1 µm, and in some embodiments the filter comprises modified polyethersulfone (mPES) with a 0.5 mm diameter and a cutoff of 750 kDa. As described above,

this TFF concentrates the product and diafiltration is then performed with diafiltration buffer DF1 (10mM Tris, 2mM MgCl<sub>2</sub>, pH 8.2), removing impurities and Denarase® endonuclease. Optionally, the system is flushed at the end of this step and the flush pooled with the filtered material in a suitable buffer.

In trial runs, these methods removed large aggregates that were present in the material after cell lysis and provided superior results at later steps and in the final product, including reduced amounts of host cell DNA and total protein. These superior results were unexpected for several reasons, including the recommendation by the Denarase® endonuclease manufacturer (c-Lecta GmbH, Leipzig, Germany) to use this endonuclease at lower salt concentrations to avoid decreasing the efficiency of the enzyme. It was also surprising that addition of relatively high amounts of NaCl early in the process could improve the removal of host cell DNA from the product at a later filtering step (see Figure 2), and that this effect could not be provided by adding NaCl later in the process. Further, adding an enzyme with trypsin-like activity early in the process also acted to reduce levels of aggregates and improved overall recovery and host cell DNA removal, which was unexpected because trypsin is typically used in upstream processes and could be considered an unnecessary contaminant when used as described here. This downstream process worked well for viral purification from different types of cultures including bioreactor cultures, perfusion bioreactor cultures, and wavebag cultures.

In some embodiments of the downstream process, a second enzymatic treatment is performed with nuclease (*e.g.*, Denarase® endonuclease) to further digest remaining host cell DNA, for example, adding Denarase® endonuclease to a concentration of 75-150 U/mL and incubating overnight (*e.g.*, about 4 to 8 hours, about 8 to 12 hours, or about 14-22 hours) at ambient temperature (~22 °C) or in a range between 25-37 °C. If performed, this step can be referred to as “Enzymatic Treatment 2.” In some cases, MgCl<sub>2</sub> is added (*e.g.*, to 2 mM) and pH adjusted to increase nuclease activity; these adjustments can be made before, after, or at the same time as addition of the nuclease to the material. Optionally, the material is held at a low temperature (*e.g.*, less than about 10°C, or less than about 4°C); this can be referred to as “Hold Up 3.” In some embodiments, the material is continually stirred or agitated during this step to avoid sedimentation and/or aggregation.

In some embodiments, a second filtration step is then performed using Tangential Flow Filtration (TFF), for example, using a 0.1 µm cutoff PESU E-screen (for example, producing a

concentration of 14-15x via ultrafiltration (including at least 2 to about 10 exchanges of diafiltration buffer)). Another suitable filter for this step, for example, is an mPES cassette with a 0.01  $\mu\text{m}$  cutoff. This filtration can be performed using diafiltration buffer (DF2) (10 mM Tris, 140 mM NaCl, pH 7.7), and the system can be flushed at the end of this step and the flush pooled with the product, resulting in a final concentration of about 4x. Alternatively, this second filtration step is performed using an HF (hollow fiber)-based TFF filtration step with any suitable filter, for example, PS, 0.5 mm diameter and 0.05  $\mu\text{m}$  cutoff hollow fiber, a mixed cellulose ester ("ME")-based filter with a 0.63 mm diameter and a cutoff of 0.1  $\mu\text{m}$ , or a modified polyethersulfone (mPES) filter with a 0.5 mm diameter and a cutoff of 750 kDa. First, the material is concentrated and then can be diafiltrated to exchange the buffer. Optionally, the system is flushed at the end of the step and the flush pooled with the filtered material, resulting in a final 10x concentrated product. Product can then be stored, for example, at about  $-20^{\circ}\text{C}$ , or at about  $-80^{\circ}\text{C}$ .

In some embodiments, during the above process, and particularly in the overnight and/or incubation steps, it is important to avoid sedimentation or settling of the material, as adverse effects on yield may be observed, possibly due to aggregation of virus product with residual debris from the host cells or other viral particles. Accordingly, in some embodiments, it is necessary to stir or otherwise agitate the material during each of the process steps to prevent settling and/or sedimentation, intermittently or continually. In some embodiments, the material is stirred or agitated during steps requiring incubation, such as during an overnight hold, but other steps may be performed without stirring or agitation. The stirring or agitation may be accomplished by any suitable means, for example, using magnetic stirrer bars, shaker platforms, circulation of gas or liquid through the material, and the like.

In some embodiments, the process can be paused or "held up" at any suitable step, or steps can be extended in time, so long as the effect of the step and/or the effect of the overall process are achieved. Thus, while some steps are indicated herein to be "Hold Up" steps, these incubations can be made shorter or longer, or "hold ups" performed at other steps, for convenience or ease of manufacturing.

The methods of the invention provide purification of active virus without the use of chromatography methods, including for example hydrophobic interaction chromatography ("HIC"), and thus have the benefit of not requiring additional materials such as HIC matrix for

purification of virus. Thus, the invention provides methods of purification that do not use hydrophobic interaction chromatography or other chromatographic techniques.

In trial runs, results of this process achieved the goals of a high overall virus (product) recovery of at least 50% and with a host cell DNA level below 100 ng per vaccine dose, as preferred. Also, the remaining host cell DNA was fragmented, making any viable coding nucleic acids in the vaccine less likely. These results were superior to those obtained with EB66 duck cells in earlier trials as well as those obtained with CEF cells.

In summary, these methods of producing vaccines comprising MVA or recombinant MVA from avian cells provide higher yields and lower impurity levels. These methods also can be used to prepare vaccines comprising MVA or recombinant MVA from infected quail cell lines, thus making possible the use of cell banks, decreasing the potential for contaminating adventitious agents, and making it possible to manufacture vaccine products without the use of antibiotics. In this manner, use of the methods of the invention in producing MVAs and recombinant MVAs can increase the quality and safety of the vaccine comprising these drug substances.

### **Certain embodiments**

Certain embodiments of the present invention also include the following items:

Item 1 is a method of culturing quail cells to produce poxviruses (or alternatively, a method of producing poxviruses in quail cells), comprising the steps of: (a) culturing quail cells in suspension in culture medium until the cell density is about  $1 \times 10^6$  cells/mL to  $5 \times 10^6$  cells/mL, preferably about  $2 \times 10^6$  cells/mL to  $4 \times 10^6$  cells/mL, more preferably about  $3 \times 10^6$  cells/mL or at least  $3 \times 10^6$  cells/mL ("cell growth stage"); (b) modifying the culture medium to induce cell aggregation; (c) adding an MVA or a recombinant MVA to said cell culture to allow infection of the cells by the MVA or the recombinant MVA ("virus infection phase"); (d) continuing to culture said cells for about one day or about one to four days so that the amount of MVA or recombinant MVA is increased ("virus production phase"); and (e) harvesting said cell cultures. In some embodiments, the method further comprises purifying said MVA or recombinant MVA as described herein.

Item 2 is the method of item 1, wherein step (a) comprises culturing the quail cells in medium comprising FS/YIE or SCGM-ac medium and step (b) comprises modifying the medium

to comprise about a 60:40 mix of FS/YIE or SCGM-ac with fresh OptiPRO™ medium. Item 3 is the method of item 2, wherein an amount of OptiPRO™ medium is added to the cell culture so that the medium comprises about a 1:1 mix of FS/YIE or SCGM-ac with OptiPRO™ medium, where each medium is either fresh or conditioned. Item 4 is the method of item 1, wherein step (a) comprises culturing the quail cells in FS/YIE or SCGM-ac medium and step (b) comprises modifying the medium to comprise FS/YIE or SCGM-ac medium and OptiPRO™ medium in a mixture between about 35:65 and 55:45 (v/v), preferably about 1:1, wherein said FS/YIE or SCGM-ac medium is partially or completely fresh or conditioned medium. Item 5 is the method of item 2, wherein step (b) comprises adding an amount of OptiPRO™ medium to the cell culture so that the medium comprises about a 40:60 mixture of fresh OptiPRO™ medium with previous medium (v/v) or about a 1:1 mixture of previous medium with fresh OptiPRO™ medium. Item 6 is the method of item 4 or item 5, wherein step (b) further comprises culturing the cells until about 50% to 70% of the cells are aggregated. Item 7 is the method of item 1, wherein cells are cultured in the virus production phase of step (d) for about 2 days, or about 24 hours to about 36 hours, about 40 hours to about 48 hours, about 60 hours to about 72 hours, about 3 days, or about 4 days. Item 8 is the method of any of items 1 through 7, wherein cells are cultured in the virus production of step (d) for about 12 to 24 hours, for about 24 to 36 hours, for about 36 to 48 hours, for about 48 to about 72 hours, or for about 72 to 96 hours, preferably for 2 to 4 days, more preferably for about 3 days. Item 9 is the method of any of items 1 through 8, wherein step (c) comprises adding MVA-BN® virus to said cell culture. Item 10 is the method of any of items 1 through 9, wherein step (c) comprises adding MVA-BN-RSV to said cell culture. Item 11 is the method of any of items 1 through 10, wherein said quail cells are CCX.E10 quail cells (Nuvonis (Vienna, Austria)).

Item 12 is a method for producing MVA or a recombinant MVA in quail cells comprising: (a) culturing a continuous quail cell suspension line to increase cell number; (b) modifying the culture medium so that aggregation of the cells in culture is induced; (c) adding said MVA or recombinant MVA to said culture to allow infection of said cells; (d) further culturing said infected cells to increase the amount of MVA or recombinant MVA in the cell culture; and (e) harvesting said cell culture for further processing. Item 13 is the method of item 12, wherein step (a) further comprises culturing the cells in medium that is FS/YIE or SCGM-ac

and step (b) comprises modifying the culture medium so that it comprises about a 1:1 mixture (v/v) FS/YIE or SCGM-ac medium and OptiPRO™ medium.

Item 14 is the method of any of items 1 through 8 or 10 through 13, wherein the recombinant MVA comprises: (i) at least one nucleotide sequence encoding an antigen of a respiratory syncytial virus (RSV) membrane glycoprotein, wherein the nucleotide sequence encodes a full-length RSV F membrane glycoprotein; and (ii) at least one nucleotide sequence encoding RSV nucleocapsid antigens, wherein the nucleotide sequence encodes both a full-length RSV N nucleocapsid protein and a full-length RSV M2 transcription elongation factor protein; and further comprising: (c) at least one nucleotide sequence encoding an antigen of an RSV G membrane glycoprotein, wherein the nucleotide sequence encodes a full-length RSV G membrane glycoprotein. Item 15 is the method of item 14, wherein the nucleotide sequence encoding the RSV G membrane glycoprotein is from RSV strain A.

Item 16 is a population of quail cells or a cell culture that is a suspension quail cell culture infected with an MVA or recombinant MVA, wherein said population or cell culture comprises about  $1 \times 10^6$  to  $1 \times 10^8$  quail cells per mL and about  $1 \times 10^3$  virus per mL of the MVA or recombinant MVA, or preferably between  $1 \times 10^6$  MVA or recombinant MVA per mL and  $1 \times 10^8$  MVA or recombinant MVA per mL. Item 17 is a cell culture that is a suspension quail cell culture or population of quail cells comprising MVA or recombinant MVA that has been cultured so that the cell culture or population comprises about  $1 \times 10^6$  cells/mL to  $5 \times 10^8$  cells/mL and about  $1 \times 10^7$  virus per mL to  $1 \times 10^9$  virus per mL. Item 18 is the cell culture that is a suspension quail cell culture of item 16 or 17 that comprises CCX.E10 quail cells or the population of quail cells of item 16 or 17 that is CCX.E10 quail cells. Item 19 is the cell culture of item 16, 17, or 18 that comprises MVA-BN® or MVA-BN-RSV.

Item 20 is a method of providing a pharmaceutical composition or making a vaccine comprising an MVA or recombinant MVA from a cell culture or population of cells (for example, of item 16, 17, 18, or 19), comprising the steps of: (a) collecting material or harvesting cell cultures comprising cells and/or virus or a population of cells to produce collected material; (b) treating the collected material with a protease having trypsin activity; (c) optionally, adjusting pH of the material to 8.0 – 8.6; (d) treating the material with a nuclease; (e) lysing cells in the material; (f) filtering the material to remove cell debris and remaining cells; (g) continuing incubation with nuclease for a period of time; and (h) concentrating the product using tangential

flow filtration. Item 21 is the method of item 20, further comprising the steps of: (j) treating the material with a nuclease; (k) concentrating the MVA or recombinant MVA product using tangential flow filtration. Optionally for each of item 20 and 21 there is an additional step of exchanging buffer with diafiltration, and optionally there is a final step of suspending the product in a suitable buffer to provide a pharmaceutical composition.

Item 22 is the method of item 20 or 21 wherein said nuclease in step (d) and/or step (j) is Denarase<sup>TM</sup> nuclease. Item 23 is the method of item 22, further comprising the addition of NaCl to a final concentration of about 1 M after step (d), during step (d), and/ or before step (e). Item 24 is the method of item 22, wherein step (d) further comprises the addition of NaCl to a final concentration of about 1 M after addition of nuclease; optionally, after NaCl is added, the material is incubated with said nuclease at between 22°C and 37°C for at least 2 hours. In some embodiments of Item 3 and Item 4, NaCl is added to a final concentration of at least 0.5M.

Item 25 is a pharmaceutical composition comprising MVA-RSV or MVA-BN-RSV made by the process of any of items 20-24 for the prevention of Lower Respiratory Tract Disease caused by RSV. Item 26 is a pharmaceutical composition comprising an MVA or recombinant MVA made by the method of any of items 20-24, optionally a vaccine containing 100 ng or less of host cell DNA per vaccine dose.

Item 27 is a recombinant modified vaccinia virus Ankara (MVA) comprising a nucleotide sequence encoding an antigen of at least one respiratory syncytial virus (RSV) membrane glycoprotein for treating or preventing disease caused by an RSV infection, for example, by intranasal administration, made by the method of any of items 20-24.

Item 28 is a recombinant modified vaccinia virus Ankara (MVA) comprising at least one nucleotide sequence encoding an antigen of a respiratory syncytial virus (RSV) membrane glycoprotein and at least one nucleotide sequence encoding an RSV nucleocapsid antigen made by the method of any of items 20-24.

Item 29 is the recombinant MVA of item 27 or 28, wherein the nucleotide sequence encoding an antigen of the RSV membrane glycoprotein encodes an RSV F antigen. Item 30 is the recombinant MVA of item 27 or 28, wherein the nucleotide sequence encoding an antigen of the RSV membrane glycoprotein encodes a full length RSV F membrane glycoprotein. Item 31 is the recombinant MVA of item 29 or 30, wherein the nucleotide sequence encoding an antigen

of the RSV F membrane glycoprotein is derived from RSV strain A, preferably from A2 and/or A<sub>long</sub>.

Item 32 is a recombinant modified vaccinia virus Ankara (MVA) made by the method of any of items 20-24 and comprising: (a) at least one nucleotide sequence encoding an antigen of a respiratory syncytial virus (RSV) membrane glycoprotein, wherein the nucleotide sequence encodes a full-length RSV F membrane glycoprotein; and (b) at least one nucleotide sequence encoding an RSV nucleocapsid antigen, wherein the nucleotide sequence encodes both a full-length RSV N nucleocapsid protein and a full-length RSV M2 matrix protein, which are encoded by a single open reading frame, wherein the single open reading frame comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:10 or comprises the nucleotide sequence of SEQ ID NO:9; and further comprising: (c) at least one nucleotide sequence encoding a full-length RSV G membrane glycoprotein.

Item 33 is the recombinant MVA of item 32 wherein the nucleotide sequence encoding the RSV F membrane glycoprotein is from RSV strain A, preferably from A2 and/or A<sub>long</sub>. Item 34 is the recombinant MVA of item 32 or 33, wherein the nucleotide sequence encoding the RSV F membrane glycoprotein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:2 or comprises the nucleotide sequence of SEQ ID NO:1. Item 35 is the recombinant MVA of any of items 27 to 32, wherein the nucleotide sequence encoding the RSV G membrane glycoprotein is from RSV strain A, preferably from strain A2, and/or B. Item 36 is the recombinant MVA of any of items 27 to 35 that comprises a nucleotide sequence encoding an RSV G membrane glycoprotein that comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:4 or comprising the nucleotide sequence of SEQ ID NO:3. Item 37 is the recombinant MVA of any one of claims 27 to 36 wherein the MVA used for generating the recombinant MVA is MVA-BN® virus as deposited at the European Collection of Cell Cultures (ECACC) under number V00083008.

Item 38 is a vial comprising the pharmaceutical composition of item 25 or 26, wherein the pharmaceutical composition comprises at least  $1 \times 10^8$  IU/mL, at least  $3 \times 10^8$  IU/mL, at least  $5 \times 10^8$  IU/mL, or at least  $7 \times 10^8$  IU/mL of MVA or recombinant MVA.

Item 39 is a pharmaceutical composition comprising the recombinant MVA of any of items 25 to 37. Item 40 is a pharmaceutical composition comprising at least  $1 \times 10^8$  IU/mL, at least  $3 \times 10^8$  IU/mL, at least  $5 \times 10^8$  IU/mL, or at least  $7 \times 10^8$  IU/mL of the recombinant MVA

of any of items 25 to 37. Item 41 is a vial comprising the pharmaceutical composition of item 39 or item 40, optionally a vaccine containing 100 ng or less of host cell DNA per vaccine dose.

Item 42 is a method comprising the steps of any of items 1 to 15 followed by the steps of any of items 20 to 24.

Item 43 is a method of culturing and processing quail cells to produce poxviruses (or alternatively, a method of producing poxviruses comprising culturing and processing quail cells), comprising the steps of: (a) culturing quail cells in suspension in culture medium until the cell density is about  $1 \times 10^6$  cells/mL to  $5 \times 10^6$  cells/mL, producing a cell culture comprising from  $1 \times 10^6$  cells/mL to  $5 \times 10^6$  cells/mL; (b) modifying said culture medium to comprise FS/YIE or SCGM-ac medium and OptiPRO™ medium in a ratio from 40:60 to 60:40 (v/v) and continuing to culture said quail cells; (c) adding MVA or recombinant MVA to said cell culture to allow infection of the cells by the MVA or the recombinant MVA; (d) continuing to culture said cells for about one to four days so that the amount of MVA or recombinant MVA is increased; (e) collecting material comprising cells and/or virus from the cell culture to produce collected material; (f) treating the collected material with a protease having trypsin activity; (g) adjusting pH of the material to 8.0 – 8.6; (h) treating the material with a nuclease; (i) mechanically lysing cells in the material; (j) filtering the material to remove cell debris and remaining cells; (k) continuing incubation with nuclease for a period of time; and (l) concentrating the product using tangential flow filtration. Item 44 is the method of item 43 wherein said nuclease in step (h) is Denarase™ nuclease. Item 45 is the method of item 43 or 44, further comprising adding NaCl to a final concentration of about 1 M after step (h), during step (h), and/ or before step (i); optionally, after NaCl is added, the material is incubated between 22°C and 37°C for at least 2 hours. Item 46 is the method of item 43, 44, or 45, further comprising the steps of: (m) after step (l), treating the material with a nuclease; and (n) concentrating the MVA or recombinant MVA product using tangential flow filtration. Optionally for each of items 43 through 46 there is an additional step of exchanging buffer with diafiltration following tangential flow filtration, thus providing the product (MVA or recombinant MVA) in a suitable solution such as a buffer.

## EXAMPLES

### Example 1: Purification of recombinant MVA from avian cell cultures

Each of MVA-BN-RSV and MVA-BN® virus were amplified (separately) in a suspension quail cell culture as described above, then purified as follows. Material (cell culture) was collected into a mixer tank and treated with TrypLE (Gibco, Fisher Scientific, Waltham, MA, USA) for an hour at ambient temperature, followed by inactivation with trypsin inhibitor for 15 minutes. The pH of the material was adjusted to 8.5,  $\text{MgCl}_2$  was added to 2mM, and 20 U/mL Denarase® endonuclease added. NaCl was added to a final concentration of 1M, and the material was incubated overnight at 5-8 °C with stirring. Cells were lysed using high pressure homogenization (HPH), and cell debris and remaining cells were removed by depth filtration using a 5  $\mu\text{m}$  depth filter. The material was then incubated at 30°C overnight with stirring and filtered using cassette-based Tangential Flow Filtration (TFF) with a 0.1  $\mu\text{m}$  cutoff PESU filter or a PS hollow fiber with a 0.05  $\mu\text{m}$  cutoff and an inner diameter of 0.5 mm. In this TFF, ultrafiltration was performed and then diafiltration using DF1 buffer (10mM Tris, 2mM  $\text{MgCl}_2$ , pH 8.2), including a flush of the system at the end that was pooled with the retained material. A second enzymatic treatment was performed with Denarase® endonuclease at 100 U/mL with 2mM  $\text{MgCl}_2$  and incubated overnight at room temperature with stirring. A second TFF step was then performed using cassette-based TFF with a 0.1  $\mu\text{m}$  cutoff PESU filter or PS hollow fiber with a 0.05  $\mu\text{m}$  cutoff and an inner diameter of 0.5 mm. In this filtration, diafiltration was performed with DF2 buffer (10 mM Tris, 140 mM NaCl, pH 7.7); optionally, a second buffer exchange was performed.

In four exemplary runs, this process yielded approximately 65% recovery with 85 ng/dose host cell DNA and 1 mg/dose total protein. This result almost meets the criteria recommended by regulatory authorities for final concentration of host cell DNA in the final product of 10 ng or less per vaccine dose; also the process met the overall recovery of more than 50% in order to be economically feasible as a vaccine production process. In addition, the remaining host cell DNA was fragmented, making any viable coding nucleic acids in the vaccine less likely.

Among the surprising aspects of these experiments was that trypsin enzymatic activity had a positive effect in later process steps and on the end product, even though the trypsin activity was only present earlier in the process even before the cells were lysed. **Figure 1** shows

the effects of Denerase® endonuclease on the amount of Host Cell DNA (“HCD”) per dose of vaccine at various stages of an exemplary downstream process. **Figure 2** shows that addition of NaCl during initial steps of the downstream process provides a surprising decrease in Host Cell DNA (HCD) per dose of vaccine at later stages of an exemplary downstream process even though at early stages of the process the amount of Host Cell DNA per dose of vaccine is increased. **Figure 7** includes a schematic showing the hypothesized effect of the first enzymatic treatment step with trypsin enzymatic activity (*e.g.*, TrypLE) and nuclease (*e.g.*, Denarase® enzyme) on the harvested cells, amplified virus, and impurities that result from lysis of the cells during the virus purification process. Figure 7 also shows transmission electron micrographs of cells with and without trypsin enzymatic treatment, where the cells in the absence of trypsin treatment form a compact clump (left-hand picture) and cells treated with trypsin enzymatic treatment appear to be much more loosely associated in a smaller group (right-hand picture) in which each cell appears to be in contact with the surrounding environment.

Another surprising aspect of the methods of the invention is that Denarase® endonuclease was able to digest the majority of Host Cell DNA (HCD) at high NaCl concentrations of about 1M, far above the manufacturer’s recommended levels of up to 150 mM NaCl, and above levels at which the manufacturer indicates this enzyme should be active.

A comparison was performed in parallel of the downstream purification process with and without adjusting the pH in the first enzymatic treatment step. The results were very similar. Specifically, in the absence of adjustment of the pH, the recovery as % of lysis was 52.9% for an obtained number of doses of 5341 doses/liter of bioreactor with HCD at 3.1 ng/dose, while in the standard process that includes pH adjustment, the recovery was 55.0% for an obtained number of doses of 5572 doses/liter of bioreactor with HCD at 1.3 ng/dose. Thus, in some embodiments, the adjustment of pH in the first enzymatic treatment may be omitted without adversely affecting the results.

#### **Example 2: Suspension quail cell lines for producing poxviruses**

Continuous suspension quail cell lines were generated from quail cells through passaging of adherent cells followed by adaptation to growth in suspension. Thereafter, single clones were selected and amplified, and adapted to serum-free culture suspension cell growth medium

(SCGM). One continuous suspension quail cell line was selected for further use (also referred to herein as “CCX.E10”), and an adherent quail cell line (CCX.2C4) was also characterized (both cell lines commercially available from Nuvonis (Vienna, Austria)).

Initial experiments were conducted to assess the suitability of quail cell suspension and adherent lines for producing viruses. Shope Fibroma Virus (SFV), Fowlpox Virus (FPV), and Vaccinia Virus (VACV) were tested. SFV, as expected, was shown to not replicate in CEF cells or quail cells. FPV and VACV produced peak titers in quail cells in the same range as those produced in CEF cells under some conditions.

### **Example 3: Virus yields from suspension quail cells cultured in various media**

Cells from the continuous suspension quail cell line CCX.E10 (Nuvonis (Vienna, Austria)) were seeded in stirrer flasks in suspension cell growth medium without anti-clumping agent (SCGM-ac medium) for the cell growth phase. Previous experiments had shown that viral infection was inhibited by the anti-clumping agent (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) normally added for routine suspension culture, so it was omitted here.

Three days later (=“Day 0”), cells were transferred to different culture media at a cell density between  $8 \times 10^5$  –  $1.1 \times 10^6$  cells/mL for the virus infection phase. The media tested were: SCGM-ac+CaCl<sub>2</sub>, OptiPRO™ medium, and a 50:50 mix of SCGM-ac + OptiPRO™ medium. In previous experiments, it was shown that viral yields were increased by the addition of CaCl<sub>2</sub> to SCGM-ac medium because CaCl<sub>2</sub> induces cell aggregation, thereby facilitating viral spread. However, OptiPRO™ also promotes aggregation of cells. Media were supplemented with GlutaMAX™ and Pluronic F68 to reduce shear forces and foaming.

Cells were incubated at 30°C or 32°C for 1 hour before infection with MVA-BN-RSV (*i.e.*, MVA-mBN294B) at a MOI (Multiplicity of Infection) of 0.1. For analysis of virus yields, cell samples were collected at days 1-4 post infection, sonicated, frozen at -20°C, and subsequently titrated (vortexed  $\geq$  1min) by the standard TCID<sub>50</sub> assay. **Figure 3** shows virus yields as geometric means (GM) of TCID<sub>50</sub>/mL with geometric standard deviation; numerical values are shown in Table 1 below. Data points represent results from nine individual experiments. Fold-differences shown below the graph are in comparison to yields obtained with SCGM-ac +CaCl<sub>2</sub>.

**Table 1: Virus yields in SCGM and OptiPro media (TCID<sub>50</sub>/mL)**

| Medium                      | Day 1                 | Day 2                 | Day 3                 | Day 4                 |
|-----------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| SCGM-ac + CaCl <sub>2</sub> | 6.1 x 10 <sup>5</sup> | 1.3 x 10 <sup>7</sup> | 5.9 x 10 <sup>6</sup> | 1.0 x 10 <sup>7</sup> |
| OptiPro                     | 1.1 x 10 <sup>6</sup> | 1.8 x 10 <sup>7</sup> | 4.1 x 10 <sup>7</sup> | 2.3 x 10 <sup>7</sup> |
| cSCGM-ac + OptiPro          | 8.3 x 10 <sup>6</sup> | 1.8 x 10 <sup>8</sup> | 8.3 x 10 <sup>7</sup> | 4.0 x 10 <sup>7</sup> |

The data in Figure 3 show that when the cell culture medium for the virus infection phase was changed completely to OptiPRO™ medium (with Pluronic F68 additive), MVA-BN-RSV yields were higher than those obtained using cell culture medium SCGM-ac+CaCl<sub>2</sub>. Cells incubated in OptiPRO™ medium produced an approximately 3-fold higher peak viral titer (d3) compared to cells incubated in SCGM-ac+CaCl<sub>2</sub> (d2; Table 1 and Figure 3; note log scale). The data shown in **Figure 3** also demonstrates that virus yields from cells incubated in medium that was a 50:50 mix of cSCGM-ac+ OptiPRO™ medium were reproducibly higher than virus yields obtained using SCGM-ac+CaCl<sub>2</sub> or OptiPRO™ medium. With cells cultured in the 50:50 mix of cSCGM-ac+ OptiPRO™ medium, the peak yields of MVA-BN-RSV at Day 2 (“d2”) post infection corresponded to a cell-specific yield of 180 TCID<sub>50</sub>/cell and were approximately 4.4-fold and 13.8-fold higher than peak yields with OptiPRO™ (d3) and SCGM-ac + CaCl<sub>2</sub> (d2) alone, respectively.

**Example 4: Virus yields from quail cells cultured in media mixtures**

Cells from the continuous suspension quail cell line CCX.E10 (Nuvonis (Vienna, Austria)) were seeded at “Day minus 3” in a stirrer flask in SCGM-ac medium (SCGM without the anti-clumping ingredient). Culturing of cells in the medium transforms the SCGM-ac medium into conditioned SCGM-ac medium (cSCGM-ac). At Day 0, 20 mL of this cell culture (with medium) was transferred to each of six different shaker flasks to be mixed with an equal volume of different media, including: (i) OptiPRO™ medium (resulting in a 50:50 mixture of cSCGM-ac and OptiPRO™ medium); (ii) OptiPRO™ medium premixed with cSCGM-ac in different amounts (resulting in proportions ranging from 60:40 to 90:10 of cSCGM-ac and OptiPRO™ medium); or (iii) cSCGM+CaCl<sub>2</sub> (containing no OptiPRO™ medium). All

conditions were tested using 40 mL of culture volume and  $1 \times 10^6$  cells/mL. Media were supplemented with GlutaMAX<sup>TM</sup> supplement (final concentration 2 mM) and Pluronic F68 (final amount 0.125%) to reduce shear forces and foaming.

Cells were transferred to 30°C for 1 h before infection with MVA-BN-RSV (co-encoding mRFP fluorescent protein) at a MOI of 0.1; subsequently, cells were cultured at 30°C. For analysis of viral yields, duplicate samples from CCX.E10 cultures were collected at Days 1-4 post infection, sonicated, and frozen at -20°C. Samples from Day 1 and 2 (d1, d2) and Day 3 and 4 (d3, d4) were titrated in parallel (vortexed  $\geq 1$  min) by the standard TCID<sub>50</sub> assay. **Figure 4A** shows viral yields as geometric means of TCID<sub>50</sub>/mL with geometric standard deviation. For analysis of viral infection and spread, mRFP expression was analyzed at Days 2-4 post infection by flow cytometry (**Figure 4B**) (GMFI, geometric mean fluorescence intensity).

As shown in **Figure 4A**, viral yields of MVA-BN-RSV were affected by the ratio of SCGM: OptiPRO<sup>TM</sup> medium used during infection of the quail cells with the recombinant MVA (*i.e.*, the virus infection stage). The highest viral yield ( $1.8 \times 10^8$  TCID<sub>50</sub>/mL) was reached at Day 3 post infection (d3) with the 50:50 mix of cSCGM: OptiPRO<sup>TM</sup> medium, although yields were already very close to this highest yield with this medium composition at Day 2 post infection (d2).

Compared to the 50:50 mixture (also referred to herein as 1:1), viral yields gradually decreased with medium containing less OptiPRO<sup>TM</sup> medium, although with the 60:40 mixture, the peak viral yield of  $1.5 \times 10^8$  TCID<sub>50</sub>/mL at Day 3 (“d3”) was still within the same range as with the 50:50 mixture. With the 70:30 mixture, the peak viral yields of  $7.5 \times 10^5$  TCID<sub>50</sub>/ml at both d3 and d4 were 2-fold lower than those obtained with the 50:50 mixture. With medium containing less than 20% OptiPRO<sup>TM</sup> medium, viral yields were clearly much lower at Day 2 post infection. TCID<sub>50</sub>/cell values were 208 for the 50:50 mix of media on Day 3; 168 for the 60:40 mix on Day 3; 78 for the 70:30 mix on Day 3 and Day 4; 75 for the 80:20 mix on Day 4; 66 for the 90:10 mix on Day 4; and 42 for the cSCGM+CaCl<sub>2</sub> medium on Day 4.

These viral yield results were echoed by the data obtained by assessing mRFP expression by the MVA-BN-RSV used here, which can be used as a marker of infected cells. Viral spread was monitored at Day 2 to Day 4 (“d2”-“d4”) post infection by analysis of mRFP. Flow cytometry results are shown in **Figure 4B**. Viral spread was most efficient with media containing 50% or 40% OptiPRO<sup>TM</sup> medium, and more than 80% of cells were infected already

at Day 2 post infection (*i.e.*, following introduction of MVA-BN-RSV to the cell culture). Levels of mRFP expression in infected cells (Figure 2B, GMFI mRFP) were higher in cell cultures infected in the presence of higher levels of OptiPRO™ medium, and the differences even increased over time. This higher mRFP expression suggested that also expression of viral proteins might be higher, which in turn might facilitate viral replication and explain higher viral yields per cell.

Viral spread was clearly slower with media containing less OptiPRO™ medium; the percentages of infected cells in media with lower levels of OptiPRO™ medium increased over time but remained below those with higher levels of OptiPRO™ medium.

Previous experiments also showed that the quail cells did not grow efficiently during the cell growth phase when cultured in OptiPRO™ medium. Possibly this is because quail cells cultured in OptiPRO™ medium tended to aggregate, which supported viral infection and yields but negatively affected quail cell growth. Thus, increased viral production can be achieved by expanding the quail cells in SCGM culture medium during the cell growth phase and switching the medium to OptiPRO™ medium or SCGM-ac + OptiPRO™ medium for the virus infection phase.

The experiments above demonstrate that virus production can be increased, for example, by a process comprising: seeding and propagating quail cells in SCGM-ac medium followed by addition of an approximately equal volume of OptiPRO™ medium to the cells culture and then infecting the cells with MVA or recombinant MVA. Later experiments showed that FS/YIE medium could be substituted for SCGM-ac medium without any decrease in viral titers or other process parameters or results.

#### **Example 5: MVA and recombinant MVA yields from quail cell cultures**

Quail cells were further examined for their ability to produce increased titers of MVA and recombinant MVAs. Additional experiments were conducted to maximize viral yield from quail suspension cells testing different media (**Figure 5**). Quail suspension cells (CCX.E10) were seeded at Day-3 in a stirrer flask in SCGM-ac medium. At Day 0, 20 ml of cell culture in cSCGM-ac was transferred to different S-125 shaker flasks and mixed with OptiPRO™ medium, or, for comparison, with DMEM-F12 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) to obtain a final concentration of 50% or 30% of OptiPRO™ medium or DMEM-F12 in

40 ml of culture volume with  $1.1 \times 10^6$  cells/mL for all conditions tested. Media additives included GlutaMAX™ supplement (final concentration 2mM) and Pluronic F68 (final amount 0.125%) to potentially reduce shear forces and foaming. Cells were transferred to 30°C for 1 h before infection with MVA-BN-RSV (co-encoding mRFP) at a MOI of 0.1. Subsequently, cells were cultured at 30°C. Duplicate samples were collected at Days 1-4 post infection, sonicated, and frozen at -20°C. Samples from Day 1/Day 2 (d1/d2) and Day 3/Day 4 (d3/d4) were titrated in parallel (vortexed  $\geq 1$  min) by the standard TCID<sub>50</sub> assay. Viral yields are shown in **Figure 5** as geometric means of TCID<sub>50</sub>/mL with geometric standard deviation. For analysis of viral infection and spread, mRFP expression was analyzed at Days 1-4 post infection by flow cytometry. Numbers in Table 2 below indicate peak viral yields = TCID<sub>50</sub>/mL for these experiments .

**Table 2: Peak virus yields in OptiPro and DMEM-F12 media (TCID<sub>50</sub>/mL)**

| Medium      | Day 1             | Day 2             | Day 3             | Day 4             |
|-------------|-------------------|-------------------|-------------------|-------------------|
| 50% OptiPro | $1.8 \times 10^7$ | $2.4 \times 10^8$ | $1.8 \times 10^8$ | $7.5 \times 10^7$ |
| 30% OptiPro | $6.5 \times 10^6$ | $6.5 \times 10^7$ | $4.9 \times 10^7$ | $4.9 \times 10^7$ |
| 50% F12     | $3.2 \times 10^6$ | $9.9 \times 10^7$ | $3.1 \times 10^7$ | $1.3 \times 10^8$ |
| 30% F12     | $6.5 \times 10^6$ | $6.5 \times 10^7$ | $4.8 \times 10^7$ | $6.5 \times 10^7$ |

Further experiments were conducted to examine the effect of cell density at the time of infection on viral yields. Suspension CCX.E10 quail cells (Nuvonis (Vienna, Austria)) were grown in SCGM+ac medium, harvested, and reseeded into OptiPRO™ medium at cell densities of  $1 \times 10^6$ ,  $2 \times 10^6$ , or  $3 \times 10^6$  cells/mL. Cells were equilibrated at 32°C for 1 h before infection with MVA-BN encoding mRFP (monomeric Red Fluorescent Protein; see, *e.g.*, Campbell *et al.* (2002) *Proc. Nat’l. Acad. Sci. USA* 99: 7877-82) at a MOI of 0.1. Subsequently, cells were cultured at 32°C in stirrer flasks. Samples of cells were taken at Days 1-4 post infection for analysis of cells, including using mRFP as a marker of viral spread (assessed by microscopy and flow cytometry). Titration of duplicate samples (vortexed  $\geq 1$  min) was assessed using the standard TCID<sub>50</sub> assay. Results (see Table 3 below) showed that increased cell densities up to  $3 \times 10^6$  cells/mL at the time of infection facilitated viral spread, leading to higher viral yields earlier after infection. Total viral yields showed a disproportionally higher viral output per cell than expected from the factor by which the starting cell numbers had been increased. At an

infection cell density of  $1 \times 10^6$  cells/mL, the viral titer peaked on Day 4 at a level that represented approximately 56 TCID<sub>50</sub>/cell, whereas at an infection cell density of  $3 \times 10^6$  cells/mL, the viral titer peaked earlier on Day 2 at a level that represented approximately 217 TCID<sub>50</sub>/cell.

**Table 3: Increased cell density affects viral titer (TCID<sub>50</sub>/mL)**

| Cell density at infection (cells/mL) | Day 1             | Day 2             | Day 3             | Day 4             |
|--------------------------------------|-------------------|-------------------|-------------------|-------------------|
| $1 \times 10^6$                      | $< 1 \times 10^6$ | $7.5 \times 10^6$ | $2.1 \times 10^7$ | $5.6 \times 10^7$ |
| $2 \times 10^6$                      | $3.2 \times 10^6$ | $6.5 \times 10^7$ | $5.6 \times 10^7$ | $1.5 \times 10^7$ |
| $3 \times 10^6$                      | $1 \times 10^7$   | $6.5 \times 10^8$ | $2.4 \times 10^8$ | $2.4 \times 10^8$ |

**Example 6: Viral yields of MVA-BN® virus from suspension quail cell cultures**

The methods of producing increased poxviral titers in suspension quail cells was further examined for MVA-BN® virus and compared to virus produced in chicken embryonic fibroblast (CEF) cells. To assess viral yields for MVA-BN® virus from suspension quail cells (CCX.E10 cells (Nuvonis, Vienna, Austria)), cells were seeded at Day -3 in SCGM-ac medium in shaker flasks. At Day 0, one hour before infection, cells were counted and mixed with an equal volume of OptiPRO™ medium (resulting in a 50:50 mix of cSCGM-ac and OptiPRO™ medium; 1:1) and supplemented with Pluronic F68 and GlutaMAX™. CEF cells were seeded at Day -1 in VP-SFM medium in six-well plates. CCX.E10 and CEF cells were infected in parallel at a MOI 0.1 and subsequently cultured at 32°C. For analysis of viral yields, triplicate samples of CEF and CCX.E10 cells were collected at Days 1-4 post infection, sonicated, frozen at -20°C, and titrated in parallel by the standard TCID<sub>50</sub> assay (vortexed  $\geq 1$  min). Data shown in **Figure 6** are viral yields as geometric means of TCID<sub>50</sub>/mL, with geometric standard deviation; TCID<sub>50</sub>/mL values are also shown in Table 4 below). Peak titers were reached on Day 4; at this time the cell-specific yield (TCID<sub>50</sub>/cell) was approximately 200 for CEF cells and approximately 900 for CCX.E10 cells.

**Table 4: Peak viral yields in CEF and CCX.E10 cells (TCID<sub>50</sub>/mL)**

| Cells   | Day 1                 | Day 2                 | Day 3                 | Day 4                 |
|---------|-----------------------|-----------------------|-----------------------|-----------------------|
| CEF     | 7.5 x 10 <sup>6</sup> | 3.8 x 10 <sup>7</sup> | 1.1 x 10 <sup>8</sup> | 1.1 x 10 <sup>8</sup> |
| CCX.E10 | 5.1 x 10 <sup>7</sup> | 3.2 x 10 <sup>8</sup> | 3.8 x 10 <sup>8</sup> | 6.2 x 10 <sup>8</sup> |

To assess the stability of MVA and recombinant MVA in quail cell lines, stability was compared following infection and culturing in chicken embryonic fibroblast (CEF) cells and suspension quail cell lines. Briefly, ten cell passages were performed for the following combinations of recombinant MVAs and cell lines, at an approximate initial MOI of 0.05: MVA-BN-RSV (MVA-mBN294B) in CCX.E10 cells in OptiPRO™ medium; MVA-BN-RSV (MVA-mBN294B) in CCX.B11 cells in OptiPRO™ medium; and MVA-BN-RSV (MVA-mBN294B) in CCX.E10 cells in SCGM medium. Stability was also examined for MVA-BN(-mRFP) in CCX.E10 cells cultured in OptiPRO™ medium; the marker protein Red Fluorescent Protein (RFP) was used to examine viral spread in the cultured cell populations. Stability was assessed by sequencing the MVA vector backbone and, for inserts in recombinant MVAs, using PCR and sequencing of the inserted genes. Results showed that the insert stability in both of the quail cell lines was not inferior to the stability exhibited in CEF cells, and that passaging did not result in mutations in the MVA vector “backbone” (*i.e.*, the non-recombinant portion of a recombinant MVA).

**Example 7: Purification of MVA-BN from avian cell cultures**

MVA-BN® virus was amplified in a suspension quail cell culture, then Bulk Drug Substance (BDS) was purified as follows. This method is similar to that described in Example 1, but comprises only one Tangential Flow Filtration (TFF) step (in some instances, referred to as a “short process”).

Material (cell culture) was collected into a mixer tank and treated with TrypLE™ enzyme (Gibco™, Fisher Scientific™, Waltham, MA, USA) for an hour at ambient temperature, followed by inactivation with trypsin inhibitor for 15 minutes. The pH of the material was adjusted to 8.5, MgCl<sub>2</sub> was added to 2mM, and 20 U/mL Denarase® endonuclease was added. NaCl was added to a final concentration of 1M, and the material was incubated for about 15 hours at 5-8 °C with stirring. Cells were lysed using high pressure homogenization (HPH) at 500

bar, and cell debris and remaining cells were removed by depth filtration using a 5  $\mu\text{m}$  depth filter (sometimes referred to as “clarification”). The material was then incubated at room temperature overnight with stirring. The material was then filtered using Tangential Flow Filtration (TFF) with a PS hollow fiber with a 0.05  $\mu\text{m}$  cutoff. Diafiltration was performed using DF2 buffer (10 mM Tris, 140 mM NaCl, pH 7.7), including a flush of the system at the end that was pooled with the retained material. An overall concentration of 4.5x was achieved. Similar results were obtained with this process when BB2 buffer was used for diafiltration.

Different runs of this process were performed on material (cell culture) collected from bioreactors comprising ~3 liter cultures and ~50 liter cultures and the results compared. In 11 runs from the 3-liter cultures, the average HCD in ng/dose was 1.3, while in a run from a 50-liter culture, the HCD in ng/dose was 5.3. Similar HCD (ng/dose) results were obtained from the “short process” in comparison to the “long process.” In these experiments, the “long process” further comprised an additional enzymatic treatment with Denarase® enzyme (100 U/mL) with 2mM  $\text{MgCl}_2$ , a room temperature incubation for ~15 hours, and a second Tangential Flow Filtration (TFF). This second TFF used 0.1 micrometer cut-off PESU, 10x overall concentration, and 10x DF in total using a 2-step DF first with DF2 buffer 5x, then BB2 buffer 5x plus a flush to produce the final Bulk Drug Substance (BDS). Because this “long process” produced approximately the same HCD in ng/dose as the “short process,” the “short process” was used for further production due to reduced cost of reagents and less time required to produce BDS. In this manner, the invention provides several processes that generate MVA-BN® Bulk Drug Substance with less than 10 ng Host Cell DNA per dose.

#### **Example 8: Purification of MVA-BN-RSV from avian cell cultures**

An exemplary MVA-BN-RSV is MVA-mBN294, a recombinant MVA that encodes an antigenic determinant of at least the RSV nucleocapsid N protein, the RSV M2 matrix protein, the RSV G membrane glycoprotein and the RSV F membrane glycoprotein (see, *e.g.*, WO 2014019718, herein incorporated by reference in its entirety).

MVA-mBN294 was amplified in a suspension quail cell culture, then purified as follows. Material (cell culture) was collected into a mixer tank and treated with TrypLE™ enzyme (Gibco™, Fisher Scientific™, Waltham, MA, USA) for an hour at ambient temperature, followed by inactivation with trypsin inhibitor for 15 minutes. The pH of the material was

adjusted to 8.5,  $\text{MgCl}_2$  was added to 2mM, and 20 U/mL Denarase® endonuclease was added. NaCl was added to a final concentration of 1M, and the material was incubated overnight at 5-8 °C with stirring. Cells were lysed using high pressure homogenization (HPH) at 500 bar, and cell debris and remaining cells were removed by depth filtration using a 5  $\mu\text{m}$  depth filter (“clarification”). The material was then incubated at room temperature overnight with stirring and filtered using Tangential Flow Filtration (TFF) with a PS hollow fiber with a 0.05  $\mu\text{m}$  cutoff. In this TFF, ultrafiltration was performed, yielding a concentration of 5.75x, then one round of diafiltration followed by a second ultrafiltration yielding a concentration of 7.65x and seven rounds of diafiltration, including a flush of the system at the end that was pooled with the retained material. All of these steps used DF1 buffer (10mM Tris, 2mM  $\text{MgCl}_2$ , pH 8.2).

A second enzymatic treatment was performed with Denarase® nuclease at 100 U/mL with 2mM  $\text{MgCl}_2$  and incubated for about 15 hours at room temperature with stirring. A second TFF step was then performed using PS hollow fibers with a 0.05  $\mu\text{m}$  cutoff and an inner diameter of 0.5 mm. In this filtration, diafiltration was performed five times with DF2 buffer (10 mM Tris, 140 mM NaCl, pH 7.7) and then five times with BB2 buffer (10 mM Tris, 140 mM NaCl, pH 7.7, 10% w/w sucrose, 2% Sorbitol, 100mM L-arginine), followed by a flush that was combined with the retained material to produce the final Bulk Drug Substance (“BDS”), providing a 10x increase in overall concentration.

This process was performed in 3L bioreactors, in 50L bioreactors, and in 200L bioreactors. In five runs with 3L bioreactors, the average HCD ng/dose was 61.6; in three runs in the 40L bioreactors, the average HCD ng/dose was 72.3, and in two runs in the 200L bioreactors, the average HCD/dose was 78.0. These results show that the process is scalable and can produce BDS with less than 100 ng/dose HCD.

#### **Example 9: Purification of MVA-BN-WEV from avian cell cultures**

MVA-BN-WEV is a recombinant MVA comprising nucleic acids encoding antigens of Eastern Equine Encephalitis Virus, Western Equine Encephalitis Virus, and Venezuelan Equine Encephalitis Virus, and is being developed for use as a vaccine against these viruses (see, *e.g.*, MVA-mBN396B as described in WO 2017129765). MVA-BN-WEV was amplified in a suspension quail cell culture, then purified as follows. Material (cell culture) was collected into a mixer tank and treated with TrypLE™ enzyme (Gibco™, Fisher Scientific™, Waltham, MA,

USA) for an hour at ambient temperature, followed by inactivation with trypsin inhibitor for 15 minutes. The pH of the material was adjusted to 8.5,  $\text{MgCl}_2$  was added to 2mM, and 20 U/mL Denarase® nuclease was added. NaCl was added to a final concentration of 1M, and the material was incubated for about 15 hours at 5-8 °C with stirring. Cells were lysed using high pressure homogenization (HPH) at about 500 bar, and cell debris and remaining cells were removed by depth filtration using a 5  $\mu\text{m}$  depth filter (“clarification”). The material was then incubated at room temperature overnight with stirring and filtered using Tangential Flow Filtration (TFF) with a filter comprising PS hollow fiber with a 0.05  $\mu\text{m}$  cutoff and an inner diameter of 0.5 mm. In this TFF, ultrafiltration was performed for an increase in concentration of 6x, then one round of diafiltration, followed by ultrafiltration for an increase of 8x and nine rounds of diafiltration including a flush of the system at the end that was pooled with the retained material. All of these steps used DF1 buffer (10mM Tris, 2mM  $\text{MgCl}_2$ , pH 8.2).

A second enzymatic treatment was performed with Denarase® nuclease at 100 U/mL with 2mM  $\text{MgCl}_2$  and incubated for about 15 hours at room temperature with stirring. A second TFF was then performed using PS hollow fiber with a 0.05  $\mu\text{m}$  cutoff and an inner diameter of 0.5 mm. Diafiltration was performed ten times, followed by a flush, all with BB2 buffer to yield a 10.5x increase in overall concentration and the final Bulk Drug Substance (BDS).

This process was performed with material produced in 3 liter (“3L”) bioreactors, 50 liter (“50L”) bioreactors, and 200 liter (“200L”) bioreactors. In 7 runs using 3L bioreactors, the average HCD in ng/dose was 16.3; in one run with a 50L bioreactor, the HCD was 40.8 ng/dose, and in one run with a 200L bioreactor, the HCD was 12.9 ng/dose. This data shows that the process is scalable and produces BDS suitable for commercial vaccines, with HCD in the range of 10 ng/dose.

These trials also showed that for MVA-BN-WEV the “short process,” which ends after one Tangential Flow Filtration, could provide similar results to the “long process,” which further includes a second Denarase® nuclease treatment and a second Tangential Flow Filtration. As the “short process” uses fewer reagents and requires less time, it provides additional benefits for commercial production.

**Example 10: Comparison of quail cell processes to CEF cell process**

Several rounds of experiments were conducted to compare downstream purification of virus from quail cells to the results obtained from CEF cells. MVA-BN® virus and recombinant MVAs were amplified in chicken embryonic fibroblast (CEF) cultures or in quail cell cultures, then purified in downstream processes of the instant invention. Cells were amplified, infected with virus, and cultured for viral amplification in wave bag cultures (for example, in a total volume of 300 liters (300L)) or in bioreactors (*e.g.*, a 250 liter (250L) bioreactor). The viruses were then purified essentially as follows.

For wavebag cultures, cell material was collected, then sonicated and clarified using a Viafuge® centrifuge (CARR Biosystems®, Clearwater, FL, US). Tangential Flow Filtration was then performed using cartridges comprising 0.1 µm PES with ultrafiltration followed by 3x diafiltration. The material was then treated with Denarase® nuclease, followed by a second round of Tangential Flow Filtration that comprised ultrafiltration followed by 15x diafiltration.

For bioreactor cultures, CEF cells were grown in VP-SFM media (Thermo Fisher Scientific®, Waltham, MA, US) with Cytodex® microcarriers (Sigma Aldrich™, St. Louis, MO, USA). Following infection with virus and virus growth, the bioreactor culture was mixed at maximum speed (~145 rpm) for approximately 5 hours to release virus from the cells and the supernatant containing the virus was passed through a Harvestainer™ system (Thermo Fisher Scientific®, Waltham, MA, US) to remove the microcarriers. Media was added to the remaining bioreactor debris, mixed to release additional virus, and the collection process repeated. The pooled collected material was then sonicated and clarified by centrifugation using a Viafuge® centrifuge (CARR Biosystems®, Clearwater, FL, US). Tangential Flow Filtration was then performed using cartridges comprising 0.1 µm PES; the first round comprised ultrafiltration and 3x diafiltration. The material was then treated with 100 U/mL Denarase for about 3 hours with rocking at ambient temperature. A second round of Tangential Flow Filtration comprised ultrafiltration and 15x diafiltration. Material was then batch centrifuged at 10,800 rcf for 45 minutes at 4 degrees C.

Results of these processes using CEF cells were compared to those from the processes using quail cells described above (see Table 5). For these calculations, a dose was considered to be  $1.58 \times 10^9$  InfU/mL in a final (vaccine) volume of 0.5 mL.

TABLE 5—MVA-BN-RSV Downstream Processing Results in CEF vs. Quail Cells

| Value                                   | CEF 250L bioreactor (n=12) | CEF 300L (wavebag culture) (n=4) | Quail cell 50 and 200L bioreactor (n=2) | Quail cell 3L bioreactor (n=7) |
|-----------------------------------------|----------------------------|----------------------------------|-----------------------------------------|--------------------------------|
| Start titer (log10 InfU/mL)             | 7.16                       | 7.69                             | 8.46                                    | 8.61                           |
| BDS titer (log10 InfU/mL)               | 9.33                       | 9.37                             | 9.63                                    | 9.66                           |
| Recovery (% relative to vessel harvest) | 107.2%                     | 34.9%                            | 151.5%                                  | 116.2%                         |
| # of doses in theoretical 500L vessel   | 6,269                      | 7,050                            | 206,982                                 | 219,800                        |
| HCD (ng/dose)                           | 19,877.0                   | 4,828.8                          | 69.0                                    | 32.3                           |

Thus, the results of the downstream processes with quail cell cultures were unexpectedly superior to those with Chicken Embryonic Fibroblast (CEF) cells, providing much higher yields of doses per amount of cell culture with a much lower level of Host Cell DNA (HCD) per dose.

TABLE 6—MVA-BN® Downstream Processing Results in CEF vs. Quail Cells

| Value                                   | CEF 300L (wavebag culture) (n=10) | Quail cell 50L bioreactor (n=1) | Quail cell 3L bioreactor (n=8) |
|-----------------------------------------|-----------------------------------|---------------------------------|--------------------------------|
| Start titer (log10 InfU/mL)             | 8.17                              | 8.11                            | 9.29                           |
| BDS titer (log10 InfU/mL)               | 8.93                              | 9.37                            | 10.03                          |
| Recovery (% relative to vessel harvest) | 57.6%                             | 406.2%                          | 123.8%                         |
| # of doses in theoretical 500L vessel   | 112,079                           | 693,627                         | 3,196,455                      |
| HCD (ng/dose)                           | 1,823.3                           | 5.3                             | 1.2                            |

As shown in Table 6 for MVA-BN®, the results of the downstream processes with quail cell cultures were unexpectedly superior to those with Chicken Embryonic Fibroblast (CEF) cells, providing much higher yields of doses per amount of cell culture with a much lower level of Host Cell DNA (HCD) per dose.

In Table 6, some of the recovery % relative to vessel harvest was above 100%. This likely reflects that much of the virus remains inside cells at the time of vessel harvest and is later released during the downstream purification process. For this reason, the post-lysis virus titer

may be more useful as a point of comparison, as that has been shown to represent peak virus titer during the process.

TABLE 7—MVA-BN-WEV Downstream Processing Results in CEF vs. Quail Cells

| Value                                   | CEF 200L (wavebag culture) (n=3) | Quail cell 50L or 200L bioreactor (n=2) | Quail cell 3L bioreactor (n=3) |
|-----------------------------------------|----------------------------------|-----------------------------------------|--------------------------------|
| Start titer (log10 InfU/mL)             | 7.76                             | 8.49                                    | 8.54                           |
| BDS titer (log10 InfU/mL)               | 8.52                             | 9.57                                    | 9.65                           |
| Recovery (% relative to vessel harvest) | 48.3%                            | 123.8%                                  | 134.9%                         |
| # of doses in theoretical 500L vessel   | 12,092                           | 177,559                                 | 219,245                        |
| HCD (ng/dose)                           | 8,678.3                          | 19.7                                    | 24.2                           |

As shown in Table 7 for MVA-BN-WEV, the results of the downstream processes with quail cell cultures were unexpectedly superior to those with Chicken Embryonic Fibroblast (CEF) cells, providing much higher yields of doses per amount of cell culture with a much lower level of Host Cell DNA (HCD) per dose.

It is to be understood that both the foregoing general and detailed description are exemplary and explanatory only and do not restrict or limit the invention as claimed. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate various embodiments of the invention and together with the description, serve to explain the principles of the invention.

## CLAIMS

We claim:

1. A method of producing a pharmaceutical composition from an avian cell culture infected with MVA or recombinant MVA, comprising the steps of:
  - (a) harvesting cell cultures comprising avian cells infected with MVA or recombinant MVA to produce collected material;
  - (b) treating the collected material with a protease having trypsin activity;
  - (c) treating the material with a nuclease
  - (d) adjusting pH and/or salt content of the material;
  - (e) lysing cells in the material;
  - (f) filtering the material to remove cell debris and remaining cells;
  - (g) continuing incubation with nuclease for a period of time;
  - (h) concentrating the product using tangential flow filtration; and
  - (i) suspending the product in a suitable buffer to provide a pharmaceutical composition.
2. The method of claim 1, further comprising after step (h) and before step (i) the steps of:
  - (A) treating the material with a nuclease; and
  - (B) concentrating the product using tangential flow filtration.
3. The method of claim 1 or 2 wherein said nuclease in step (c) and/or step (A) is Denarase® endonuclease.
4. The method of claim 3, further comprising the addition of NaCl to a final concentration of at least 0.5M or about 1 M during step (d).
5. The method of claim 1, wherein step (i) comprises the use of diafiltration to exchange the buffer in which the product is suspended.

6. The method of claim 1, wherein said avian cells are quail cells.
7. The method of claim 6, wherein said quail cells are CCX.E10 cells.
8. The method of claim 1, wherein said MVA is MVA-BN® virus.
9. The method of claim 1, wherein said recombinant MVA is MVA-BN-RSV.
10. The method of claim 1, wherein said avian cell culture infected with MVA-BN® virus or recombinant MVA is prepared by a method comprising the steps of:
  - (i) culturing quail cells in suspension in culture medium comprising FS/YIE or SCGM-ac medium until the cell density is from  $1 \times 10^6$  cells/mL to  $5 \times 10^6$  cells/mL, producing a cell culture comprising from  $1 \times 10^6$  cells/mL to  $5 \times 10^6$  cells/mL;
  - (ii) modifying said culture medium to comprise FS/YIE or SCGM-ac medium and OptiPRO™ medium in a ratio from 40:60 to 60:40 (v/v) and continuing to culture said quail cells;
  - (iii) adding MVA or recombinant MVA to said cell culture to allow infection of the cells by the MVA or the recombinant MVA;
  - (iv) continuing to culture said cells for about one to four days so that the amount of MVA or recombinant MVA is increased.
11. The method of claim 10, wherein step (ii) comprises modifying the culture medium to comprise FS/YIE or SCGM-ac medium with OptiPRO™ medium in a ratio of 50:50 (v/v).
12. The method of claim 10, wherein step (ii) is continued until about 50% to 70% of the cells are aggregated.
13. A pharmaceutical composition comprising MVA-BN® virus or MVA-BN-RSV made by the method of any of claims 1-7 or 10-12.