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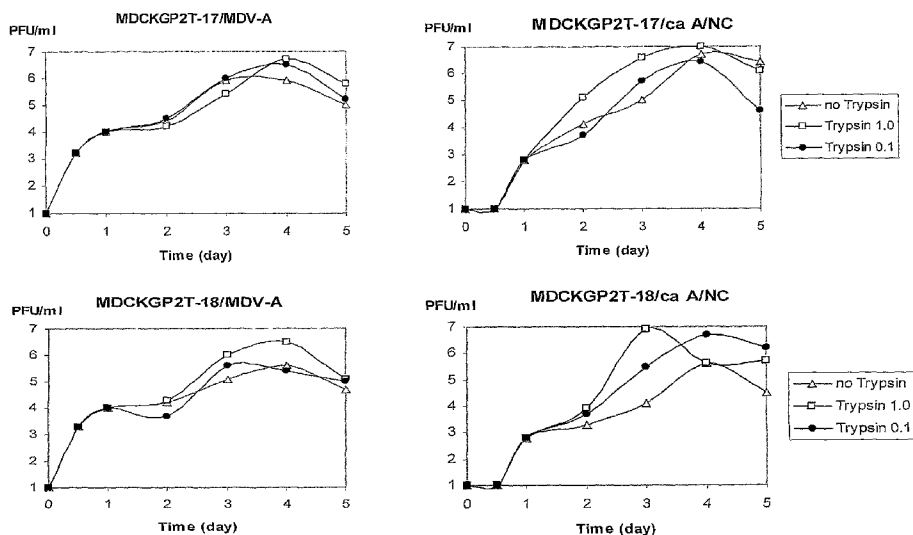
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(54) Title: METHODS AND COMPOSITIONS FOR EXPRESSING A HETEROLOGOUS PROTEASE

MDCK_GP2Trypsinogen



(57) Abstract: The present invention provides methods and compositions for expressing a protease or pro-protease in cells that do not naturally express the protease or pro-protease. The invention further provides methods of producing viruses, *e.g.*, influenza viruses, in such cells. The invention also provides methods for increasing the titer of influenza viruses grown in cells that express such a heterologous protease or pro-protease. Additionally, the invention provides a protease from *Streptomyces griseus* useful in the methods and compositions.



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METHODS AND COMPOSITIONS FOR EXPRESSING A HETEROLOGOUS PROTEASE
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1. Field of the Invention

5 [0001] In one aspect, the present invention provides methods and compositions for expressing a protease or pro-protease in cells that do not naturally express the protease or pro-protease. In other aspects, the invention provides methods of producing viruses, *e.g.*, influenza viruses, in such cells. In other aspects, the invention provides methods for increasing the titer of influenza viruses grown in cells that express such a heterologous
10 protease or pro-protease. In still other aspects, the invention provides a heterologous protease from *Streptomyces griseus* useful in the methods and compositions.

2. Background

[0002] Influenza pandemics are defined by a dramatic global increase in morbidity and mortality due to influenza illness. Several factors combine to modulate the severity and
15 extent of the pandemic including the low degree of immunity in the population and the efficiency with which the virus can transmit among humans. The latter is generally influenced not only by the virus itself but the density of the population and ease of travel into and out of a region. The virus responsible for the pandemic is generally a recently emerged antigenic variant that the majority of the population have not had prior experience with and,
20 therefore, have little or no immunity to. In addition, efficient human to human transmission is a prerequisite for rapid spread and, in the case of zoonotic introduction of animal viruses into human populations, the virus must adapt to replication in humans and be capable of efficient transmission.

[0003] Pandemic influenza spreads very quickly and can have devastating impact. The most
25 severe pandemic of the 20th century, the 1918 pandemic, killed over 500,000 U.S. citizens and between 20 to 40 million people worldwide. The pandemic may produce waves of disease, with peaks of incidence separated by several weeks to months. The relatively rapid onset and spread of pandemic influenza presents several problems for responding to a global attack of this magnitude and imposes overwhelming burdens on emergency responders and
30 health care workers. Rapid identification and response to the emerging pandemic is clearly a necessary element of the solution; several programs are currently in place worldwide to monitor emerging influenza viruses including avian influenza viruses that infrequently cause

disease in humans. These surveillance data are used in conjunction with predefined pandemic alert levels in order to identify the likelihood of the threat and provide guidance for an effective response.

[0004] Vaccination is the most important public health measure for preventing disease caused by annual epidemics of influenza. The short interval between identification of a potential pandemic and the onset of significantly increased disease levels present significant challenges for producing sufficient vaccine to protect a large segment of the population. Having vaccine technology and manufacturing infrastructure in place prior to the emergence of the next pandemic will be critical in ameliorating a significant amount of illness and death. The short response times needed to produce a “pandemic vaccine” will not allow for prolonged research or process development to be conducted in order to provide an effective response.

[0005] To date, all commercially available influenza vaccines in the United States have been propagated in embryonated hen’s eggs. Although influenza virus grows well in hen’s eggs, production of vaccine is dependent on the availability of eggs. Supplies of eggs must be organized, and strains for vaccine production selected months in advance of the next flu season, limiting the flexibility of this approach, and often resulting in delays and shortages in production and distribution. Unfortunately, some influenza vaccine strains, such as the prototype A/Fujian/411/02 strain that circulated during the 2003-04 season, do not replicate well in embryonated chicken eggs, and have to be isolated by cell culture in a costly and time consuming procedure.

[0006] Systems for producing influenza viruses in cell culture have also been developed in recent years (See, e.g., Furminger. *Vaccine Production*, in Nicholson *et al.* (eds) Textbook of Influenza pp. 324-332; Merten *et al.* (1996) *Production of influenza virus in cell cultures for vaccine preparation*, in Cohen & Shafferman (eds) Novel Strategies in Design and Production of Vaccines pp. 141-151). Typically, these methods involve the infection of suitable immortalized host cells with a selected strain of virus. While eliminating many of the difficulties related to vaccine production in hen’s eggs, not all pathogenic strains of influenza grow well and can be produced according to established tissue culture methods. In addition, many strains with desirable characteristics, e.g., attenuation, temperature sensitivity and cold adaptation, suitable for production of live attenuated vaccines, have not been successfully grown in tissue culture using established methods.

[0007] Thus, there is a need for 1) manufacturing facilities and procedures needed to produce influenza vaccine from cell culture and 2) development of an effective vaccine technology from cell culture to prevent illness caused by seasonal epidemics of influenza. These procedures and technologies can be rapidly applied to the production and distribution of pandemic vaccine in the event of an imminent pandemic.

[0008] One of the many obstacles to be overcome in licensing a cell culture based influenza vaccine is the need for proteolytic cleavage of the hemagglutinin (HA) protein for a newly-formed virus to productively infect a new cell. During infection of an animal, the HA protein is cleaved by a trypsin-like serine protease endogenous to the animal. In culture, endopeptidase activity is frequently insufficient to allow robust replication of the influenza virus. Accordingly, proteases such as trypsin are frequently added to the culture medium following infection of the cells in culture with an influenza virus of interest to increase viral yield. *See, e.g.*, U.S. Patent No. 5,698,433. However, addition of exogenous proteases to the cell culture medium introduces additional components to the culture medium, adding complexity and the need for additional regulatory review. In addition, the addition of exogenous trypsin increases the costs associated with making vaccine using cell culture methods. Thus, new methods and compositions are needed for growing influenza virus in culture without the need for addition of exogenous proteases. Further, such methods and compositions must overcome the inherent toxicity of expressing active proteases in cells. These and other unmet needs are provided by the present invention.

[0009] Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention. In addition, citation of a patent shall not be construed as an admission of its validity.

3. Summary

[0010] The present invention provides cells, referred to herein as "cell(s) of the invention," comprising a nucleic acid that encodes a protease or pro-protease, such that the cell expresses a higher level of the protease or pro-protease than would be ordinarily expressed in the cell in the absence of the nucleic acid. In certain embodiments, the cell does not normally express the protease or pro-protease. In certain embodiments, the cell expresses the protease or pro-protease at low levels, for example, at levels less than optimal or desired for a particular biological activity, *e.g.*, culture of viruses, *e.g.*, influenza viruses. In certain aspects, the invention provides a cell comprising a nucleic acid encoding a protease or pro-protease, wherein the nucleic acid encoding the protease or pro-protease is stably integrated into the

cell's genome. In other aspects, the invention provides a cell comprising a nucleic acid encoding a protease or pro-protease, wherein the nucleic acid encoding the protease or pro-protease is maintained extrachromasomally. In other aspects, the invention provides a cell comprising a nucleic acid encoding a protease or pro-protease, wherein the nucleic acid encoding the protease or pro-protease is transiently expressed in the cell. In certain embodiments, the cell expresses the protease or pro-protease. In certain embodiments, the cell constitutively expresses the protease or pro-protease. In certain embodiments, the cell inducibly expresses the protease or pro-protease. In certain embodiments, the cell secretes the protease or pro-protease. In certain embodiments, the cell expresses the protease or pro-protease in the cytosol of the cell.

[0011] In certain embodiments, the protease is a serine protease. In certain embodiments, the serine protease is an S1 family protease. In certain embodiments, the protease is trypsin. In certain embodiments, the serine protease is a bacterial subtilisin. In certain embodiments, the protease is SPRT from *Streptomyces griseus*. In certain embodiments, the protease is a protease listed in Table 1. In certain embodiments, the protease is a pro-protease. In certain embodiments, the pro-protease is trypsinogen. In certain embodiments, the pro-protease is processed into an active protease.

[0012] In certain embodiments, expression of the protease or pro-protease is under the control of an inducible promoter. In certain embodiments, the inducible promoter is induced by interferon or a downstream signaling molecule induced by interferon. In certain embodiments, the inducible promoter is induced by a tetracycline-regulated expression system. In certain embodiments, expression of the protease or pro-protease is under the control of a constitutively active promoter. In certain embodiments, the nucleic acid encoding the protease or pro-protease comprises sequence encoding a secretion signal that directs secretion of the protease or pro-protease.

[0013] In certain embodiments, the cell expresses between about 0.1 ng and about 50 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses between about 1 ng and about 50 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses between about 10 ng and about 50 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses between about 100 ng and about 50 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses between about 1 µg and about 50 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses between about 0.1

ng and about 5 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses between about 0.1 ng and about 100 ng of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses between about 0.1 ng and about 10 ng of the protease or pro-protease per ml of cell culture. In certain
5 embodiments, the cell expresses between about 0.1 ng and about 1 ng of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses an amount of protease or pro-protease sufficient to increase the titer of virus grown in a culture of the cells expressing the protease or pro-protease. In certain embodiments, the molecular weight of the protease or pro-protease is calculated based on the pro-protease form of the enzyme. In
10 certain embodiments, the molecular weight of the protease or pro-protease is calculated based on the mature, active form of the protease.

[0014] In certain embodiments, the cell expresses at least about 0.1 ng of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses at least about 1 ng of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell
15 expresses at least about 10 ng of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses at least about 100 ng of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses at least about 1 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses at least about 10 µg of the protease or pro-protease per ml of cell culture. In certain embodiments,
20 the cell expresses at least about 20 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses at least about 30 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses at least about 40 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses at least about 50 µg of the protease or pro-protease per ml of cell culture.

[0015] In certain embodiments, the cell is a bacterial cell. In certain embodiments, the bacterial cells is an *E. coli* cell. In certain embodiments, the cell is a mammalian cell. In certain embodiments, the mammalian cell is a canine cell. In certain embodiments, the canine cell is an MDCK cell. In certain embodiments the MDCK cell is non-tumorigenic. In certain embodiments, the mammalian cell is a primate cell. In certain embodiments, the
30 primate cell is an African green monkey or human cell. In certain embodiments, the cell is an avian cell. In certain embodiments, the avian cell is a chicken cell.

[0016] In another aspect, the invention provides a method of producing a virus, comprising infecting a cell of the invention with an virus, culturing the cell under conditions that allow

replication of the virus, and collecting virus from the cell culture. In a specific embodiment, the virus is an influenza virus.

[0017] In another aspect, the invention provides a method of producing a virus, comprising transfecting a cell of the invention with nucleic acids comprising a viral genome, culturing
5 the cell under conditions that allow replication of the virus, and collecting virus from the cell culture. In a specific embodiment, the viral genome is an influenza genome and the virus is an influenza virus. In some embodiments, the influenza virus correspond to an influenza B virus. In some embodiments, the influenza virus correspond to an influenza A virus. In certain embodiments, the viruses include an attenuated influenza virus, a cold adapted
10 influenza virus, a temperature sensitive influenza virus, or a virus with any combination of these desirable properties. In one embodiment, the influenza virus is an influenza B/Ann Arbor/1/66 strain virus, *e.g.*, a cold adapted, temperature sensitive, attenuated strain of B/Ann Arbor/1/66. In another embodiment, the influenza virus is an influenza A/Ann Arbor/6/60 strain virus, *e.g.*, a cold adapted, temperature sensitive, attenuated strain of A/Ann
15 Arbor/6/60.

[0018] In certain embodiments, the methods include recovering influenza viruses and using the viruses in the preparation of an immunogenic composition, *e.g.*, a vaccine. In one embodiment the virus is capable of eliciting an immune response upon administration, *e.g.*, intranasal administration, to a subject. In some embodiments, the viruses used to prepare a
20 vaccine are inactivated prior to administration, in other embodiments, live-attenuated viruses are used to prepare a vaccine. In certain embodiments, recombinant and reassortant influenza A and influenza B viruses are produced according to the methods of the invention. In one embodiment, a vaccine is prepared comprising a live, inactivated, or killed virus derived from a virus produced by the methods of the invention. In one embodiment, viruses produced by
25 the methods of the invention are used to replicate other viruses in cell culture or eggs. In one embodiment, a vaccine is provided that comprises immunogenic polypeptides derived from a virus produced by the methods of the invention.

[0019] In certain embodiments, the cell expresses a pro-protease, and the method further comprises adding an exogenous protease to the culture medium. In certain embodiments, the
30 exogenous protease is added to a maximum concentration of about 0.1 µg/ml. In certain embodiments, the exogenous protease is trypsin.

[0020] In certain embodiments, the virus is a DNA virus. In certain embodiments, the virus is an RNA virus. In certain embodiments, the virus is a single-stranded DNA virus. In certain embodiments, the virus is a double-stranded DNA virus. In certain embodiments, the virus is a positive-sense single-stranded RNA virus. In certain embodiments, the virus is a negative-sense single-stranded RNA virus. In certain embodiments, the virus is a double-stranded RNA virus. In certain embodiments, the virus is a reverse-transcribing virus.

[0021] In another aspect, the invention provides a method of replicating an influenza virus, comprising infecting a cell of the invention with an influenza virus, culturing the cell under conditions that allow replication of the influenza virus, and collecting influenza virus from the cell culture. In certain embodiments, such conditions do not include exogenously added proteases or pro-proteases, *e.g.*, trypsin or trypsinogen.

[0022] In still another aspect, the invention provides a method of replicating an influenza virus, comprising transfecting a cell with nucleic acids encoding an influenza genome, culturing the cell under conditions that allow replication of the influenza virus, and collecting influenza virus from the cell culture. In certain embodiments, such conditions do not include exogenously added proteases or pro-proteases, *e.g.*, trypsin or trypsinogen.

[0023] In certain embodiments, the cell expresses a pro-protease or enzymatically active protease not normally expressed by the cell. In certain embodiments, the cell is a mammalian cell. In certain embodiments, the cell is an avian cell. In certain embodiments, the cell is a primate cell, canine cell, hamster cell, mouse cell, or rat cell. In certain embodiments, the cell is an MDCK cell. In certain embodiments, the cell is a Vero cell. In certain embodiments, the cell is a chicken cell.

[0024] In yet another aspect, the invention provides a method of increasing the titer of influenza virus grown in cell culture, comprising culturing the influenza virus in a cell culture, wherein the cells in the cell culture express a protease or pro-protease that i) is heterologous to the cell, and ii) cleaves a hemagglutinin of the influenza virus, thereby increasing the titer of the influenza virus grown in the cell culture relative to the titer obtained by culturing the influenza virus in cells that do not express a heterologous protease or pro-protease.

[0025] In certain embodiments, the cells stably express the heterologous protease. In certain embodiments, the cells constitutively express the heterologous protease. In certain embodiments, the cells inducibly express the heterologous protease. In certain embodiments,

the heterologous protease is trypsin. In certain embodiments, the cells constitutively express the heterologous pro-protease. In certain embodiments, the cells inducibly express the heterologous pro-protease. In certain embodiments, the pro-protease is trypsinogen. In certain embodiments, the protease is SPRT protease from *Streptomyces griseus*. In certain
5 embodiments, the pro-protease is prepro-SPRT protease from *Streptomyces griseus*.

[0026] One indication of the ability of a cell to support viral replication is the yield of virus obtained from an infected cell culture. Viral yield can be determined by numerous methods known to one skilled in the art. For example, viral yield can be quantified by determining the concentration of virus present in a sample according to a median tissue culture infectious
10 dose (TCID₅₀) assay that measures infectious virions. The TCID₅₀ values are often reported as the log₁₀ TCID₅₀/mL. In one embodiment, the cells expressing a heterologous protease support the replication of influenza viruses (*e.g.*, *ca/ts* strains) to a log₁₀ TCID₅₀/mL of at least 6.0, or at least 6.2, or at least 6.4, or at least 6.6, or at least 6.8, or at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4,
15 or at least 8.6, or at least 8.8, or at least 9.0, or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8. In a specific embodiment, the cells expressing a heterologous protease support the replication of influenza viruses (*e.g.*, *ca/ts* strains) to commercially reasonable titers (>10⁷ Log TCID₅₀/mL).

[0027] In certain embodiments, the titer of the influenza virus that is produced in a cell
20 culture of cells expressing a heterologous protease is increased by a log₁₀ TCID₅₀/mL of at least about 0.1, or at least about 0.2, or at least about 0.3, or at least about 0.4, or at least about 0.5, or at least about 0.6, or at least about 0.7, or at least about 0.8, or at least about 0.9, or at least about 1.0, or at least about 1.2, or at least about 1.4, or at least about 1.6, or at least about 1.8, or at least about 2.0, or at least about 2.2, or at least about 2.4, or at least about 2.6,
25 or at least about 2.8, or at least about 3.0, or at least about 3.2, or at least about 3.4, or at least about 3.6, or at least about 3.8, or at least about 4.0, or at least about 4.2, or at least about 4.4, or at least about 4.6, or at least about 4.8, or at least about 5.0, relative to the titer of influenza virus produced in a culture of corresponding cells that do not express a heterologous protease and to which no exogenous protease, *e.g.*, trypsin has been added.

[0028] In certain embodiments, the titer of the influenza virus that is produced in a cell
30 culture of cells expressing a heterologous protease is increased by at least about 10%, relative to the titer of influenza virus produced in a culture of corresponding cells that do not express a heterologous protease and to which no exogenous protease, *e.g.*, trypsin has been added. In

certain embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 25%. In certain embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 50%. In certain
5 embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 100%. In certain embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 500%. In certain
10 embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 1000%. In certain embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 5000%. In certain
15 embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 30,000%. In certain embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 50,000%. In certain
20 embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 70,000%. In certain embodiments, the titer of the influenza virus that is produced in a cell culture of cells expressing a heterologous protease is increased by at least about 100,000%.

[0029] In certain embodiments, the cell culture medium in which a cell of the invention is cultured is serum-free. In certain embodiments, the cell culture medium in which a cell of the
25 invention is cultured contains serum (*e.g.*, fetal calf serum). In certain embodiments, the cell culture medium in which a cell of the invention is cultured contains no exogenously added animal proteins, such media is often referred to “animal protein free” or “APF” media. In certain embodiments, the cell culture medium in which a cell of the invention is cultured contains exogenous protease (*e.g.*, porcine trypsin).

30 [0030] In still another aspect, the invention provides a method for producing a heterologous protease or pro-protease in a cell, wherein said cell is capable of supporting influenza replication, comprising culturing a cell comprising a nucleic acid encoding a protease or pro-

protease not normally expressed in the cell under conditions that permit expression of said protease or pro-protease, thereby producing the protease or pro-protease in the cell.

[0031] In certain embodiments, the cell expresses a protease. In certain embodiments, the cell stably expresses a protease. In certain embodiments, the cell expresses a pro-protease. In certain embodiments, the cell stably expresses a pro-protease. In certain embodiments, the cell secretes the protease or pro-protease into the cell culture medium. In certain embodiments, the cell expresses between about 0.1 ng and about 50 µg of the protease or pro-protease per ml of cell culture. In certain embodiments, the cell expresses an amount of protease or pro-protease sufficient to increase the titer of virus grown in a culture of the cells expressing the protease or pro-protease. In certain embodiments, expression of the protease or pro-protease is inducible. In certain embodiments, expression of the protease or pro-protease is constitutive.

[0032] In still another aspect, the invention provides a method for making a cell that expresses a protease or pro-protease not normally expressed by the cell, comprising introducing a nucleic acid encoding a protease or pro-protease operably linked to regulatory elements effective to express the protease or pro-protease in the cell (which may or may not be secreted or released from the cell), thereby making a cell that expresses a protease or pro-protease not normally expressed by the cell.

[0033] Any suitable technique and/or vector known by one skilled in the art, without limitation, can be used to introduce the nucleic acid into the cell. In certain embodiments, the nucleic acid is introduced into the cell as a plasmid, a cosmid, a viral vector, a bacteriophage, a phagemid, a transposon, or an artificial chromosome. In certain embodiments, the nucleic acid is introduced into the cell as a retroviral vector. In certain embodiments, the nucleic acid is stably maintained in the cell. In other embodiments, the nucleic acid is transiently maintained.

[0034] In certain embodiments, the nucleic acid encoding the protease or pro-protease further comprises a selectable marker. Methods utilizing a selectable marker to select for those cell stably expressing the nucleic acid encoding the protease or pro-protease are known to one skilled in the art. Any selectable marker known to one skilled in the art to be effective in the cell into which the nucleic acid is introduced can be used in such embodiments. Thus, in certain embodiments, the selectable marker is an antibiotic resistance gene. In certain embodiments, the selectable marker is a gene in an anabolic pathway that complements a

deficiency in the cell. For example, the nucleic acid encoding the protease or pro-protease can be introduced into a cell deficient in synthesis of, for example, an amino acid. The nucleic acid can comprise a gene that complements the deficiency of the cell. By culturing the cell in medium that does not comprise the amino acid, the presence of the nucleic acid comprising the synthesis gene is selected. Any such gene known to one skilled in the art, without limitation, can be used according to the present invention.

[0035] In yet another aspect, the invention provides an isolated nucleic acid that is at least about 90% identical to SEQ ID NO.:1. In certain embodiments, the isolated nucleic acid comprises or alternatively consists of SEQ ID NO.:1. In certain embodiments, the isolated nucleic acid hybridizes under hybridization conditions to a nucleic acid encoding SEQ ID NO.:1 (or the complement thereof). In certain embodiments, the hybridization conditions are stringent hybridization conditions. In certain embodiments, the hybridization conditions are highly stringent hybridization conditions.

[0036] In yet another aspect, the invention provides an isolated polypeptide comprising or alternatively consisting of the amino acid sequence that is SEQ ID NO.:2.

[0037] In still another aspect, the invention provides an isolated nucleic acid encoding a polypeptide comprising or alternatively consisting of the amino acid sequence that is SEQ ID NO.:2.

[0038] In yet another aspect, the invention provides an expression vector comprising a nucleic acid of the invention.

[0039] In still another aspect, the invention provides a cell transfected with an expression vector of the invention.

4. Brief Description of the Figures

[0040] Figure 1 presents replication of ca A/Vietnam/1203/2004 (H5N1) in MDCK cells.

[0041] Figure 2 presents a table showing amounts of luciferase activity observed in cells transfected with different retroviral vectors.

[0042] Figure 3 presents a graphical representation comparing luciferase activity observed in MDCK cells with the concentration of retroviral particles used to infect the MDCK cells.

[0043] Figure 4 presents a table showing luciferase expression in 12 different single MDCK clones and two different mixtures of MDCK clones.

[0044] Figure 5 presents a table showing a summary of MDCK clones obtained from viral particles produced in two different packaging cell lines and transfected with three different vectors.

5 [0045] Figures 6A-6C present graphical representations of influenza viral titers obtained by infecting different MDCK clones (panel A control clone, panel B AmphoTrypsinogen clone, panel C GP2Trypsinogen clone) with MDV-A or ca A/NC influenza strains. Exogenous trypsin added: 0.0 µg/ml, open triangles; 0.1 µg/ml on day 1 at day 1, closed circles; 1.0 µg/ml at days 1-5 open squares.

10 [0046] Figures 7A-7B present western blots showing expression of 6xHis-labeled trypsinogen in 12 of 16 MDCK clones.

[0047] Figure 8 presents a table showing inducible luciferase expression in 15 different MDCK clones.

[0048] Figure 9 presents the nucleotide sequence (SEQ ID NO:1) of the *sprT* gene, encoding a serine protease from *Streptomyces griseus*.

15 [0049] Figure 10 presents the amino acid sequence (SEQ ID NO:2) of a serine protease from *Streptomyces griseus* encoded by the *sprT* gene.

[0050] Figure 11 presents the nucleotide sequence (SEQ ID NO:3) encoding trypsinogen.

[0051] Figure 12 presents the Forward and Reverse primers used to clone the *sprT* gene (SEQ ID NOS: 5 and 5, respectively).

20 [0052] Figure 13 presents the schematic map of the pT-Rex-DEST30/Luciferase and pT-Rex-DEST30/Trypsin plasmids transfected into R3/7 clones.

5. Detailed Description of the Invention

5.1 Definitions

25 [0053] Unless defined otherwise, all scientific and technical terms are understood to have the same meaning as commonly used in the art to which they pertain. For the purpose of the present invention the following terms are defined below.

[0054] The terms “nucleic acid,” “polynucleotide,” “polynucleotide sequence” and “nucleic acid sequence” refer to single-stranded or double-stranded deoxyribonucleotide or ribonucleotide polymers, or chimeras or analogues thereof. As used herein, the term
30 optionally includes polymers of analogs of naturally occurring nucleotides having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids

in a manner similar to naturally occurring nucleotides (*e.g.*, peptide nucleic acids). Unless otherwise indicated, a particular nucleic acid sequence of this invention encompasses complementary sequences, in addition to the sequence explicitly indicated.

5 [0055] The term “gene” is used broadly to refer to any nucleic acid associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. The term “gene” applies to a specific genomic sequence, as well as to a cDNA or an mRNA encoded by that genomic sequence.

10 [0056] Genes also include non-expressed nucleic acid segments that, for example, form recognition sequences for other proteins. Non-expressed regulatory sequences include “promoters” and “enhancers,” to which regulatory proteins such as transcription factors bind, resulting in transcription of adjacent or nearby sequences. A “tissue specific” promoter or enhancer is one which regulates transcription in a specific tissue type or cell type, or types.

15 [0057] The term “vector” refers to plasmids, viral vectors, recombinant nucleic acids and cDNA. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not autonomously replicating. Most commonly, the vectors of the present invention are plasmids.

20 [0058] An “expression vector” is a vector, such as a plasmid, which is capable of promoting expression, as well as replication of a nucleic acid incorporated therein. Typically, the nucleic acid to be expressed is “operably linked” to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer.

25 [0059] A “bi-directional expression vector” is typically characterized by two alternative promoters oriented in the opposite direction relative to a nucleic acid situated between the two promoters, such that expression can be initiated in both orientations resulting in, *e.g.*, transcription of both plus (+) or sense strand, and negative (-) or antisense strand RNAs. Alternatively, the bi-directional expression vector can be an ambisense vector, in which the viral mRNA and viral genomic RNA (as a cRNA) are expressed from the same strand.

30 [0060] In the context of the invention, the term “isolated” refers to a biological material, such as a nucleic acid or a protein, which is substantially free from components that normally accompany or interact with it in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment, *e.g.*, a

cell. For example, if the material is in its natural environment, such as a cell, the material has been placed at a location in the cell (*e.g.*, genome or genetic element) not native to a material found in that environment. For example, a naturally occurring nucleic acid (*e.g.*, a coding sequence, a promoter, an enhancer, etc.) becomes isolated if it is introduced by non-naturally occurring means to a locus of the genome (*e.g.*, a vector, such as a plasmid or virus vector, or amplicon) not native to that nucleic acid. Such nucleic acids are also referred to as “heterologous” nucleic acids.

[0061] The term “recombinant” indicates that the material (*e.g.*, a nucleic acid or protein) has been artificially or synthetically (non-naturally) altered by human intervention. The alteration can be performed on the material within, or removed from, its natural environment or state. Specifically, when referring to a virus, *e.g.*, an influenza virus, the virus is recombinant when it is produced by the expression of a recombinant nucleic acid.

[0062] The term “reassortant,” when referring to a virus, indicates that the virus includes genetic and/or polypeptide components derived from more than one parental viral strain or source. For example, a 7:1 reassortant includes 7 viral genomic segments (or gene segments) derived from a first parental virus, and a single complementary viral genomic segment, *e.g.*, encoding hemagglutinin or neuraminidase, from a second parental virus. A 6:2 reassortant includes 6 genomic segments, most commonly the 6 internal genes from a first parental virus, and two complementary segments, *e.g.*, hemagglutinin and neuraminidase, from a different parental virus.

[0063] The term “introduced” when referring to a heterologous or isolated nucleic acid refers to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid can be incorporated into the genome of the cell (*e.g.*, chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (*e.g.*, transfected mRNA). The term includes such methods as “infection,” “transfection,” “transformation” and “transduction.” In the context of the invention a variety of methods can be employed to introduce nucleic acids into prokaryotic cells, including electroporation, calcium phosphate precipitation, lipid mediated transfection (lipofection), etc.

[0064] The term “host cell” means a cell which contains a heterologous nucleic acid, such as a vector, and supports the replication and/or expression of the nucleic acid, and optionally production of one or more encoded products including a polypeptide and/or a virus. Host cells can be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect,

amphibian, avian or mammalian cells, including human cells. Exemplary host cells in the context of the invention include Vero (African green monkey kidney) cells, Per.C6 cells (human embryonic retinal cells), BHK (baby hamster kidney) cells, primary chick kidney (PCK) cells, Madin-Darby Canine Kidney (MDCK) cells, Madin-Darby Bovine Kidney (MDBK) cells, 293 cells (*e.g.*, 293T cells), and COS cells (*e.g.*, COS1, COS7 cells). The term host cell encompasses combinations or mixtures of cells including, *e.g.*, mixed cultures of different cell types or cell lines (*e.g.*, Vero and CEK cells). A co-cultivation of electroporated sf vero cells is described for example in PCT/US04/42669 filed December 22, 2004, which is incorporated by reference in their entirety.

10 **[0065]** The expression “artificially engineered” is used herein to indicate that the virus, viral nucleic acid or virally encoded product, *e.g.*, a polypeptide, a vaccine, comprises at least one mutation introduced by recombinant methods, *e.g.*, site directed mutagenesis, PCR mutagenesis, etc. The expression “artificially engineered” when referring to a virus (or viral component or product) comprising one or more nucleotide mutations and/or amino acid
15 substitutions indicates that the viral genome or genome segment encoding the virus (or viral component or product) is not derived from naturally occurring sources, such as a naturally occurring or previously existing laboratory strain of virus produced by non-recombinant methods (such as progressive passage at 25 °C), *e.g.*, a wild type or cold adapted A/Ann Arbor/6/60 or B/Ann Arbor/1/66strain.

20 **[0066]** The term “% sequence identity” is used interchangeably herein with the term “% identity” and refers to the level of amino acid sequence identity between two or more peptide sequences or the level of nucleotide sequence identity between two or more nucleotide sequences, when aligned using a sequence alignment program. For example, as used herein, 80% identity means the same thing as 80% sequence identity determined by a
25 defined algorithm, and means that a given sequence is at least 80% identical to another length of another sequence. Exemplary levels of sequence identity include, but are not limited to, 60, 70, 80, 85, 90, 95, 98% or more sequence identity to a given sequence.

[0067] The term “% sequence homology” is used interchangeably herein with the term “% homology” and refers to the level of amino acid sequence homology between two or
30 more peptide sequences or the level of nucleotide sequence homology between two or more nucleotide sequences, when aligned using a sequence alignment program. For example, as used herein, 80% homology means the same thing as 80% sequence homology determined by a defined algorithm, and accordingly a homologue of a given sequence has greater than 80%

sequence homology over a length of the given sequence. Exemplary levels of sequence homology include, but are not limited to, 60, 70, 80, 85, 90, 95, 98% or more sequence homology to a given sequence.

5 [0068] Exemplary computer programs which can be used to determine identity between two sequences include, but are not limited to, the suite of BLAST programs, *e.g.*, BLASTN, BLASTX, and TBLASTX, BLASTP and TBLASTN, publicly available on the Internet at the NCBI website. *See also* Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-10 (with special reference to the published default setting, *i.e.*, parameters $w=4$, $t=17$) and Altschul *et al.*, 1997, *Nucleic Acids Res.*, 25:3389-3402. Sequence searches are typically carried out using
10 the BLASTP program when evaluating a given amino acid sequence relative to amino acid sequences in the GenBank Protein Sequences and other public databases. The BLASTX program is preferred for searching nucleic acid sequences that have been translated in all reading frames against amino acid sequences in the GenBank Protein Sequences and other public databases. Both BLASTP and BLASTX are run using default parameters of an open
15 gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix. *See id.*

[0069] A preferred alignment of selected sequences in order to determine “% identity” between two or more sequences, is performed using for example, the CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of
20 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

[0070] "Hybridizing specifically to" or "specific hybridization" or "selectively hybridize to", refers to the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.*, total cellular) DNA or RNA.

25 [0071] The term "stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental
30 parameters. An extensive guide to the hybridization of nucleic acids can be found in Tijssen, 1993, *Laboratory Techniques in Biochemistry and Molecular Biology - Hybridization with Nucleic Acid Probes*, part I, chapter 2, "Overview of principles of hybridization and the

strategy of nucleic acid probe assays", Elsevier, NY; Sambrook *et al.*, 2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 3rd ed., NY; and Ausubel *et al.*, eds., Current Edition, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY.

5 [0072] Generally, highly stringent hybridization and wash conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

10 [0073] One example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than about 100 complementary residues on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42° C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72° C for about 15 minutes. An example of stringent wash conditions is a
15 0.2X SSC wash at 65° C for 15 minutes. *See* Sambrook *et al.* for a description of SSC buffer. A high stringency wash can be preceded by a low stringency wash to remove background probe signal. An exemplary medium stringency wash for a duplex of, *e.g.*, more than about 100 nucleotides, is 1x SSC at 45° C for 15 minutes. An exemplary low stringency wash for a duplex of, *e.g.*, more than about 100 nucleotides, is 4-6x SSC at 40° C for 15 minutes. In
20 general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

[0074] The term "about," as used herein, unless otherwise indicated, refers to a value that is no more than 10% above or below the value being modified by the term. For example, the term "about 5 µg/kg" means a range of from 4.5 µg/kg to 5.5 µg/kg. As another example,
25 "about 1 hour" means a range of from 48 minutes to 72 minutes.

[0075] The term "stably integrated," as used herein in reference to a nucleic acid, refers to a nucleic acid that has recombined with a host cell's genomic nucleic acids and thus become a part of a cell's genome. Stably integrated nucleic acids can comprise a selectable marker to ensure that the stably integrated nucleic acids remain a part of the cells genome. Stably
30 integrated nucleic acids need not necessarily remain integrated into the genome at a single location; the nucleic acids can integrate at more than one location and can move from location to location within the genome.

5.2 Cells Expressing a Heterologous Protease or Pro-protease

[0076] Influenza virus that contains the precursor hemagglutinin molecule (HA0) on its surface is not capable of fusing with a cell and initiating infection. The HA0 must be proteolytically cleaved, separating the HA1 and HA2 subunits, to achieve its active form.

5 Virions with mature HA on the surface actively fuse with a host cell and initiate infection. Several cell types *in vivo*, including cells in the airway, contain proteases that activate HA; however, many cell types used *in vitro*, including MDCK, do not contain active proteases that can efficiently cleave HA. For these cells, exogenous trypsin has been added to the culture at a concentration that does not negatively impact the cell but allows the HA to be cleaved and
10 activated. *See, e.g.*, US Patent Nos. 5,698,433 and 5,756,341.

[0077] Porcine trypsin has been shown to effectively activate HA and is used routinely, by several influenza investigators for this purpose. In the examples described below, recombinant cells expressing, *e.g.*, porcine trypsin or trypsinogen have been evaluated and selected for their ability to support influenza replication in, *e.g.*, MDCK cells.

15 [0078] Thus, in certain embodiments, an active protease or pro-protease expressed from a recombinant system or from the cell itself is contemplated in connection with the invention. Expression of a cloned protease or pro-protease from a recombinant cell line enables the reduction or removal of animal derived products as well as provides the protease or pro-protease *in situ* enabling the most effective cleavage of the HA molecule. Alternately, a
20 protease or pro-protease encoded in the cell's genome that is not normally expressed by the cell can be expressed by altering the regulation of expression of the protease or pro-protease. For example, an promoter, *e.g.*, an inducible promoter, that directs transcription and translation of the protease or pro-protease can be introduced by, *e.g.*, homologous recombination in the region of the cell's genome that regulates expression of the protease or
25 pro-protease. By selecting a promoter (and/or other regulatory sequences) that is/are active in the selected cell type, the protease or pro-protease can be expressed in a cell that does not normally express the protease or pro-protease.

[0079] Any cell known to be useful for culturing influenza known to one skilled in the art without limitation can be used to generate a cell of the invention. For example, suitable host
30 cells for the replication of influenza virus include, *e.g.*, Vero cells, Per.C6 cells, BHK cells, MDCK cells, 293 cells and COS cells, including 293T cells, COS7 cells. Further, co-cultures including two or more of the above cell lines, *e.g.*, MDCK cells and either 293T or COS cells

can be employed at a ratio, *e.g.*, of 1:1, to improve replication efficiency. In such embodiments, either or both of the co-cultured cells can express the heterologous protease.

5.2.1 Proteases Expressed by Cells

[0080] Any protease known to one skilled in the art to be useful in cleaving the HA0 influenza protein can be expressed by a cell according to the present invention.

[0081] Several proteases can be evaluated for their ability to be produced from a recombinant system or engineered into a cell, *e.g.*, an MDCK cell, itself. Suitable proteases and pro-proteases include, but are not limited to, trypsin, trypsinogen, and SPRT. Additional exemplary proteases that can be used are listed in Table 1, below. Further, active fragments of the proteases can also be used in the cells and methods of the present invention.

[0082] The skilled artisan can routinely determine whether a particular protease is suitable for use in the cells and methods of the invention. Typically, such assays involve an assessment of cleavage of a viral protein wherein such cleavage is an important step in the virus's life cycle. Cleavage can be assessed directly, *e.g.*, by monitoring production of two smaller proteins from a larger protein, or indirectly, *e.g.*, by monitoring viral titers produced in the presence of the protease. For example, proteases suitable for cells and/or methods for influenza virus culture can be identified by, *e.g.*, assessing cleavage of the HA0 protein or by monitoring viral titer in cell culture comprising the protease.

Table 1: Proteases

S1 family - SA clan	Enzyme Classification	Length (AA)	SwissProt Accession No.	GenBank Accession No.
achelase I protease: giant silkworm moth, saturnid moth	3.4.21.-	213	(P23604) ACH1_LONAC	N/A
achelase II protease: giant silkworm moth, saturnid moth	3.4.21.-	214	(P23605) ACH2_LONAC	N/A
acrosin: goat	3.4.21.10	60	(P10626) ACRO_CAPHI	N/A
acrosin: human	3.4.21.10	421	(P10323) ACRO_HUMAN	Y00970
acrosin: mouse	3.4.21.10	436	(P23578) ACRO_MOUSE	S66245
acrosin : pig	3.4.21.10	415	(P08001) ACRO_PIG	J04950
acrosin : rabbit	3.4.21.10	431	(P48038) ACRO_RABIT	U05204
acrosin: rat	3.4.21.10	437	(P29293) ACRO_RAT	X59254

ancrod: malayan pit viper	3.4.21.74	234	(P26324) ANC1_AGKRH	N/A
ancrod: malayan pit viper	3.4.21.74	258	(P47797) ANC2_AGKRH	L07308
ancrod: cantil, tropical mocassin	3.4.21.74	20	(P33588) ANCR_AGKBI	N/A
ancrod: southern copperhead	3.4.21.74	231	(P09872) ANCR_AGKCO	N/A
apolipoprotein(A): human	3.4.21.-	4548	(P08519) APOA_HUMAN	X06290
apolipoprotein(A): rhesus macaque	3.4.21.-	1420	(P14417) APOA_MACMU	J04635
batroxobin: barba amaril, fer-de-lance	3.4.21.74	255	(P04971) BATX_BOTAT	J02684
complement C1R component: human	3.4.21.41	705	(P00736) C1R_HUMAN	X04701
complement C1S component: human	3.4.21.42	688	(P09871) C1S_HUMAN	X06596
proproteinase E (procarboxypeptidaseA complex: bovine	N/A	253	(P05805) CAC3_BOVIN	N/A
azurocidin(cathionic antimicrobial protein) : human	N/A	251	(P20160) CAP7_HUMAN	M96326
azurocidin(cathionic antimicrobial protein CAP37) : pig	N/A	219	(P80015) CAP7_PIG	N/A
calcium-dependent serine proteinase : golden hamster	3.4.21.-	695	(P15156) CASP_MESAU	X16160
cathepsin G: human	3.4.21.20	255	(P08311) CATG_HUMAN	M16117
cathepsin G: mouse	3.4.21.20	261	(P28293) CATG_MOUSE	M96801
cathepsin G: rat	3.4.21.20	26	(P17977) CATG_RAT	N/A
cerastotin: horned desert viper	3.4.21.-	98	(P81038) CERA_CERCE	N/A
cerastobin: sahara sand viper	3.4.21.-	35	(P18692) CERA_CERVI	N/A
cercarial protease: blood fluke	3.4.21.-	264	(P12546) CERC_SCHMA	J03946
complement factor B: bovine	3.4.21.47	16	(P81187) CFAB_BOVIN	N/A
complement factor B: human	3.4.21.47	764	(P00751) CFAB_HUMAN	X72875
complement factor B: mouse	3.4.21.47	761	(P04186) CFAB_MOUSE	M60646
complement factor B : pig	3.4.21.47	151	(Q03710) CFAB_PIG	M59240
complement factor D: human	3.4.21.46	253	(P00746)	M84526

			CFAD_HUMAN	
complement factor D: mouse	3.4.21.46	259	(P03953) CFAD_MOUSE	M11768
complement factor D : pig	3.4.21.46	259	(P51779) CFAD_PIG	U29948
complement factor D: rat	3.4.21.46	263	(P32038) CFAD_RAT	S73894
complement factor I: human	3.4.21.45	583	(P05156) CFAI_HUMAN	Y00318
complement factor B-like protease: chicken	3.4.21.-	250	(P81475) CFBL_CHICK	N/A
caldecrin: rat	3.4.21.-	268	(P55091) CLCR_RAT	S80379
complement C2: human	3.4.21.43	752	(P06681) CO2_HUMAN	M15082
complement C2: mouse	3.4.21.43	760	(P21180) CO2_MOUSE	M60579
cocoonase: atlantic horseshoe crab	3.4.21.-	14	(P35586) COCO_LIMPO	N/A
collagenolytic protease 25 KD II/III: crab-beetle	3.4.21.32	20	(P34153) COG1_CHIOP	N/A
collagenolytic protease 28 KD : red king crab	3.4.21.32	20	(P20731) COG1_PARCM	N/A
collagenolytic protease 35 KD II: crab- beetle	3.4.21.32	20	(P34154) COG2_CHIOP	N/A
collagenolytic protease 36 KD: crab- beetle	3.4.21.32	20	(P34155) COG3_CHIOP	N/A
collagenolytic protease 36 KD A : red king crab	3.4.21.32	20	(P20732) COGA_PARCM	N/A
collagenolytic protease36 KD B : red king crab	3.4.21.32	20	(P20733) COGB_PARCM	N/A
collagenolytic protease36 KD C : red king crab	3.4.21.32	20	(P20734) COGC_PARCM	N/A
collagenase: cattle grub	3.4.21.-	260	(P08897) COGS_HYPLI	X74306
brachyurin: atlantic sand fiddler crab	3.4.21.32	226	(P00771) COGS_UCAPU	N/A
complement -activating component of RA-reactive factor : human	N/A	699	(P48740) CRAR_HUMAN	D17525
complement -activating component of RA-reactive factor : mouse	N/A	704	(P98064) CRAR_MOUSE	D16492
chymotrypsin1: african malaria mosquito	3.4.21.1	259	(Q27289) CTR1_ANOGA	Z18887
chymotrypsin BI: penoied shrimp, european white shrimp	3.4.21.1	271	(Q00871) CTR1_PENVA	X66415
chymotrypsin2: african malaria mosquito	3.4.21.1	258	(Q17025) CTR2_ANOGA	Z18888

chymotrypsin2: dog	3.4.21.1	263	(P04813) CTR2_CANFA	K01173
chymotrypsinBII: penoied shrimp, european white shrimp	3.4.21.1	271	(P36178) CTR2_PENVA	N/A
chymotrypsinII: european hornet	3.4.21.1	218	(P00769) CTR2_VESCR	N/A
chymotrypsinII : oriental hornet	3.4.21.1	216	(P00768) CTR2_VESOR	N/A
chymotrypsinA: bovine	3.4.21.1	245	(P00766) CTRA_BOVIN	N/A
chymotrypsinA: atlantic cod	3.4.21.1	263	(P47796) CTRA_GADMO	X78490
chymotrypsinB: bovine	3.4.21.1	245	(P00767) CTRB_BOVIN	N/A
chymotrypsinB : atlantic cod	3.4.21.1	245	(P80646) CTRB_GADMO	N/A
chymotrypsinB: human	3.4.21.1	263	(P17538) CTRB_HUMAN	M24400
chymotrypsinB: rat	3.4.21.1	263	(P07338) CTRB_RAT	K02298
chymotrypsin-like serine proteinase : california red abalone	3.4.21.-	254	(P35003) CTRL_HALRU	X71438
chymotrypsin-like protease CTRL-1: human	3.4.21.-	264	(P40313) CTRL_HUMAN	X71874
chymotrypsin: penoeid shrimp.	3.4.21.1	31	(P35002) CTRP_PENMO	N/A
duodenase I: bovine	3.4.21.-	226	(P80219) DDN1_BOVIN	N/A
mite allergen der. F 3: house-dust mite	3.4.21.-	259	(P49275) DEF3_DERFA	D63858
mite allergen der. F 6: house-dust mite	3.4.21.-	20	(P49276) DEF6_DERFA	N/A
mite allergen der. P 3: house-dust mite	3.4.21.-	261	(P39675) DER3_DERPT	U11719
mite allergen der. P 6 : house-dust mite	3.4.21.-	20	(P49277) DER6_DERPT	N/A
serine protease easter: fruit fly	3.4.21.-	392	(P13582) EAST_DROME	J03154
elastase 1: bovine	3.4.21.36	266	(Q28153) EL1_BOVIN	M80838
elastase 1 : human	3.4.21.36	68	(P11423) EL1_HUMAN	N/A
elastase 1: pig	3.4.21.36	266	(P00772) EL1_PIG	X04036
elastase 1: rat	3.4.21.36	266	(P00773) EL1_RAT	V01234
neutrophil elastase 2A : horse	3.4.21.-	85	(P37357) EL2A_HORSE	N/A

elastase 2A: human	3.4.21.71	269	(P08217) EL2A_HUMAN	M16631
neutrophil elastase 2B : horse	3.4.21.-	73	(P37358) EL2B_HORSE	N/A
elastase 2B: human	3.4.21.71	269	(P08218) EL2B_HUMAN	M16653
elastase 2: bovine	3.4.21.71	269	(Q29461) EL2_BOVIN	X97635
elastase 2: mouse	3.4.21.71	271	(P05208) EL2_MOUSE	X04573
elastase 2: pig	3.4.21.71	269	(P08419) EL2_PIG	M16651
elastase 2: rat	3.4.21.71	271	(P00774) EL2_RAT	V01233
elastase IIIA: human	3.4.21.70	270	(P09093) EL3A_HUMAN	M18700
elastase IIIB: human	3.4.21.70	270	(P08861) EL3B_HUMAN	M16630
elastase : atlantic cod	3.4.21.-	20	(P32197) ELAS_GADMO	N/A
leukocyteelastase: human	3.4.21.37	267	(P08246) ELNE_HUMAN	J03545
enteropeptidase: bovine	3.4.21.9	1035	(P98072) ENTK_BOVIN	U09859
enteropeptidase: human	3.4.21.9	1019	(P98073) ENTK_HUMAN	U09860
enteropeptidase: mouse	3.4.21.9	1069	(P97435) ENTK_MOUSE	U73378
enteropeptidase: pig	3.4.21.9	1034	(P98074) ENTK_PIG	D30799
arginine esterase: dog	3.4.21.35	260	(P09582) ESTA_CANFA	Y00751
coagulation factor X: bovine	3.4.21.6	492	(P00743) FA10_BOVIN	X00673
coagulation factor X: chicken	3.4.21.6	475	(P25155) FA10_CHICK	D00844
coagulation factor X: human	3.4.21.6	488	(P00742) FA10_HUMAN	K03194
coagulation factor X: rabbit	3.4.21.6	490	(O19045) FA10_RABIT	AF003200
coagulation factor XI: human	3.4.21.27	625	(P03951) FA11_HUMAN	M13142
coagulation factor XII: bovine	3.4.21.38	593	(P98140) FA12_BOVIN	S70164
coagulation factor XII: guinea pig	3.4.21.38	603	(Q04962) FA12_CAVPO	X68615
coagulation factor XII: human	3.4.21.38	615	(P00748) FA12_HUMAN	M31315

coagulation factor VII: bovine	3.4.21.21	407	(P22457) FA7_BOVIN	N/A
coagulation factor VII: human	3.4.21.21	466	(P08709) FA7_HUMAN	M13232
coagulation factor VII: mouse	3.4.21.21	446	(P70375) FA7_MOUSE	U66079
coagulation factor VII: rabbit	3.4.21.21	444	(P98139) FA7_RABIT	U77477
coagulation factor IX: bovine	3.4.21.22	416	(P00741) FA9_BOVIN	J00007
coagulation factor IX: dog	3.4.21.22	452	(P19540) FA9_CANFA	M21757
coagulation factor IX : guinea pig	3.4.21.22	285	(P16295) FA9_CAVPO	M26237
coagulation factor IX: human	3.4.21.22	461	(P00740) FA9_HUMAN	K02402
coagulation factor IX: mouse	3.4.21.22	459	(P16294) FA9_MOUSE	M23109
coagulation factor IX : pig	3.4.21.22	271	(P16293) FA9_PIG	M26235
coagulation factor IX : rabbit	3.4.21.22	275	(P16292) FA9_RABIT	M26234
coagulation factor IX : rat	3.4.21.22	282	(P16296) FA9_RAT	M26247
coagulation factor IX : sheep	3.4.21.22	274	(P16291) FA9_SHEEP	M26233
flavoxobin: habu	3.4.21.-	260	(P05620) FLVB_TRIFL	D67078
gilatoxin: beaded lizard	3.4.21.-	245	(P43685) GILX_HELHO	N/A
granzyme A: human	3.4.21.78	262	(P12544) GRAA_HUMAN	M18737
granzyme A: mouse	3.4.21.78	260	(P11032) GRAA_MOUSE	X14799
granzyme B: human	3.4.21.79	247	(P10144) GRAB_HUMAN	M17016
granzyme B (G,H): mouse	3.4.21.79	247	(P04187) GRAB_MOUSE	X04072
granzyme C: mouse	3.4.21.-	248	(P08882) GRAC_MOUSE	M22527
granzyme D: mouse	3.4.21.-	248	(P11033) GRAD_MOUSE	J03255
granzyme E: mouse	3.4.21.-	248	(P08884) GRAE_MOUSE	M36901
granzyme F: mouse	3.4.21.-	248	(P08883) GRAF_MOUSE	M36902
granzyme G: mouse	3.4.21.-	248	(P13366) GRAG_MOUSE	M36900

granzyme H: human	3.4.21.-	246	(P20718) GRAH_HUMAN	J02907
granzyme K: human	3.4.21.-	264	(P49863) GRAK_HUMAN	U35237
granzyme K: mouse	3.4.21.-	263	(O35205) GRAK_MOUSE	AF011446
granzyme K: rat	3.4.21.-	258	(P49864) GRAK_RAT	L19694
granzyme M: human	3.4.21.-	257	(P51124) GRAM_HUMAN	L36936
granzyme M: rat	3.4.21.-	258	(Q03238) GRAM_RAT	L05175
granzyme-like protein I: rat	3.4.21.-	248	(Q06605) GRL1_RAT	X66693
granzyme-like protein II: rat	3.4.21.-	248	(Q06606) GRL2_RAT	X68657
halistase: gloydius blomhoffii	3.4.21.-	238	(P81176) HAYS_AGKHA	N/A
serine protease hepsin : human	3.4.21.-	417	(P05981) HEPS_HUMAN	M18930
serine protease hepsin : mouse	3.4.21.-	416	(O35453) HEPS_MOUSE	AF030065
serine protease hepsin : rat	3.4.21.-	416	(Q05511) HEPS_RAT	X70900
hepatocyte growth factor activator: human	3.4.21.-	655	(Q04756) HGFA_HUMAN	D14012
hepatocyte growth factor -like protein: human	N/A	711	(P26927) HGFL_HUMAN	M74178
hepatocyte growth factor -like protein: mouse	N/A	716	(P26928) HGFL_MOUSE	M74180
hepatocyte growth factor (scatter factor) : human	N/A	728	(P14210) HGF_HUMAN	D90334
hepatocyte growth factor (scatter factor) : mouse	N/A	728	(Q08048) HGF_MOUSE	D10212
hepatocyte growth factor (scatter factor) : rat	N/A	728	(P17945) HGF_RAT	D90102
hypodermin A: cattle grub	3.4.21.-	256	(P35587) HYPA_HYPLI	X74303
hypodermin B: cattle grub	3.4.21.-	256	(P35588) HYPB_HYPLI	L24915
plasma kallikrein: human	3.4.21.34	638	(P03952) KAL_HUMAN	M13143
plasma kallikrein: mouse	3.4.21.34	638	(P26262) KAL_MOUSE	M58588
plasma kallikrein: rat	3.4.21.34	638	(P14272) KAL_RAT	M62357
glandular kallikrein, submandibular :	3.4.21.35	31	(P12322)	N/A

guinea pig			KLK1_CAVPO	
glandular kallikrein1: human	3.4.21.35	262	(P06870) KLK1_HUMAN	M25629
glandular kallikrein1: crab-eating macaque, cynomolgus monkey	3.4.21.35	257	(Q07276) KLK1_MACFA	L10039
glandular kallikrein K1: mouse	3.4.21.35	261	(P15947) KLK1_MOUSE	M13500
glandular kallikrein1 hamadryas baboon	3.4.21.35	258	(Q28773) KLK1_PAPHA	L43121
glandular kallikrein, pancreatic 1: rat	3.4.21.35	261	(P00758) KLK1_RAT	J00758
glandular kallikrein, prostatic : guinea pig	3.4.21.35	239	(P12323) KLK2_CAVPO	N/A
glandular kallikrein 2: human	3.4.21.35	261	(P20151) KLK2_HUMAN	M18156
tonin: rat	3.4.21.35	259	(P00759) KLK2_RAT	M11565
glandular kallikrein K3: mouse	3.4.21.35	261	(P00756) KLK3_MOUSE	X01389
glandular kallikrein 3, submandibular : rat	3.4.21.35	188	(P15950) KLK3_RAT	M26534
7S nerve growth factor alpha chain: mouse	N/A	256	(P00757) KLK4_MOUSE	X01800
glandular kallikrein K5: mouse	3.4.21.35	261	(P15945) KLK5_MOUSE	Y00500
glandular kallikrein K6: mouse	3.4.21.35	261	(P00755) KLK6_MOUSE	V00829
glandular kallikrein 7, submandibular/renal: rat	3.4.21.35	261	(P36373) KLK7_RAT	M19647
glandular kallikrein K8: mouse	3.4.21.35	261	(P07628) KLK8_MOUSE	X03994
glandular kallikrein 8, prostatic: rat	3.4.21.35	261	(P36374) KLK8_RAT	M27217
glandular kallikrein K9: mouse	3.4.21.35	261	(P15949) KLK9_MOUSE	M17962
glandular kallikrein 9, submandibular: rat	3.4.21.35	259	(P07647) KLK9_RAT	M11566
glandular kallikrein K11: mouse	3.4.21.35	261	(P15946) KLKA_MOUSE	X13215
glandular kallikrein 10: rat	3.4.21.35	244	(P36375) KLKA_RAT	S48142
glandular kallikrein 12, submandibular/renal: rat	3.4.21.35	259	(P36376) KLKB_RAT	M19648
glandular kallikrein K13: mouse	3.4.21.35	261	(P36368) KLKC_MOUSE	M17982
gamma-renin, submandibular gland: mouse	3.4.21.54	261	(P04071) KLKF_MOUSE	J03877

glandular kallikrein K22: mouse	3.4.21.35	259	(P15948) KLKL_MOUSE	M17979
glandular kallikrein, renal: african soft furred rat	3.4.21.35	263	(P32824) KLKR_PRANA	X17352
glandular kallikrein K26: mouse	3.4.21.35	261	(P36369) KLKZ_MOUSE	K01831
glandular kallikrein: pig	3.4.21.35	232	(P00752) KLK_PIG	N/A
chymotrypsin-like serine proteinase : human	3.4.21.-	26	(P34168) LCLP_HUMAN	N/A
limulus clotting factor C: japanese horseshoe crab	3.4.21.84	1019	(P28175) LFC_TACTR	D90271
chymase: dog	3.4.21.39	249	(P21842) MCT1_CANFA	J02904
chymase: human	3.4.21.39	247	(P23946) MCT1_HUMAN	M69137
chymase: crab-eating macaque, cynomolgus monkey	3.4.21.39	247	(P56435) MCT1_MACFA	AB000823
mast cell protease 1: mongolian jird	3.4.21.-	246	(P50340) MCT1_MERUN	D45173
mast cell protease 1: mouse	3.4.21.-	246	(P11034) MCT1_MOUSE	S44609
chymase hamadryas baboon	3.4.21.39	247	(P52195) MCT1_PAPHA	U38521
mast cell protease I: rat	3.4.21.39	260	(P09650) MCT1_RAT	U67915
mast cell protease 1A: sheep	3.4.21.-	245	(P80931) MCT1_SHEEP	Y14654
mast cell protease 2: mongolian jird	3.4.21.-	247	(P50341) MCT2_MERUN	D45174
mast cell protease 2: mouse	3.4.21.-	244	(P15119) MCT2_MOUSE	J05177
mast cell protease II: rat	3.4.21.-	247	(P00770) MCT2_RAT	J02712
mast cell protease 3 : mouse	3.4.21.-	21	(P21843) MCT3_MOUSE	N/A
mast cell protease III: rat	3.4.21.-	247	(P50339) MCT3_RAT	D38495
mast cell protease 4: mouse	3.4.21.-	246	(P21812) MCT4_MOUSE	M55617
mast cell protease 5: mouse	3.4.21.-	247	(P21844) MCT5_MOUSE	X68805
mast cell protease 6: mouse	3.4.21.-	276	(P21845) MCT6_MOUSE	M57626
mast cell protease 7: mouse	3.4.21.-	273	(Q02844) MCT7_MOUSE	L00654
mast cell protease 7: rat	3.4.21.-	273	(P27435)	U67910

			MCT7_RAT	
mast cell protease 8: mouse	3.4.21.-	247	(P43430) MCT8_MOUSE	X78545
mast cell protease 9: mouse	3.4.21.-	246	(O35164) MCT9_MOUSE	AF007119
mast cell protease-like protein: mouse	3.4.21.-	246	(Q00356) MCTX_MOUSE	M57401
serine protease nudel: fruit fly	3.4.21.-	2616	(P98159) NDL_DROME	U29153
natural killer cell protease1: rat	3.4.21.-	248	(P18291) NKP1_RAT	M34097
neutrophil proteinase 4 : human	3.4.21.-	25	(P18078) NP4_HUMAN	N/A
okimaxobin I hime-habu	3.4.21.-	20	(P20005) OKI1_TRIOK	N/A
proclotting enzyme: japanese horseshoe crab	3.4.21.86	375	(P21902) PCE_TACTR	M58366
plasmin: bovine	3.4.21.7	812	(P06868) PLMN_BOVIN	X79402
plasmin : dog	3.4.21.7	333	(P80009) PLMN_CANFA	N/A
plasmin: western european hedgehog	3.4.21.7	810	(Q29485) PLMN_ERIEU	U33171
plasmin: horse	3.4.21.7	338	(P80010) PLMN_HORSE	N/A
plasmin: human	3.4.21.7	810	(P00747) PLMN_HUMAN	X05199
plasmin: rhesus macaque	3.4.21.7	810	(P12545) PLMN_MACMU	J04697
plasmin: mouse	3.4.21.7	812	(P20918) PLMN_MOUSE	J04766
plasmin: sea lamprey	3.4.21.7	325	(P33574) PLMN_PETMA	N/A
plasmin : pig	3.4.21.7	790	(P06867) PLMN_PIG	N/A
plasmin: rat	3.4.21.7	169	(Q01177) PLMN_RAT	M62832
plasmin: sheep	3.4.21.7	343	(P81286) PLMN_SHEEP	N/A
myeloblastin: human	3.4.21.76	256	(P24158) PRN3_HUMAN	X56132
prostate specific antigen: human	3.4.21.77	261	(P07288) PROS_HUMAN	X14810
prostate specific antigen: rhesus macaque	3.4.21.35	261	(P33619) PROS_MACMU	X73560
vitamin-K dependent protein C: bovine	3.4.21.69	456	(P00745) PRTC_BOVIN	K02435

vitamin-K dependent protein C : dog	3.4.21.69	157	(Q28278) PRTC_CANFA	D43751
vitamin-K dependent protein C : goat	3.4.21.69	157	(Q28315) PRTC_CAPHI	D43752
vitamin-K dependent protein C : cat	3.4.21.69	157	(Q28412) PRTC_FELCA	D43750
vitamin-K dependent protein C : horse	3.4.21.69	157	(Q28380) PRTC_HORSE	D43753
vitamin-K dependent protein C: human	3.4.21.69	461	(P04070) PRTC_HUMAN	M11228
vitamin-K dependent protein C: rhesus macaque	3.4.21.69	161	(Q28506) PRTC_MACMU	D43754
vitamin-K dependent protein C: mouse	3.4.21.69	461	(P33587) PRTC_MOUSE	D10445
vitamin-K dependent protein C: rabbit	3.4.21.69	458	(Q28661) PRTC_RABIT	U49933
vitamin-K dependent protein C: rat	3.4.21.69	461	(P31394) PRTC_RAT	X64336
protease serine-like 1: human	3.4.21.-	276	(O43240) PSL1_HUMAN	AF024605
prostasin: human	3.4.21.-	343	(Q16651) PSS8_HUMAN	L41351
protease M: human	3.4.21.-	244	(Q92876) PSS9_HUMAN	U62801
proteinase RVV-V alpha: russell viper	3.4.21.95	236	(P18964) RVVA_DABRU	N/A
proteinase RVV-V gamma: russell viper	3.4.21.95	236	(P18965) RVVG_DABRU	N/A
serine protease SP24D: african malaria mosquito	3.4.21.-	271	(Q17004) S24D_ANOGA	U21917
stratum corneum chymotryptic enzyme: human	3.4.21.-	253	(P49862) SCCE_HUMAN	L33404
serine protease S 1 and 2: fruit fly	3.4.21.-	265	(P17205) SER1_DROME	M24379
serine protease 3 : fruit fly	3.4.21.-	61	(P17207) SER3_DROME	M24380
serine protease snake: fruit fly	3.4.21.-	430	(P05049) SNAK_DROME	X04513
serine proteinase stubble : fruit fly	3.4.21.-	786	(Q05319) STUB_DROME	L11451
Subtilisin: <i>Bacillus subtilis</i>	3.4.21.-	378	N/A	M28537
thrombin: bovine	3.4.21.5	625	(P00735) THRB_BOVIN	V00135
thrombin: human	3.4.21.5	622	(P00734) THRB_HUMAN	M17262
thrombin: mouse	3.4.21.5	618	(P19221) THRB_MOUSE	X52308

thrombin: rat	3.4.21.5	617	(P18292) THRB RAT	X52835
gyroxin analog: bushmaster	3.4.21.74	228	(P33589) THRL LACMU	N/A
transmembrane protease, serine2 : human	3.4.21.-	492	(O15393) TMS2 HUMAN	U75329
trypsin I: african malaria mosquito	3.4.21.4	274	(P35035) TRY1 ANOGA	Z18889
trypsin, cationic: bovine	3.4.21.4	243	(P00760) TRY1 BOVIN	D38507
trypsin, cationic: dog	3.4.21.4	246	(P06871) TRY1 CANFA	M11590
trypsin I-P1 : chicken	N/A	248	(Q90627) TRY1 CHICK	U15155
trypsin I: atlantic cod	3.4.21.4	241	(P16049) TRY1 GADMO	X76886
trypsin I: human	3.4.21.4	247	(P07477) TRY1 HUMAN	M22612
trypsin I, anionic: rat	3.4.21.4	246	(P00762) TRY1 RAT	V01273
trypsin I: atlantic salmon	3.4.21.4	242	(P35031) TRY1 SALSA	X70075
trypsin: yellowfever mosquito	3.4.21.4	243	(P19799) TRY1 XENLA	X53458
trypsin 2: african malaria mosquito	3.4.21.4	277	(P35036) TRY2 ANOGA	Z18890
trypsin, anionic: bovine	3.4.21.4	247	(Q29463) TRY2 BOVIN	X54703
trypsin, anionic: dog	3.4.21.4	247	(P06872) TRY2 CANFA	M11589
trypsin I-P38 : chicken	N/A	248	(Q90628) TRY2 CHICK	U15156
trypsin II: human	3.4.21.4	247	(P07478) TRY2 HUMAN	M27602
trypsin II, anionic: rat	3.4.21.4	246	(P00763) TRY2 RAT	V01274
trypsin II: atlantic salmon	3.4.21.4	231	(P35032) TRY2 SALSA	X70073
trypsin: yellowfever mosquito	N/A	244	(P70059) TRY2 XENLA	U72330
trypsin 3A1: yellowfever mosquito	3.4.21.4	254	(P29786) TRY3 AEDAE	X64362
trypsin3: african malaria mosquito	3.4.21.4	275	(P35037) TRY3 ANOGA	Z22930
trypsin II-P29 : chicken	N/A	248	(Q90629) TRY3 CHICK	U15157
trypsin III: human	3.4.21.4	247	(P15951)	X15505

			TRY3_HUMAN	
trypsin alpha-3: green-bottle fly, australian sheep blowfly	3.4.21.4	165	(P35043) TRY3_LUCCU	L15632
trypsin III, cationic: rat	3.4.21.4	247	(P08426) TRY3_RAT	M16624
trypsin III: atlantic salmon	3.4.21.4	238	(P35033) TRY3_SALSA	X70074
trypsin 4: african malaria mosquito	3.4.21.4	275	(P35038) TRY4_ANOGA	Z22930
trypsin 4A: human	3.4.21.4	304	(P35030) TRY4_HUMAN	X72781
trypsin alpha-4: green-bottle fly, australian sheep blowfly	3.4.21.4	255	(P35044) TRY4_LUCCU	L15632
trypsin IV: rat	3.4.21.4	247	(P12788) TRY4_RAT	X15679
trypsin 5G1: yellowfever mosquito	3.4.21.4	238	(P29787) TRY5_AEDAE	X64363
trypsin 5: african malaria mosquito	3.4.21.4	274	(P35039) TRY5_ANOGA	Z22930
trypsin 6: african malaria mosquito	3.4.21.4	273	(P35040) TRY6_ANOGA	Z22930
trypsin 7: african malaria mosquito	3.4.21.4	267	(P35041) TRY7_ANOGA	Z22930
trypsin alpha:fruit fly	3.4.21.4	256	(P54624) TRYA_DROER	U40653
trypsin alpha: fruit fly	3.4.21.4	256	(P04814) TRYA_DROME	X02989
alpha-tryptase: human	3.4.21.59	275	(P15157) TRYA_HUMAN	M30038
trypsin, alkaline A : tobacco hawkmoth, horworm	N/A	256	(P35045) TRYA_MANSE	L16805
trypsin V-A: rat	3.4.21.4	246	(P32821) TRYA_RAT	X59012
trypsin beta:fruit fly	3.4.21.4	253	(P54625) TRYB_DROER	U40653
trypsin beta: fruit fly	3.4.21.4	253	(P35004) TRYB_DROME	M96372
beta-tryptase: human	3.4.21.59	275	(P20231) TRYB_HUMAN	M37488
trypsin, alkaline B : tobacco hawkmoth, horworm	N/A	256	(P35046) TRYB_MANSE	L16806
trypsin V-B: rat	3.4.21.4	246	(P32822) TRYB_RAT	X59013
trypsin, alkaline C : tobacco hawkmoth, horworm	N/A	256	(P35047) TRYC_MANSE	L16807
trypsin delta/gamma: fruit fly	3.4.21.4	253	(P54626) TRYD_DROER	U40653

trypsin delta: fruit fly	3.4.21.4	253	(P42276) TRYD_DROME	U04853
trypsin epsilon: fruit fly	3.4.21.4	256	(P54627) TRYE_DROER	U40653
trypsin epsilon: fruit fly	3.4.21.4	256	(P35005) TRYE_DROME	M96372
trypsin gamma: fruit fly	3.4.21.4	253	(P42277) TRYG_DROME	U04853
trypsin iota: fruit fly	3.4.21.4	252	(P52905) TRYI_DROME	U41476
Trypsin precursor: streptomyces griseus	3.4.21.-	259	(P00775) TRYP_STRGR	N/A
trypsin-like protease: rat	3.4.21.59	23	(P27436) TRYL_RAT	N/A
mastocytoma protease: dog	3.4.21.-	269	(P19236) TRYM_CANFA	M24665
mast cell trypsin: rat	3.4.21.59	274	(P50343) TRYM_RAT	D38455
trypsin I , : broad-fingered crayfish	3.4.21.4	237	(P00765) TRYP_ASTFL	N/A
trypsin CFT-1: spruce budworm	3.4.21.4	256	(P35042) TRYP_CHOFU	L04749
trypsin: cat	3.4.21.4	16	(P81071) TRYP_FELCA	N/A
trypsin: fusarium oxysporum	3.4.21.4	248	(P35049) TRYP_FUSOX	S63827
trypsin: mouse	3.4.21.4	246	(P07146) TRYP_MOUSE	X04574
trypsin: penaeid shrimp.	3.4.21.4	32	(P35050) TRYP_PENMO	N/A
trypsin: pig	3.4.21.4	231	(P00761) TRYP_PIG	N/A
trypsin: plaice	3.4.21.4	250	(P35034) TRYP_PLEPL	X56744
trypsin: marbled lungfish	3.4.21.4	21	(P35051) TRYP_PROAT	N/A
trypsin: streptomyces erythraeus	3.4.21.4	227	(P24664) TRYP_SACER	N/A
trypsin: grey flesh fly	3.4.21.4	254	(P51588) TRYP_SARBU	X94691
trypsin: black fly	3.4.21.4	247	(P35048) TRYP_SIMVI	L08428
trypsin: spiny dogfish	3.4.21.4	229	(P00764) TRYP_SQUAC	N/A
trypsin-like protease: streptomyces glaucescens	3.4.21.-	268	(Q54179) TRYP_STRGA	U13770
trypsin: streptomyces griseus	3.4.21.4	259	(P00775)	M64471

			TRYP_STRGR	
tryptase: dog	3.4.21.59	275	(P15944) TRYT_CANFA	M24664
trypsin theta: fruit fly	3.4.21.4	262	(P54628) TRYT_DROER	U40653
trypsin theta: fruit fly	3.4.21.4	262	(P42278) TRYT_DROME	U04853
mast cell tryptase: mongolian jird	3.4.21.59	270	(P50342) TRYT_MERUN	D31789
trypsineta: fruit fly	3.4.21.4	258	(P54629) TRYU_DROER	U40653
trypsineta: fruit fly	3.4.21.4	262	(P42279) TRYU_DROME	U04853
trypsinX: atlantic cod	3.4.21.4	241	(Q91041) TRYX_GADMO	X76887
trypsin zeta: fruit fly	3.4.21.4	281	(P54630) TRYZ_DROER	U40653
trypsin zeta: fruit fly	3.4.21.4	280	(P42280) TRYZ_DROME	U04853
urokinase-type plasmin activator: bovine	3.4.21.73	433	(Q05589) UROK_BOVIN	L03546
urokinase-type plasmin activator: chicken	3.4.21.73	434	(P15120) UROK_CHICK	J05187
urokinase-type plasmin activator: human	3.4.21.73	431	(P00749) UROK_HUMAN	X02419
urokinase-type plasmin activator: mouse	3.4.21.73	433	(P06869) UROK_MOUSE	X02389
urokinase-type plasmin activator: yellow baboon	3.4.21.73	433	(P16227) UROK_PAPCY	X51935
urokinase-type plasmin activator: pig	3.4.21.73	442	(P04185) UROK_PIG	X01648
urokinase-type plasmin activator: rat	3.4.21.73	432	(P29598) UROK_RAT	X63434
tissue plasmin activator: bovine	3.4.21.68	566	(Q28198) UROT_BOVIN	X85800
tissue plasmin activator: human	3.4.21.68	562	(P00750) UROT_HUMAN	X07393
tissue plasmin activator: mouse	3.4.21.68	559	(P11214) UROT_MOUSE	J03520
tissue plasmin activator: rat	3.4.21.68	559	(P19637) UROT_RAT	M23697
salivary plasmin activator alpha 1: vampire bat	3.4.21.68	477	(P98119) URT1_DESRO	M63987
salivary plasmin activator alpha 2: vampire bat	3.4.21.68	477	(P15638) URT2_DESRO	M63988
salivary plasmin activator beta: vampire bat	3.4.21.68	431	(P98121) URTB_DESRO	M63989

salivary plasmin activator gamma: vampire bat	3.4.21.68	394	(P49150) URTG DESRO	M63990
vitellin-degrading protease: silk moth	3.4.21.-	264	(Q07943) VDP BOMMO	D16232

5.2.2 Vectors Encoding a Protease

[0083] Any suitable method known to one skilled in the art without limitation for expressing a heterologous gene in a cell can be used to express the heterologous protease or pro-protease. Typically, a recombinant nucleic acid construct comprising a nucleic acid encoding the protease or pro-protease is constructed using conventional molecular biology techniques, then the construct is introduced into a host cell line of interest. Cells comprising the desired construct are identified, then screened to identify cells that express the heterologous protein to the desired concentration. Methods for performing each of these operations are legion.

[0084] Numerous vectors suitable for this purpose are publicly available, including, but not limited to, plasmids, bacteriophage, viral vectors, retroviral vectors, artificial chromosomes and episomal vectors. Methods by which a construct can be selected and used such vectors are well known to those skilled in the art. Such vectors may be used for simple cloning and mutagenesis; however, gene expression vectors should be employed when introducing a nucleic acid encoding a protease or pro-protease into a suitable cell. The vector may be selected to accommodate a protease or pro-protease coding sequence of any desired size, typically from 0.25 kilobases (kb) to 40 kb or more in length.

[0085] Vectors typically contain various functional components, including a cloning (or "polylinker") site, an origin of replication and at least one selectable marker gene. Further, to express the protease or pro-protease, the vectors typically possess one or more of the following: enhancer element, promoter, transcription termination and signal sequences, each positioned in the vicinity of the cloning site, such that they are operatively linked to the nucleic acid encoding a protease or pro-protease.

[0086] The expression of a protease or pro-protease protein may be controlled by any promoter or enhancer element known in the art. Suitable promoters which may be used include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences

of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42), the tetracycline (Tet) promoter (Gossen et al., 1995, *Proc. Nat. Acad. Sci. USA* 89:5547-5551), or a CMV promoter (such as the human immediate-early CMV promoter). Further, should temporal control of the expression of the protease or pro-protease be desirable, any inducible promoter known to one skilled in the art without limitation can be used according to the present invention. For example, the regulatory element can be an interferon-responsive inducible promoter or regulatory element. *See, e.g.,* Levy et al., 1988, *Genes & Devel.* 2:383-393, Reich et al., 1987, *P.N.A.S. USA* 84:6394-6398, and Pellegrini et al., 1989, *Mol Cell Biol.* 9:4605-4612. Vectors may also be inducible because they contain hormone response elements, such as the glucocorticoid response element (GRE) and the estrogen response element (ERE), which can confer hormone inducibility where vectors are used for expression in cells having the respective hormone receptors. To reduce background levels of expression, elements responsive to ecdysone, an insect hormone, can be used instead, with coexpression of the ecdysone receptor.

[0087] Vectors generally contain nucleic acid sequences that enable the vector to replicate in one or more selected host cells. However, when the vector is intended to integrate into the host cell's genome, the vector need not be able to autonomously replicate, and, in fact, desirably does not do so. Typically, this sequence enables the vector to replicate independently of the host chromosomal DNA and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, viruses, and mammalian cells.

[0088] The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micron plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication is not needed for mammalian expression vectors unless these are used in mammalian cells able to replicate high levels of DNA, such as COS cells.

[0089] Advantageously, the vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will therefore not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available in the growth media. Since the vectors encoding

protease or pro-protease are most commonly introduced into mammalian cells, a mammalian selectable marker, for example, a G418 resistance gene, can advantageously be employed.

[0090] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk-, hgp^{rt}- or ap^{rt}-cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hyg^{ro}, which confers resistance to hygromycin (Santerre et al., 1984, Gene 30:147) genes. Other antibiotic-resistance genes, such as those conferring resistance to ampicillin, Claforan, gentamycin, G418, hygromycin, rifampicin, kanamycin, neomycin, spectinomycin, or tetracycline, may also find use as selectable markers.

[0091] Expression vectors typically contain a promoter that is recognized by the host organism and is operably linked to the coding sequence of interest. Such a promoter may be inducible or constitutive. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

[0092] Construction of vectors encoding a protease or pro-protease employs conventional ligation techniques. Isolated vectors or DNA fragments are cleaved, tailored, and religated in the form desired to generate the required vector. If desired, analysis to confirm that the correct sequences are present in the constructed vector can be performed in a known fashion. Suitable methods for constructing expression vectors, preparing *in vitro* transcripts, introducing DNA into host cells, and performing analyses for assessing expression and function are known to those skilled in the art. The presence of a gene sequence in a sample is detected, or its amplification and/or expression quantified by conventional methods, such as Southern or Northern analysis, Western blotting, dot blotting of DNA, RNA or protein, *in situ* hybridization, immunocytochemistry or sequence analysis of nucleic acid or protein

molecules. Those skilled in the art will readily envisage how these methods may be modified, if desired.

[0093] In cases where an adenovirus is used as an expression vector, the protease or pro-protease coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the protease or pro-protease in infected hosts (e.g., see Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:355-359).

Alternatively, a retroviral expression vector containing the Harvey murine sarcoma virus (Ha-MSV) long terminal repeats (LTRs) flanking the promoter and nucleic acid encoding the modified ABC transporter polypeptide may be used. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

[0094] Artificial chromosomes may also be employed to deliver larger fragments of DNA than can be contained in and expressed from a plasmid or retroviral vector. Artificial chromosomes of about 6 kb to 10 Mb can be constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes. (See, e.g., Harrington, J. J. et al. (1997) Nat. Genet. 15:345-355.)

[0095] Specific initiation signals may also be required for efficient translation of inserted protease or pro-protease coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon should be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see, e.g., Bittner et al., 1987, Methods in Enzymol. 153:516-544).

[0096] In certain embodiments, cells that stably express a protease or pro-protease are provided. To produce such cell lines, rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign

DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci that in turn can be cloned and expanded into cell lines.

5 **5.2.3 Methods for Making a Cell Expressing a Heterologous Protease**

[0097] The methods chosen for introducing the vector encoding a protease or pro-protease to a desired cell will typically depend on the vector and the cell.

[0098] Transformation and other methods of introducing nucleic acids into a host cell (e.g., conjugation, protoplast transformation or fusion, transfection, electroporation, liposome
10 delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion) can be accomplished by a variety of methods that are well known in the art (See, for instance, Ausubel, *infra*, and Sambrook et al., *infra*). Bacterial, yeast, plant or mammalian cells can be transformed or transfected with an expression vector, such as a plasmid, a cosmid, or the like, wherein the expression vector comprises the nucleic acid of
15 interest, as described above. Alternatively, the cells may be infected by a viral expression vector comprising the nucleic acid of interest. Depending upon the host cell, vector, and method of transformation used, transient or stable expression of the polypeptide will be constitutive or inducible. One having ordinary skill in the art will be able to decide whether to express a polypeptide transiently or stably, and whether to express the protein constitutively
20 or inducibly, as described above.

[0099] Mammalian cells can be directly infected by packaged viral vectors, or transfected by chemical or electrical means. For chemical transfection, DNA can be coprecipitated with CaPO_4 or introduced using liposomal and nonliposomal lipid-based agents. Commercial kits are available for CaPO_4 transfection (CalPhos™ Mammalian Transfection Kit, Clontech
25 Laboratories, Palo Alto, Calif., USA), and lipid-mediated transfection can be practiced using commercial reagents, such as LIPOFECTAMIN® 2000, LIPOFECTAMINE™ Reagent, CELLFECTIN® Reagent, LIPOFECTIN® Reagent (Invitrogen, Carlsbad, Calif., USA), DOTAP Liposomal Transfection Reagent, FuGENE 6, X-tremeGENE Q2, DOSPER, (Roche Molecular Biochemicals, Indianapolis, Ind. USA), Effectene™, PolyFect®, and Superfect
30 (Qiagen, Inc., Valencia, Calif., USA). Protocols for electroporating mammalian cells can be found in, for example,; Norton et al. (eds.), *Gene Transfer Methods: Introducing DNA into Living Cells and Organisms*, BioTechniques Books, Eaton Publishing Co. (2000). Other transfection techniques include transfection by particle bombardment and microinjection.

See, e.g., Cheng et al., Proc. Natl. Acad. Sci. USA 90(10): 4455-9 (1993); Yang et al, Proc. Natl. Acad. Sci. USA 87(24): 9568-72 (1990).

5.2.4 Culturing Cells Expressing a Heterologous Protease

[0100] The cells expressing a heterologous protease can be cultured in any suitable culture medium known to one skilled in the art without limitation. Typically, cells are cultured in a standard commercial culture medium, such as Dulbecco's modified Eagle's medium supplemented with serum (e.g., 10% fetal bovine serum), or in serum free medium, under controlled humidity and CO₂ concentration suitable for maintaining neutral buffered pH (e.g., at pH between 7.0 and 7.2). Optionally, the medium contains antibiotics to prevent bacterial growth, e.g., penicillin, streptomycin, etc., and/or additional nutrients, such as L-glutamine, sodium pyruvate, non-essential amino acids, additional supplements to promote favorable growth characteristics, e.g., trypsin, β -mercaptoethanol, and the like.

[0101] Procedures for maintaining mammalian cells in culture have been extensively reported, and are known to those of skill in the art. General protocols are provided, e.g., in Freshney (1983) *Culture of Animal Cells: Manual of Basic Technique*, Alan R. Liss, New York; Paul (1975) *Cell and Tissue Culture*, 5th ed., Livingston, Edinburgh; Adams (1980) *Laboratory Techniques in Biochemistry and Molecular Biology-Cell Culture for Biochemists*, Work and Burdon (eds.) Elsevier, Amsterdam. Additional details regarding tissue culture procedures of particular interest in the production of influenza virus in vitro include, e.g., Merten et al. (1996) *Production of influenza virus in cell cultures for vaccine preparation*. In Cohen and Shafferman (eds) *Novel Strategies in Design and Production of Vaccines*, which is incorporated herein in its entirety. Additionally, variations in such procedures adapted to the present invention are readily determined through routine experimentation.

[0102] In one embodiment, the cells of the invention are cultivated as adherent cells on a surface to which they attach. Adherent surfaces on which tissue culture cells can be grown on are well known in the art. Adherent surfaces include but are not limited to, surface modified polystyrene plastics, protein coated surfaces (e.g., fibronectin and/or collagen coated glass/plastic) as well as a large variety of commercially available microcarriers (e.g., DEAE-Dextran microcarrier beads, such as Dormacell, Pfeifer & Langen; Superbead, Flow Laboratories; styrene copolymer-tri-methylamine beads, such as Hillex, SoloHill, Ann Arbor).

[0103] In one embodiment, the cells of the invention are cultivated as suspension cells without a carrier. Methods for adapting cells to for growth in suspension without a carrier are known in the art (*see, e.g.*, U.S. Patent Nos. 6,825,036 and 6,455,298). Alternatively, or optionally, the level of protease or pro-protease expressed by the cells of the invention may be regulated so as to result in the growth of the cells in suspension without any adaptation. In certain embodiments, the cells of the invention expression sufficient levels of protease or pro-protease to grow in suspension without adaptation. In other embodiments, the cells of the invention grown as suspension cells without adaptation are used for the replication of viruses. In a specific embodiment, the cells of the invention grown as suspension cells without adaptation are used for the replication of influenza viruses.

[0104] In one embodiment, the cells of the invention grow as suspension cells without adaptation due to expression of a protease or pro-protease. In certain embodiments, the protease or pro-protease is a serine protease. In other embodiments, the protease or pro-protease is trypsin or trypsinogen. In still other embodiments, the protease or pro-protease is a mammalian trypsin or trypsinogen. In yet other embodiments, the protease or pro-protease is a bacterial trypsin or trypsinogen.

[0105] In some embodiments, the level of protease or pro-protease expressed by the cells of the invention growing in suspension is between about 0.1 ng and about 50 µg per ml of cell culture. In other embodiments, the level of protease or pro-protease expressed by the cells of the invention growing in suspension is at least 0.1 ng, or at least 0.5 ng, or at least 1.0 ng, or at least 5.0 ng, or at least 10 ng, or at least 20 ng, or at least 30 ng, or at least 40 ng, or at least 50 ng, or at least 60 ng, or at least 70 ng, or at least 80 ng, or at least 90 ng, or at least 1 µg, or at least 2 µg, or at least 5 µg, or at least 10 µg, or at least 20 µg, or at least 30 µg, or at least 40 µg, or at least 50 µg per ml of cell culture.

[0106] Cells for production of influenza virus can be cultured in serum-containing or serum free medium. In some case, *e.g.*, for the preparation of purified viruses, it is desirable to grow the host cells in serum free conditions. Appropriate serum free media are described in U.S. Provisional Application No. 60/638,166, filed December 23, 2004, U.S. Provisional Application No. 60/641,139, filed January 5, 2005 and U.S. Patent Application No. 11/304,589, filed December 16, 2005, each of which is hereby incorporated by reference in its entirety.

[0107] It will be appreciated by one of skill in the art that the use of serum or animal extracts in tissue culture applications may have drawbacks (Lambert, K.J. et al., In: Animal Cell Biotechnology, Vol 1, Spier, R.E. et al., Eds., Academic Press New York, pp. 85-122 (1985)). For example, the chemical composition of these supplements may vary between lots, even
5 from a single manufacturer. In addition, supplements of animal or human origin may also be contaminated with adventitious agents (e.g., mycoplasma, viruses, and prions). These agents can seriously undermine the health of the cultured cells when these contaminated supplements are used in cell culture media formulations. Further, these agents may pose a health risk when substances produced in cultures contaminated with adventitious agents are
10 used in cell therapy and other clinical applications. A major fear is the presence of prions which cause spongiform encephalopathies in animals and Creutzfeld-Jakob disease in humans. Thus, in certain embodiments, the culture media is completely serum free. Advantageously, the culture medium can be completely free of animal products. Accordingly, in certain embodiments, the culture media is animal protein free (APF). In
15 certain embodiments, no exogenous animal-derived protease is added to the culture medium. In certain embodiments, no animal derived product is added to the culture medium. Specific media formulations are disclosed in, for example, U.S. Patent Application No. 11/304,589, filed December 16, 2005.

[0108] Cells can be cultured in small scale, e.g., less than 25 ml medium, culture tubes or
20 flasks or in large flasks with agitation, in rotator bottles, or on microcarrier beads (e.g., DEAE-Dextran microcarrier beads, such as Dormacell, Pfeiffer & Langen; Superbead, Flow Laboratories; styrene copolymer-tri-methylamine beads, such as Hillex, SoloHill, Ann Arbor) in flasks, bottles or reactor cultures. Microcarrier beads are small spheres (in the range of 100-200 microns in diameter) that provide a large surface area for adherent cell growth per
25 volume of cell culture. For example a single liter of medium can include more than 20 million microcarrier beads providing greater than 8000 square centimeters of growth surface. For commercial production of viruses, e.g., for vaccine production, it is often desirable to culture the cells in a bioreactor or fermenter. Bioreactors are available in volumes from under 1 liter to in excess of 100 liters, e.g., Cyto3 Bioreactor (Osmonics, Minnetonka, MN);
30 NBS bioreactors (New Brunswick Scientific, Edison, N.J.); laboratory and commercial scale bioreactors from B. Braun Biotech International (B. Braun Biotech, Melsungen, Germany).

[0109] Regardless of the culture volume, in certain embodiments, it is important that the cultures be maintained at a temperature less than or equal to 35 °C, to insure efficient

recovery of recombinant and/or reassortant influenza virus that are to some extent cold-adapted. For example, in certain embodiments, the cells are cultured at a temperature between about 32 °C and 35 °C, typically at a temperature between about 32 °C and about 34 °C, usually at about 33 °C.

- 5 [0110] Typically, a regulator, *e.g.*, a thermostat, or other device for sensing and maintaining the temperature of the cell culture system can be employed to insure that the temperature does not exceed 35 °C during the period of virus replication.

5.3 SPRT Protease from *Streptomyces griseus*

- 10 [0111] In addition to the proteases described above, the present invention provides a novel bacterial protease, termed SPRT. The gene encoding this protease was cloned out of *Streptomyces griseus* as described in the examples below. The SPRT protease is suitable for expressing in cells to be used for culturing viruses, as described above.

- [0112] The nucleotide sequence of the *sprT* gene is presented as Figure 9 (SEQ ID NO:1), while the amino acid sequence of the SPRT protease is presented as Figure 10 (SEQ ID NO:2). In addition to the native nucleic acid encoding the *sprT* gene, nucleic acids encoding sequences homologous to the nucleic acid sequence of the *sprT* gene are also contemplated according to the present invention. Thus, in certain embodiments, the invention provides a nucleic acid encoding a nucleotide sequence that is about 99%, about 95%, about 90%, about 85%, about 80%, about 75%, about 70%, about 65%, about 60%, about 55%, or about 50% identical to the nucleic acid sequence of Figure 9. In another aspect, the invention provides a nucleic acid that encodes a polypeptide that has a sequence that is about 99%, about 95%, about 90%, about 85%, about 80%, about 75%, about 70%, about 65%, about 60%, about 55%, or about 50% identical to the sequence of the polypeptide encoded by the nucleic acid sequence of Figure 9.

- 25 [0113] In another embodiment, the invention provides a nucleic acid that hybridizes to a nucleic acid comprising the nucleotide sequence presented as Figure 9 (or encoding a polypeptide of Table 1). In certain embodiments, the nucleic acid hybridizes to the nucleic acid comprising the nucleotide sequence presented as Figure 9 under defined hybridization conditions. In certain embodiments, the hybridization conditions are stringent hybridization conditions. In certain embodiments, the hybridization conditions are highly stringent hybridization conditions.
- 30

[0114] Furthermore, the nucleic acids of the invention also encompass derivative versions of nucleic acids encoding an SPRT protease. Such derivatives can be made by any method known by one of skill in the art without limitation. For example, derivatives can be made by site-specific mutagenesis, including substitution, insertion, or deletion of one, two, three, five, ten or more nucleotides, of the nucleic acids. Alternatively, derivatives can be made by random mutagenesis. One method for randomly mutagenizing a nucleic acid comprises amplifying the nucleic acid in a PCR reaction in the presence of 0.1 mM MnCl₂ and unbalanced nucleotide concentrations. These conditions increase the misincorporation rate of the polymerase used in the PCR reaction and result in random mutagenesis of the amplified nucleic acid.

[0115] In certain embodiments, the derivative nucleic acids encoding derivatives of the SPRT protease have improved properties relative to the wild-type enzyme described herein. For example, in some embodiments, the derivative nucleic acids encode a derivative SPRT protease that has a greater activity, *e.g.*, greater specific activity than the wild-type enzyme.

In some embodiments, the derivative SPRT protease can have a derivative secretion signal that increases secretion of the SPRT protease relative to the wild-type protease. In some embodiments, the derivative SPRT protease can have a derivative prepropeptide sequence that increases cleavage of the prepropeptide from the SPRT protease relative to the wild-type protease. In some embodiments, the derivative SPRT protease exhibits an activity maximum at a different pH from that of the wild-type protease. In certain embodiments, the pH is the pH preferred for culturing viruses, *e.g.*, influenza viruses.

[0116] In other aspects, the invention provides a SPRT polypeptide. In certain embodiments, the amino acid sequence of the SPRT polypeptide is at least about 99%, about 95%, about 90%, about 85%, about 80%, about 75%, about 70%, about 65%, about 60%, about 55%, or about 50% identical to the amino acid sequence of Figure 10. In other aspects, the invention provides a protein having an amino acid sequence of the polypeptide that is at least about 99%, about 95%, about 90%, about 85%, about 80%, about 75%, about 70%, about 65%, about 60%, about 55%, or about 50% identical to the amino acid sequence of Table 1.

Further, the invention provides active fragments of a SPRT polypeptide or protease of the invention. In certain embodiments, the active fragments comprise at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, or 320 contiguous amino acids selected from the amino acid sequence of Figure 10 (or Table 1) while retaining protease activity. The skilled

artisan can routinely identify such active fragments by, for example, expressing the fragment and testing for protease activity using conventional techniques.

[0117] Accordingly, in another aspect, the present invention relates to artificial variants comprising a conservative substitution, deletion, and/or insertion of one or more amino acids of the mature polypeptide of SEQ ID NO:2 (or Table 1); or a homologous sequence thereof. In a specific embodiment, the amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

[0118] Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R. L. Hill, 1979, In, The Proteins, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

[0119] In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-M-methyl lysine, 2-aminoisobutyric acid, isovaline, and alpha-methyl serine) may be substituted for amino acid residues of a wild-type polypeptide. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, and are commercially available, and include pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

[0120] Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

5 [0121] Essential amino acids in the parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (i.e., lipase activity) to identify amino acid
10 residues that are critical to the activity of the molecule. See also, Hilton et al., 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et
15 al., 1992, *Science* 255: 306-312; Smith et al., 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver et al., 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides which are related to a polypeptide according to the invention.

[0122] Single or multiple amino acid substitutions can be made and tested using known
20 methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman et al., 1991, *Biochem.* 30: 10832-10837; U.S. Pat. No. 5,223,409; WO 92/06204),
25 and region-directed mutagenesis (Derbyshire et al., 1986, *Gene* 46: 145; Ner et al., 1988, *DNA* 7: 127).

[0123] Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness et al., 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules
30 that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

5.4 Expression Vectors

[0124] In still another aspect, the invention provides expression vectors for expressing the SPRT protease or another protease of the invention (See, e.g., Table 1). Generally, expression vectors are recombinant polynucleotide molecules comprising expression control sequences operatively linked to a nucleotide sequence encoding a polypeptide. Expression vectors can readily be adapted for function in prokaryotes or eukaryotes by inclusion of appropriate promoters, replication sequences, selectable markers, *etc.* to result in stable transcription and translation of mRNA. Techniques for construction of expression vectors and expression of genes in cells comprising the expression vectors are well known in the art. *See, e.g.,* Sambrook *et al.*, 2001, *Molecular Cloning -- A Laboratory Manual*, 3rd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, and Ausubel *et al.*, eds., Current Edition, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY.

[0125] Useful promoters for use in expression vectors include, but are not limited to, a metallothionein promoter, a constitutive adenovirus major late promoter, a dexamethasone-inducible MMTV promoter, a SV40 promoter, a MRP pol III promoter, a constitutive MPSV promoter, a tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), a constitutive CMV promoter, and an interferon-responsive promoter.

[0126] The expression vectors should contain expression and replication signals compatible with the cell in which the SPRT protease is expressed. Suitable expression vectors include, but are not limited to, viral vectors such as retroviruses, adenoviruses and adenoassociated viruses, plasmid vectors, cosmids, and the like. Viral and plasmid vectors are preferred for transfecting the expression vectors into mammalian cells. For example, the expression vector pcDNA1 (Invitrogen, San Diego, CA), in which the expression control sequence comprises the CMV promoter, provides good rates of transfection and expression into such cells. Further examples of expression vectors that can be used are provided in the Examples, below.

[0127] The expression vectors can be introduced into a cell by any method known to one of skill in the art without limitation. Such methods include, but are not limited to, *e.g.*, direct uptake of the molecule by a cell from solution; facilitated uptake through lipofection using, *e.g.*, liposomes or immunoliposomes; particle-mediated transfection; *etc.* *See, e.g.,* U.S. Patent No. 5,272,065; Goeddel *et al.*, eds, 1990, *Methods in Enzymology*, vol. 185, Academic Press, Inc., CA; Krieger, 1990, *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, NY; Sambrook *et al.*, 1989, *Molecular Cloning -- A Laboratory Manual*,

Cold Spring Harbor Laboratory, NY; and Ausubel *et al.*, eds., Current Edition, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY.

[0128] The expression vectors can also contain a purification moiety that simplifies isolation of the delivery construct. For example, a polyhistidine moiety of, *e.g.*, six histidine residues, can be incorporated at the amino terminal end of the protein. The polyhistidine moiety allows convenient isolation of the protein in a single step by nickel-chelate chromatography. In certain embodiments, the purification moiety can be cleaved from the remainder of the delivery construct following purification. In other embodiments, the moiety does not interfere with the function of the functional domains of the delivery construct and thus need not be cleaved.

5.5 Additional Methods for Manipulation of Nucleic Acids and Proteins

[0129] In the context of the invention, nucleic acids, including viral nucleic acids, nucleic acids encoding a protease or pro-protease, and the like, can be manipulated according to well known molecular biology techniques. Detailed protocols for numerous such procedures, including amplification, cloning, mutagenesis, transformation, and the like, are described in, *e.g.*, in Ausubel *et al.* Current Protocols in Molecular Biology (supplemented through 2006) John Wiley & Sons, New York ("Ausubel"); Sambrook *et al.* Molecular Cloning - A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2001 ("Sambrook"), and Berger and Kimmel Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152, Academic Press, Inc., San Diego, CA ("Berger").

[0130] In addition to the above references, protocols for in vitro amplification techniques, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), Q β -replicase amplification, and other RNA polymerase mediated techniques (*e.g.*, NASBA), useful *e.g.*, for amplifying cDNA probes of the invention, are found in Mullis *et al.* (1987) U.S. Patent No. 4,683,202; PCR Protocols A Guide to Methods and Applications (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) ("Innis"); Arnheim and Levinson (1990) C&EN 36; The Journal Of NIH Research (1991) 3:81; Kwoh *et al.* (1989) Proc Natl Acad Sci USA 86, 1173; Guatelli *et al.* (1990) Proc Natl Acad Sci USA 87:1874; Lomell *et al.* (1989) J Clin Chem 35:1826; Landegren *et al.* (1988) Science 241:1077; Van Brunt (1990) Biotechnology 8:291; Wu and Wallace (1989) Gene 4: 560; Barringer *et al.* (1990) Gene 89:117, and Sooknanan and Malek (1995) Biotechnology 13:563. Additional methods, useful for cloning nucleic acids in the context of the present invention, include Wallace *et al.* U.S. Pat. No.

5,426,039. Improved methods of amplifying large nucleic acids by PCR are summarized in Cheng *et al.* (1994) Nature 369:684 and the references therein.

[0131] Certain polynucleotides of the invention, *e.g.*, oligonucleotides can be synthesized utilizing various solid-phase strategies including mononucleotide- and/or trinucleotide-based phosphoramidite coupling chemistry. For example, nucleic acid sequences can be synthesized by the sequential addition of activated monomers and/or trimers to an elongating polynucleotide chain. *See e.g.*, Caruthers, M.H. *et al.* (1992) Meth Enzymol 211:3.

[0132] In lieu of synthesizing the desired sequences, essentially any nucleic acid can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company, The Great American Gene Company, ExpressGen, Inc., Operon Technologies, Inc., and many others.

[0133] In addition, substitutions of selected amino acid residues in viral polypeptides, proteases, or pro-proteases, can be accomplished by, *e.g.*, site directed mutagenesis. For example, viral polypeptides with amino acid substitutions functionally correlated with desirable phenotypic characteristic, *e.g.*, an attenuated phenotype, cold adaptation, temperature sensitivity, cleavage by a particular protease, *etc.* can be produced by introducing specific mutations into a viral nucleic acid segment encoding the polypeptide. Methods for site directed mutagenesis are well known in the art, and described, *e.g.*, in Ausubel, Sambrook, and Berger, *supra*. Numerous kits for performing site directed mutagenesis are commercially available, *e.g.*, the Chameleon Site Directed Mutagenesis Kit (Stratagene, La Jolla), and can be used according to the manufacturers instructions to introduce, *e.g.*, one or more amino acid substitutions into a genome segment encoding a influenza A or B polypeptide, respectively, or into a nucleic acid encoding a protease or pro-protease.

5.6 Use of Cells Expressing a Heterologous Protease to Culture Influenza

[0134] Methods for replicating influenza viruses in cell culture are known to one of skill in the art (*See*, section entitled "Culturing Cells Expressing a Heterologous Protease," *supra*). Typically, these methods involve the infection of suitable host cells with a selected strain of virus. Alternatively, recombinant methods are used to introduce the viral genome into the host (*e.g.*, plasmid rescue detailed in section entitled "Influenza Genomic Vectors in Host Cells Expressing a Protease or Pro-Protease," *infra*). As discussed extensively above, exogenous proteases have typically been added to culture medium to efficiently produce influenza viruses in cell culture for cells that do not express a protease that efficiently cleaves

the HA0 protein (see, *e.g.*, Appleyard, et al., 1974, J.Gen Virol. 25:351-357; U.S. Patents 5,824,536; 4,500,513; and Patent Publication WO 96/15232). One of the objects of the present invention is to provide a cell that expresses a heterologous protease to replicate influenza, and in a specific embodiment, improve the efficiency of influenza infection to increase viral titers in cell culture.

[0135] Accordingly, in certain embodiments, exogenous protease, *e.g.*, porcine trypsin, is not added to the cell culture medium in which viruses, *e.g.*, influenza viruses, are to be replicated. In certain embodiments, the titer of the influenza virus yielded from a cell culture expressing a heterologous protease without exogenous protease is equal or substantially equal to the titer that would be achieved from a cell culture not expressing a heterologous protease with the addition of exogenous protease. In other embodiments, the titer of the influenza virus yielded from a cell culture expressing a heterologous protease without exogenous protease is greater than the titer that would be achieved from a cell culture not expressing a heterologous protease with the addition of exogenous protease. In still other embodiments, a cell culture expressing a heterologous protease is capable of propagating influenza virus to a commercially reasonable titer ($>10^7$ Log TCID₅₀/mL).

[0136] In yet other embodiments, exogenous protease, *e.g.*, porcine trypsin or SPRT protease, can be added to a cell culture comprising cells of the invention in which viruses, *e.g.*, influenza viruses, are to be replicated. Such embodiments are useful, for example, to culture viruses in cells of the invention that express a protease at low levels, *e.g.*, levels insufficient to propagate the virus to commercially reasonable titer. Such embodiments are also useful in methods where the cells of the invention expresses a pro-protease that is activated by proteolytic cleavage.

[0137] In one embodiment, methods of generating infectious viral particles of a negative-strand RNA virus in cultured cells are provided, wherein no exogenous protease, *e.g.*, trypsin, is added to the cell culture medium. In a specific embodiment, methods of replicating negative-strand RNA viruses to a titer ($\log_{10}$ TCID₅₀/mL) of at least about 6.0, or at least 6.2, or at least 6.4, or at least 6.6, or at least 6.8, or at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8, or at least 9.0, or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8 in a population of cells is provided, wherein no exogenous protease is added to the cell culture medium.

[0138] In certain embodiments, the titer ($\log_{10}$ TCID₅₀/mL) of influenza virus yielded from a cell culture of cells expressing a heterologous protease without exogenous protease is at least 6.0, or at least 6.2, or at least 6.4, or at least 6.6, or at least 6.8, or at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8, or at least 9.0, or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8.

[0139] In a specific embodiment, the titer of the influenza virus yielded from a cell culture of cells expressing a heterologous protease without exogenous protease has a $\log_{10}$ TCID₅₀/mL that is at least about 0.1, or at least about 0.2, or at least about 0.3, or at least about 0.4, or at least about 0.5, or at least about 0.6, or at least about 0.7, or at least about 0.8, or at least about 0.9, or at least about 1.0, or at least about 1.2, or at least about 1.4, or at least about 1.6, or at least about 1.8, or at least about 2.0, or at least about 2.2, or at least about 2.4, or at least about 2.6, or at least about 2.6, or at least about 2.8, or at least about 3.0, or at least about 3.2, or at least about 3.4, or at least about 3.6, or at least about 3.8, or at least about 4.0, or at least about 4.2, or at least about 4.4, or at least about 4.6, or at least about 4.8, or at least about 5.0, greater than the titer of influenza virus produced in a culture of corresponding cells that do not express a heterologous protease and to which no exogenous protease, *e.g.*, trypsin has been added.

[0140] In other embodiments, less protease than would otherwise be added to the cell culture medium can be added to the cell culture medium. Accordingly, in certain embodiments, methods of generating infectious viral particles of a negative-strand RNA virus in cultured cells are provided, wherein a minimal amount of exogenous protease, *e.g.*, trypsin, is added to the cell culture medium are provided. In a specific embodiment, methods of replicating negative-strand RNA viruses to a titer ($\log_{10}$ TCID₅₀/mL) of at least about 6.0, or at least 6.2, or at least 6.4, or at least 6.6, or at least 6.8, or at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8, or at least 9.0, or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8 in a population of cells are provided, wherein about 0.1 ng/ml to about 100 μ g/ml exogenous protease is added to the cell culture medium.

[0141] In another a specific embodiment, methods of replicating negative-strand RNA viruses to a titer ($\log_{10}$ TCID₅₀/mL) of at least about 6.0, or at least 6.2, or at least 6.4, or at least 6.6, or at least 6.8, or at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least

7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8, or at least 9.0 , or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8 in a population of cells are provided, wherein about 1 mU/ml to about 5000 mU/ml exogenous protease is added to the cell culture medium.

5 [0142] In still another embodiment, the titer ($\log_{10}$ TCID₅₀/mL) of influenza virus yielded from a cell culture of cells expressing a heterologous protease, to which about 0.1 ng/ml to about 100 μ g/ml of exogenous protease has been added, is at least 6.0, or at least 6.2, or at least 6.4, or at least 6.6, or at least 6.8, or at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8,
10 or at least 9.0 , or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8. In a specific embodiment, the titer ($\log_{10}$ TCID₅₀/mL) of influenza virus yielded from a cell culture of cells expressing a heterologous protease, to which about 0.1 ng/ml to about 10 μ g/ml of exogenous protease has been added, is at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8, or at least
15 9.0 , or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8. In another specific embodiment, the titer ($\log_{10}$ TCID₅₀/mL) of influenza virus yielded from a cell culture of cells expressing a heterologous protease, to which about 0.1 ng/ml to about 1.0 μ g/ml of exogenous protease has been added, is at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8,
20 or at least 9.0 , or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8.

[0143] In certain embodiments, the titer ($\log_{10}$ TCID₅₀/mL) of influenza virus yielded from a cell culture of cells expressing a heterologous protease, to which about 1 mU/ml to about 5000 mU/ml of exogenous protease has been added, is at least 6.0, or at least 6.2, or at least 6.4, or at least 6.6, or at least 6.8, or at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6,
25 or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8, or at least 9.0 , or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8. In a specific embodiment, the titer ($\log_{10}$ TCID₅₀/mL) of influenza virus yielded from a cell culture of cells expressing a heterologous protease, to which about 1 mU/ml to about 1000 mU/ml of exogenous protease has been added, is at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8,
30 or at least 9.0 , or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8. In another specific embodiment, the titer ($\log_{10}$ TCID₅₀/mL) of influenza virus yielded from a cell culture of cells expressing a heterologous protease, to which about 1 mU/ml to about 500 mU/ml of

exogenous protease has been added, is at least 7.0, or at least 7.2, or at least 7.4, or at least 7.6, or at least 7.8, or at least 8.0, or at least 8.2, or at least 8.4, or at least 8.6, or at least 8.8, or at least 9.0, or at least 9.2, or at least 9.4, or at least 9.6, or at least 9.8.

[0144] In certain embodiments, the titer of virus is obtained after incubation of the cells for about about 2 days to about 10 days, or optionally about 3 days to about 7 days. In a specific embodiment, the titer of virus is obtained after incubation of the cells for 2 days, or 3 days, or 4 days, or 5 days, or after 6 days, or 7 days, or 8 days, or 9 days, or 10 days, or 12 days, or 14 days.

[0145] In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of less than about 0.1 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 0.1 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 0.5 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 1 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 5 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 10 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 50 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 100 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 250 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 500 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 750 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 1 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 5 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 10 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 25 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 50 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration of about 100 µg/ml.

[0146] In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 0.01 ng/ml and about 100 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 1 ng/ml and about 100 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 10 ng/ml and about 100 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 100 ng/ml and about 100 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 1 µg/ml and about 100 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 10 µg/ml and about 100 µg/ml.

[0147] In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 0.01 ng/ml and about 10 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 0.01 ng/ml and about 1 µg/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 0.01 ng/ml and about 100 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 0.01 ng/ml and about 10 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 0.01 ng/ml and about 1 ng/ml. In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 0.01 ng/ml and about 0.1 ng/ml.

[0148] In certain embodiments, exogenous protease is added to the cell culture medium to a final concentration between about 1 to 5000 mU/ml, or 5 to 1000 mU/ml, or 100 to 500 mU/ml. In one embodiment, exogenous protease is added to the cell culture medium to a final concentration of less than about 1 mU/ml. In one embodiment, exogenous protease is added to the cell culture medium to a final concentration of less than about 5 mU/ml. In one embodiment, exogenous protease is added to the cell culture medium to a final concentration of less than about 10 mU/ml. In one embodiment, exogenous protease is added to the cell culture medium to a final concentration of less than about 25 mU/ml. In one embodiment, exogenous protease is added to the cell culture medium to a final concentration of less than about 50 mU/ml. In one embodiment, exogenous protease is added to the cell culture medium to a final concentration of less than about 100 mU/ml. In one embodiment, exogenous

protease is added to the cell culture medium to a final concentration of less than about 250 mU/ml. In one embodiment, exogenous protease is added to the cell culture medium to a final concentration of less than about 500 mU/ml. In one embodiment, exogenous protease is added to the cell culture medium to a final concentration of less than about 1000 mU/ml.

5 [0149] These embodiments where less protease than would typically be required for efficient production of influenza virus is added to the culture medium are particularly useful when the cell cultured expresses a pro-protease. Addition of the exogenous protease to the culture medium can “prime” the pro-protease by cleaving off the protective pro-peptide, converting the pro-protease into its active form. Thereafter, the activated protease can cleave pro-
10 proteases subsequently expressed by the cell, thereby converting these newly produced pro-proteases to active proteases. Such embodiments thus allow greatly reduced usage of exogenously added proteases, such as porcine trypsin.

[0150] Accordingly, in certain embodiments, exogenous protease is added to the cell culture in a single application. In certain embodiments, the exogenous protease is added at or near
15 the same time that the virus is used to infect the cells in the cell culture. In certain embodiments, the exogenous protease is added on the same day that the virus is used to infect the cells in the cell culture. In other embodiments, the exogenous protease is added prior to the addition of the virus used to infect the cells in the cell culture. In certain embodiments, the exogenous protease is added at or near the same time that vectors are introduced into the
20 cells in the cell culture for production of the virus. In certain embodiments, the exogenous protease is added on the same day that vectors are introduced into the cells in the cell culture for production of the virus. In other embodiments, the exogenous protease is added prior to the addition of the vectors that are introduced into the cells in the cell culture for production of the virus.

25 [0151] Alternatively, in certain embodiments, the cell can express both a protease and a pro-protease. In certain of such embodiments, expression of the protease can be under the control of an inducible promoter to permit the transient expression of the protease. Such embodiments allow the protease to be temporarily expressed, thereby activating the pro-protease. Thereafter, the activated pro-protease can continue to activate subsequently
30 expressed pro-protease. Alternately, in certain embodiments, the cell can express two or more proteases or pro-proteases. In certain embodiments, the two or more pro-proteases can be expressed under the same or different regulatory systems, *e.g.*, constitutive or inducible

expression. For inducible expression embodiments, the inducible system selected can be the same or different for each inducibly expressible protease or pro-protease.

[0152] In certain embodiments, it is useful to temporally control the expression of the heterologous protease in the cell culture. For example, it may prove useful to induce expression of the protease at a defined time, *e.g.*, about 1, 2, 3, 4, or 5 days following infection of the cell culture with an influenza virus. In other embodiments, the expression of the protease is induced prior to infection of the cell culture with an influenza virus. In still other embodiments, the protease is induced at the same time as infection of the cell culture with an influenza virus. In yet other embodiments, the protease is induced prior to, at the same time or after the addition of the vectors that are introduced into the cells in the cell culture for production of the virus. Thus, in certain embodiments, the heterologous protease can be under the control of an inducible promoter or otherwise under the control of a genetic regulatory element, as described above.

5.7 Influenza Genomic Vectors in Host Cells Expressing a Protease or Pro-Protease

[0153] In addition to cell culture-based methods that rely on infecting the cell culture with live virus, fully infectious influenza viruses can be produced in cell culture using recombinant DNA technology, *e.g.* plasmid rescue. *See, e.g.*, Neumann et al. (1999) *Generation of influenza A virus entirely from cloned cDNAs*. Proc Natl Acad Sci USA 96:9345-9350; Fodor et al. (1999) *Rescue of influenza A virus from recombinant DNA*. J. Virol 73:9679-9682; Hoffmann et al. (2000) *A DNA transfection system for generation of influenza A virus from eight plasmids* Proc Natl Acad Sci USA 97:6108-6113; WO 01/83794; Hoffmann and Webster (2000), *Unidirectional RNA polymerase I-polymerase II transcription system for the generation of influenza A virus from eight plasmids*, 81:2843-2847; Hoffmann et al. (2002), *Rescue of influenza B viruses from 8 plasmids*, 99(17): 11411-11416; U.S. patent nos. 6,649,372 and 6,951,754; U.S. publication nos. 20050003349 and 20050037487, which are incorporated by reference herein.

[0154] In certain embodiments, the methods of the invention comprise introducing a plurality of vectors, each of which encodes a portion of an influenza virus genome into cells expressing a heterologous protease or pro-protease to obtain influenza viruses. The cells expressing a heterologous protease or pro-protease are then cultured under conditions permissive for viral growth, and influenza viruses are recovered. In some embodiments, the

influenza viruses are attenuated viruses, cold adapted viruses and/or temperature sensitive viruses. For example, in certain embodiments, the vector-derived recombinant influenza viruses can be attenuated, cold adapted, temperature sensitive viruses, such as are suitable for administration as a live attenuated vaccine, *e.g.*, in a intranasal vaccine formulation. In an exemplary embodiment, the viruses are produced by introducing a plurality of vectors incorporating all or part of an influenza B/Ann Arbor/1/66 virus genome, *e.g.*, a ca B/Ann Arbor/1/66 virus genome.

[0155] In some embodiments, a plurality of vectors comprising cDNA encoding at least the 6 internal genome segments of one influenza strain and cDNA encoding one or more genome segments (*e.g.*, HA and NA vRNA segments) of a different influenza strain can be introduced into cells expressing a heterologous protease or pro-protease to obtain influenza viruses. For example, at least the 6 internal genome segments (“the backbone”) of an attenuated, cold adapted and/or temperature sensitive influenza A or B strain, *e.g.*, a ca, att, ts strain of B/Ann Arbor/1/66, can be introduced into cells expressing a heterologous protease or pro-protease along with one or more segments encoding immunogenic antigens derived from another virus strain. Typically the immunogenic surface antigens include either or both of the hemagglutinin (HA) and/or neuraminidase (NA) antigens. In embodiments where a single segment encoding an immunogenic surface antigen is introduced, the 7 complementary segments of the selected virus are also introduced into the host cells.

[0156] In some embodiments, a plurality of vectors comprising cDNA encoding 1-7 internal genome segments of one influenza strain and cDNA encoding 1-7 genome segments (*e.g.*, HA and NA vRNA segments) of a different influenza strain can be introduced into cells expressing a heterologous protease or pro-protease to obtain influenza viruses.

[0157] In certain embodiments, expression vectors encoding influenza vRNA are cotransfected into the cells expressing a heterologous protease or pro-protease by electroporation. In certain embodiments, the expression vectors are introduced into cells expressing a heterologous protease or pro-protease by transfection into cells in the presence of a liposomal transfection reagent or by means of calcium phosphate precipitation. In certain embodiments, the expression vectors are plasmids. In certain embodiments, the expression vectors comprise a separate expression vector for expression of each genomic RNA segment of said virus or the corresponding coding RNAs.

[0158] In certain embodiments, a plurality of plasmid vectors encoding each influenza virus vRNA are introduced into a population of host cells. For example, in certain embodiment, 8 plasmids, each of which encodes a different vRNA segment can be utilized to introduce a complete influenza genome into the host cells. In one embodiment, plasmid vectors also
5 encode mRNA of at least one influenza polypeptide. Alternatively, a greater number of plasmids, incorporating smaller genomic subsequences can be employed.

[0159] In accordance with the present invention, viral genomic RNA corresponding to each of the eight genomic segments of influenza can be inserted into a recombinant expression vector for manipulation and production of influenza viruses. A variety of vectors, including
10 viral vectors, plasmids, cosmids, phage, and artificial chromosomes, can be employed in the context of the invention. Typically, for ease of manipulation, the viral genomic segments are inserted into a plasmid vector, providing one or more origins of replication functional in bacterial and eukaryotic cells, and, optionally, a marker convenient for screening or selecting cells incorporating the plasmid sequence. These vectors can then be introduced into cells
15 expressing a heterologous protease or pro-protease to obtain influenza viruses.

[0160] In another embodiment, the invention provides a method for producing a recombinant influenza virus, comprising introducing into cells of the invention a plurality of expression vectors comprising an RNA pol I promoter operably linked to one or more cDNAs encoding each influenza genomic RNA and one or more expression vectors that express viral mRNA
20 that encodes one or more influenza polypeptides: PB2, PB1, PA, HA, NP, NA, M1, M2, and NS2; and isolating, i.e., harvesting said recombinant influenza virus from the cells.

[0161] In one embodiment, the present invention provides for methods of generating infectious recombinant influenza virus in host cells of the invention using expression vectors to express the vRNA segments or corresponding cRNAs and influenza virus proteins, in
25 particular PB1, PB2, PA and NA. In accordance with this embodiment, helper virus may or may not be included to generate the infectious recombinant influenza viruses.

[0162] The present invention provides a method for generating in cultured cells of the invention infectious viral particles of a negative-strand RNA virus, said method comprising:
(a) introducing into a population of said cells a set of expression vectors capable of both
30 expressing in said cells genomic vRNA to provide the complete genomic vRNA of said virus and capable of expressing mRNA encoding one or more polypeptides of said virus; (b) culturing said cells whereby said viral particles are produced. In certain embodiments, the

cells are canine cells. In certain embodiments, the cells are MDCK cells. In certain
embodiments, the virus is influenza B virus. In certain embodiments, the set of expression
vectors is contained in 1-17 plasmids. In certain embodiments, the set of expression vectors
is contained in 1-8 plasmid. In certain embodiments, the set of expression vectors is
5 contained in 1-3 plasmids. In certain embodiments, the sets of expression vectors are
introduced by electroporation. In certain embodiments, the set of expression vectors encode
each vRNA segment of an influenza virus. In certain embodiments, the set of expression
vectors encode the mRNA of one or more influenza polypeptide. In certain embodiments, the
set of expression vectors encode each vRNA segment of an influenza virus and the mRNA of
10 one or more influenza polypeptide. In certain embodiments, the set of expression vectors
encode a vRNA or mRNA of a second virus. For instance, the set of vectors comprises one
or more vectors encoding the HA and/or NA mRNA and/or vRNA of a second influenza
virus. In one embodiment, helper virus is used in the method. In one embodiment, the
cultured cells used in the method are canine cells.

15 **[0163]** The present invention further provides a method for generating in cultured cells of the
invention infectious recombinant viral particles of a negative-strand RNA virus, said method
comprising: (a) introducing into a population of said cells a first set of expression vectors
capable of expressing in said cells genomic vRNA to provide the complete genomic vRNA of
said virus; (b) introducing into said cells a second set of expression vectors capable of
20 expressing mRNA encoding one or more polypeptides of said virus; and (c) culturing said
cells whereby said viral particles are produced. In certain embodiments, the cells are canine
cells. In certain embodiments, the cells are MDCK cells. In certain embodiments, the virus
is influenza B virus. In certain embodiments, the first set of expression vectors is contained
in 1-8 plasmids. In certain embodiments, the first set of expression vectors is contained in
25 one plasmid. In certain embodiments, the second set of expression vectors is contained in 1-8
plasmids. In certain embodiments, the second set of expression vectors is contained in one
plasmid. In certain embodiments, the first, second, or both sets of expression vectors are
introduced by electroporation. In certain embodiments, the first set of expression vectors
encode each vRNA segment of an influenza virus. In certain embodiments, the second set of
30 expression vectors encode the mRNA of one or more influenza polypeptide. In certain
embodiments, the first set or second set of expression vectors (or both sets) comprise a
nucleic acid of the invention. In one embodiment, helper virus is used in the method.

[0164] The present invention also provides a method for generating in cultured cells of the invention infectious recombinant viral particles of a segmented negative-strand RNA virus having greater than 3 genomic vRNA segments, for example an influenza virus such as an influenza A virus, said method comprising: (a) introducing into a population of said cells a first set of expression vectors capable of expressing in said cells genomic vRNA segments to provide the complete genomic vRNA segments of said virus; (b) introducing into said cells a second set of expression vectors capable of expressing mRNA encoding one or more polypeptides of said virus; and (c) culturing said cells whereby said viral particles are produced. In certain embodiments, the cells are canine cells. In certain embodiments, the cells are MDCK cells. In certain embodiments, the recombinant virus is influenza A or B virus. In certain embodiments, the first set of expression vectors is contained in 1-8 plasmids. In certain embodiments, the first set of expression vectors is contained in one plasmid. In certain embodiments, the second set of expression vectors is contained in 1-8 plasmids. In certain embodiments, the second set of expression vectors is contained in one plasmid. In certain embodiments, the first, second, or both sets of expression vectors are introduced by electroporation. In certain embodiments, the first set of expression vectors encode each vRNA segment of an influenza virus. In certain embodiments, the second set of expression vectors encode the mRNA of one or more or all influenza polypeptides. In certain embodiments, the first set or second set of expression vectors (or both sets) encode a vRNA or mRNA of a second virus. For instance, a set of vectors comprises one or more vectors encoding the HA and/or NA mRNA and/or vRNA of a second influenza virus. In one embodiment, helper virus is used in the method.

[0165] The present invention further provides a method for generating in cultured cells of the invention infectious recombinant viral particles of a segmented negative-strand RNA virus having greater than 3 genomic vRNA segments, for example an influenza virus such as an influenza A virus, said method comprising: (a) introducing into a population of said cells a set of expression vectors capable of both expressing in said cells genomic vRNA segments to provide the complete genomic vRNA segments of said virus and capable of expressing mRNA encoding one or more polypeptides of said virus; (b) culturing said cells whereby said viral particles are produced. In certain embodiments, the cells are canine cells. In certain embodiments, the cells are MDCK cells. In certain embodiments, the virus is influenza A or B virus. In certain embodiments, the set of expression vectors is contained in 1-17 plasmids. In certain embodiments, the set of expression vectors is contained in 1-8 plasmid. In certain

embodiments, the set of expression vectors is contained in 1-3 plasmids. In certain embodiments, the set of expression vectors is contained in one plasmid. In certain embodiments, the sets of expression vectors are introduced by electroporation. In certain embodiments, the set of expression vectors encode each vRNA segment of an influenza virus.

5 In certain embodiments, the set of expression vectors encode the mRNA of one or more influenza polypeptide. In certain embodiments, the set of expression vectors encode each vRNA segment of an influenza virus and the mRNA of one or more influenza polypeptide. In certain embodiments, the set of expression vectors comprise a nucleic acid of the invention. In certain embodiments, the set of expression vectors encode a vRNA or mRNA of
10 a second virus. For instance, the set of vectors comprises one or more vectors encoding the HA and/or NA mRNA and/or vRNA of a second influenza virus. In certain embodiments, the first set or second set of expression vectors (or both sets) encode a vRNA or mRNA of a second virus. For instance, a set of vectors comprises one or more vectors encoding the HA and/or NA mRNA and/or vRNA of a second influenza virus. In one embodiment, helper virus
15 is used in the method.

[0166] The plasmid expression vectors may be bi-directional expression vectors capable of initiating transcription of the inserted viral genomic segment in either direction, that is, giving rise to both (+) strand and (-) strand viral RNA molecules. To effect bi-directional transcription, each of the viral genomic segments is inserted into a vector having at least two
20 independent promoters, such that copies of viral genomic RNA are transcribed by a first RNA polymerase promoter (*e.g.*, a RNA pol I promoter), from one strand, and viral mRNAs are synthesized from a second RNA polymerase promoter (*e.g.*, a RNA Pol II promoter or other promoter that can initiate transcription by RNA pol II in cells). Accordingly, the two promoters can be arranged in opposite orientations flanking at least one cloning site (*i.e.*, a
25 restriction enzyme recognition sequence) preferably a unique cloning site, suitable for insertion of viral genomic RNA segments. Alternatively, an “ambisense” vector can be employed in which the (+) strand mRNA and the (-) strand viral RNA (as a cRNA) are transcribed from the same strand of the vector.

[0167] To ensure the correct 3' end of each expressed vRNA or cRNA, each vRNA or cRNA
30 expression vector can incorporate a ribozyme sequence or appropriate terminator sequence downstream of the RNA coding sequence. This may be, for example, the hepatitis delta virus genomic ribozyme sequence or a functional derivative thereof, or the murine rDNA terminator sequence (Genbank Accession Number M12074). Alternatively, for example, a

Pol I terminator may be employed (Neumann *et al.*, 1994, Virology 202:477-479). The RNA expression vectors may be constructed in the same manner as the vRNA expression vectors described in Pleschka *et al.*, 1996, J. Virol. 70:4188-4192; Hoffmann and Webster, 2000, J. Gen Virol. 81:2843-2847; Hoffmann *et al.*, 2002, Vaccine 20:3165-3170; Fodor *et al.*, 1999, J. Virol. 73:9679-9682; Neumann *et al.*, 1999, P.N.A.S.USA 96:9345-9350; and Hoffmann *et al.*, 2000, Virology 267:310-317; U.S. patent nos. 6,649,372 and 6,951,754; U.S. publication nos. 20050003349 and 20050037487, each of which is hereby incorporated by reference in its entirety.

[0168] In other systems, the viral sequences transcribed by the pol I and pol II promoters can be transcribed from different vectors. In these embodiments, vectors encoding each of the viral genomic segments under the control of a pol I promoter and vectors encoding at least PA, PB1, PB2, and NP under the control of a pol II promoter can be used.

[0169] In either case, with regard to the pol II promoter, the influenza virus genome segment to be expressed can be operably linked to an appropriate transcription control sequence (promoter) to direct mRNA synthesis. A variety of promoters are suitable for use in expression vectors for regulating transcription of influenza virus genome segments. In certain embodiments, the cytomegalovirus (CMV) DNA dependent RNA Polymerase II (Pol II) promoter is utilized. If desired, *e.g.*, for regulating conditional expression, other promoters can be substituted which induce RNA transcription under the specified conditions, or in the specified tissues or cells. Numerous viral and mammalian, *e.g.*, human promoters are available, or can be isolated according to the specific application contemplated. For example, alternative promoters obtained from the genomes of animal and human viruses include such promoters as the adenovirus (such as Adenovirus 2), papilloma virus, hepatitis-B virus, and polyoma virus, and various retroviral promoters. Mammalian promoters include, among many others, the actin promoter, immunoglobulin promoters, heat-shock promoters, and the like. In a specific embodiment, the regulatory sequence comprises the adenovirus 2 major late promoter linked to the spliced tripartite leader sequence of human adenovirus 2, as described by Berg *et al.*, Bio Techniques 14:972-978. In addition, bacteriophage promoters can be employed in conjunction with the cognate RNA polymerase, *e.g.*, the T7 promoter.

[0170] Expression vectors used to express viral proteins, in particular viral proteins for RNP complex formation, will preferably express viral proteins homologous to the desired virus. The expression of viral proteins by these expression vectors may be regulated by any

regulatory sequence known to those of skill in the art. The regulatory sequence may be a constitutive promoter, an inducible promoter or a tissue-specific promoter.

[0171] Further examples of promoters which may be used to control the expression of viral proteins in protein expression vectors include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Kamaroff et al., 1978, *Proc. Natl. Acad. Sci. USA* 75:3727-3731), or the tac promoter (DeBoer et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:21-25); *see also* "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., *Nature* 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, *Nucl. Acids Res.* 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, *Nature* 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, *Cell* 38:639-646; Omitz et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409; MacDonald, 1987, *Hepatology* 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, *Cell* 38:647-658; Adames et al., 1985, *Nature* 318:533-538; Alexander et al., 1987, *Mol. Cell. Biol.* 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, *Cell* 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, *Genes and Devel.* 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, *Mol. Cell. Biol.* 5:1639-1648; Hammer et al., 1987, *Science* 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, *Genes and Devel.* 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogam et al., 1985, *Nature* 315:338-340; Kollias et al., 1986, *Cell* 46:89-94; myelin basic protein gene control

region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

5 [0172] In a specific embodiment, the protein expression vector comprises a promoter operably linked to a nucleic acid sequence, one or more origins of replication, and, optionally, one or more selectable markers (e.g., an antibiotic resistance gene). In another embodiment, a protein expression vector that is capable of producing bicistronic mRNA may be produced by inserting bicistronic mRNA sequence. Certain internal ribosome entry site
10 (IRES) sequences may be utilized. Preferred IRES elements include, but are not limited to the mammalian BiP IRES and the hepatitis C virus IRES.

[0173] Expression vectors containing gene inserts can be identified, e.g., by three general approaches: (a) nucleic acid hybridization; (b) presence or absence of "marker" gene functions; and (c) expression of inserted sequences. In the first approach, the presence of the
15 viral gene inserted in an expression vector(s) can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to the inserted gene(s). In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., resistance to antibiotics or transformation phenotype) caused by the insertion of the gene(s) in the vector(s). In the third
20 approach, expression vectors can be identified by assaying the gene product expressed. Such assays can be based, for example, on the physical or functional properties of the viral protein in *in vitro* assay systems, e.g., binding of viral proteins to antibodies.

[0174] In a specific embodiment, one or more protein expression vectors encode and express the viral proteins necessary for the formation of RNP complexes. In another embodiment, one
25 or more protein expression vectors encode and express the viral proteins necessary to form viral particles. In yet another embodiment, one or more protein expression vectors encode and express the all of the viral proteins of a particular negative-strand RNA virus.

[0175] Transcription can optionally be increased by including an enhancer sequence. Enhancers are typically short, e.g., 10-500 bp, cis-acting DNA elements that act in concert
30 with a promoter to increase transcription. Many enhancer sequences have been isolated from mammalian genes (hemoglobin, elastase, albumin, alpha.-fetoprotein, and insulin), and eukaryotic cell viruses. The enhancer can be spliced into the vector at a position 5' or 3' to

the heterologous coding sequence, but is typically inserted at a site 5' to the promoter.

Typically, the promoter, and if desired, additional transcription enhancing sequences are chosen to optimize expression in the host cell type into which the heterologous DNA is to be introduced (Scharf et al. (1994) *Heat stress promoters and transcription factors* Results Probl Cell Differ 20:125-62; Kriegler et al. (1990) *Assembly of enhancers, promoters, and splice signals to control expression of transferred genes* Methods in Enzymol 185: 512-27).

Optionally, the amplicon can also contain a ribosome binding site or an internal ribosome entry site (IRES) for translation initiation.

[0176] The vectors can also include sequences for the termination of transcription and for stabilizing the mRNA, such as a polyadenylation site or a terminator sequence. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. In some embodiments, the SV40 polyadenylation sequences provide a polyadenylation signal.

[0177] In addition, as described above, the expression vectors optionally include one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells, in addition to genes previously listed, markers such as dihydrofolate reductase or neomycin resistance are suitable for selection in eukaryotic cell culture.

[0178] The vector containing the appropriate DNA sequence as described above, as well as an appropriate promoter or control sequence, can be employed to transform a host cell permitting expression of the protein. While the vectors can be replicated in bacterial cells, most frequently it will be desirable to introduce them into mammalian cells, e.g., Vero cells, BHK cells, MDCK cell, 293 cells, COS cells, for the purpose of expression. In a specific embodiment, MDCK cells are used for the purpose of expression.

[0179] The expression vectors can be used to directing the expressing of genomic vRNA(s) or corresponding cRNA(s) which have one or more mutations. These mutations may result in the attenuation of the virus. For example, the vRNA segments may be the vRNA segments of an influenza A virus having an attenuated base pair substitution in a pan-handle duplex promoter region, in particular, for example, the known attenuating base pair substitution of A for C and U for G at position 11-12' in the duplex region of the NA-specific vRNA (Fodor et al., 1998, J. Virol. 69:23-6290). By using the methods to produce recombinant negative-strand RNA virus, new attenuating mutations may be identified.

[0180] Further, any of the expression vectors described in U.S. Patent Nos. 6,951,754, 6,887,699, 6,649,372, 6,544,785, 6,001,634, 5,854,037, 5,824,536, 5,840,520, 5,820,871, 5,786,199, and 5,166,057 and U.S. Patent Application Publication Nos. 20060019350, 20050158342, 20050037487, 20050266026, 20050186563, 20050221489, 20050032043, 20040142003, 20030035814, and 20020164770 can be used in accordance with the present invention.

[0181] Still further, the expression vectors can also be used to make chimeric viruses that express sequences heterologous to a viral genome. Expression vectors directing the expression of vRNA(s) or corresponding cRNA(s) and introduced into host cells along with expression vectors direct the expression of viral proteins to generate novel infectious recombinant negative-strand RNA viruses or chimeric viruses. Heterologous sequences which may be engineered into these viruses include antisense nucleic acids and nucleic acid such as a ribozyme. Alternatively, heterologous sequences which express a peptide or polypeptide may be engineered into these viruses. Heterologous sequences encoding the following peptides or polypeptides may be engineered into these viruses include: 1) antigens that are characteristic of a pathogen; 2) antigens that are characteristic of autoimmune disease; 3) antigens that are characteristic of an allergen; and 4) antigens that are characteristic of a tumor. For example, heterologous gene sequences that can be engineered into the chimeric viruses of the invention include, but are not limited to, epitopes of human immunodeficiency virus (HIV) such as gp160; hepatitis B virus surface antigen (HBsAg); the glycoproteins of herpes virus (e.g., gD, gE); VP1 of poliovirus; and antigenic determinants of nonviral pathogens such as bacteria and parasites to name but a few.

[0182] Yet further, the methods of the present invention may be modified to incorporate aspects of methods known to those skilled in the art, in order to improve efficiency of rescue of infectious viral particles. For example, the reverse genetics technique involves the preparation of synthetic recombinant viral RNAs that contain the non-coding regions of the negative strand virus RNA which are essential for the recognition by viral polymerases and for packaging signals necessary to generate a mature virion. The recombinant RNAs are synthesized from a recombinant DNA template and reconstituted in vitro with purified viral polymerase complex to form recombinant ribonucleoprotein (RNPs) which can be used to transect cells. A more efficient transfection is achieved if the viral polymerase proteins are present during transcription of the synthetic RNAs either in vitro or in vivo. The synthetic recombinant RNPs can be rescued into infectious virus particles. The foregoing techniques

are described in U.S. Pat. No. 5,166,057 issued Nov. 24, 1992; in U.S. Pat. No. 5,854,037 issued Dec. 29, 1998; in U.S. Pat. No. 5,789,229 issued Aug. 4, 1998; in European Patent Publication EP 0702085A1, published Feb. 20, 1996; in U.S. Pat. application Ser. No. 09/152,845; in International Patent Publications PCR WO97/12032 published Apr. 3, 1997; WO96/34625 published Nov. 7, 1996; in European Patent Publication EP-A780475; WO99/02657 published Jan. 21, 1999; WO98/53078 published Nov. 26, 1998; WO98/02530 published Jan. 22, 1998; WO99/15672 published Apr. 1, 1999; WO98/13501 published Apr. 2, 1998; WO97/06720 published Feb. 20, 1997; and EPO 780 47SA1 published Jun. 25, 1997, each of which is incorporated by reference herein in its entirety.

5 [0183] Vectors comprising influenza genome segments can be introduced (*e.g.*, transfected) into host cells of the invention according to methods well known in the art for introducing heterologous nucleic acids into eukaryotic cells, including, *e.g.*, calcium phosphate co-precipitation, electroporation, microinjection, lipofection, and transfection employing polyamine transfection reagents. For example, vectors, *e.g.*, plasmids, can be transfected into 10 host cells, such as COS cells, 293T cells, MDCK cells, or combinations of COS or 293T cells and MDCK cells, using the polyamine transfection reagent TransIT-LT1 (Mirus) according to the manufacturer's instructions. Approximately 1 µg of each vector to be introduced into the population of host cells with approximately 2 µl of TransIT-LT1 diluted in 160 µl medium, (*e.g.*, serum-free medium), in a total vol. of 200 µl. The DNA:transfection reagent mixtures 15 are incubated at room temperature for 45 min followed by addition of 800 µl of medium. The transfection mixture is added to the host cells, and the cells are cultured as described above. Accordingly, for the production of recombinant or reassortant viruses in cell culture, vectors incorporating each of the 8 genome segments, (PB2, PB1, PA, NP, M, NS, HA and NA) are mixed with approximately 20 µl TransIT-LT1 and transfected into host cells. Optionally, 20 serum-containing medium is replaced prior to transfection with serum-free medium, *e.g.*, Opti-MEM I, and incubated for 4-6 hours.

[0184] Alternatively, electroporation can be employed to introduce vectors incorporating influenza genome segments into host cells of the invention. For example, plasmid vectors incorporating an influenza A or influenza B virus are favorably introduced into Vero cells 30 using electroporation according to the following procedure. In brief, 5×10^6 Vero cells, *e.g.*, grown in Modified Eagle's Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS) are resuspended in 0.4 ml OptiMEM and placed in an electroporation cuvette. Twenty micrograms of DNA in a volume of up to 25 µl is added to the cells in the cuvette, which is

then mixed gently by tapping. Electroporation is performed according to the manufacturer's instructions (e.g., BioRad Gene Pulser II with Capacitance Extender Plus connected) at 300 volts, 950 microFarads with a time constant of between 28-33 msec. The cells are remixed by gently tapping and approximately 1-2 minutes following electroporation 0.7 ml MEM
5 with 10% FBS is added directly to the cuvette. The cells are then transferred to two wells of a standard 6 well tissue culture dish containing 2 ml MEM, 10% FBS or OPTI-MEM without serum. The cuvette is washed to recover any remaining cells and the wash suspension is divided between the two wells. Final volume is approximately 3.5 mls. The cells are then incubated under conditions permissive for viral growth, e.g., at approximately 33 °C for cold
10 adapted strains.

5.8 Recovery of Viruses from Cell Culture

[0185] Viruses can be typically recovered from the culture medium, in which infected (transfected) cells have been grown. Typically crude medium is clarified prior to concentration of influenza viruses. Common methods include filtration, ultrafiltration,
15 adsorption on barium sulfate and elution, and centrifugation. For example, crude medium from infected cultures can first be clarified by centrifugation at, e.g., 1000-2000 x g for a time sufficient to remove cell debris and other large particulate matter, e.g., between 10 and 30 minutes. Alternatively, the medium is filtered through a 0.8 µm cellulose acetate filter to remove intact cells and other large particulate matter. Optionally, the clarified medium
20 supernatant is then centrifuged to pellet the influenza viruses, e.g., at 15,000 x g, for approximately 3-5 hours. Following resuspension of the virus pellet in an appropriate buffer, such as STE (0.01 M Tris-HCl; 0.15 M NaCl; 0.0001 M EDTA) or phosphate buffered saline (PBS) at pH 7.4, the virus is concentrated by density gradient centrifugation on sucrose (60%-12%) or potassium tartrate (50%-10%). Either continuous or step gradients, e.g., a
25 sucrose gradient between 12% and 60% in four 12% steps, are suitable. The gradients are centrifuged at a speed, and for a time, sufficient for the viruses to concentrate into a visible band for recovery. Alternatively, and for most large scale commercial applications, virus is elutriated from density gradients using a zonal-centrifuge rotor operating in continuous mode. Additional details sufficient to guide one of skill through the preparation of influenza viruses
30 from tissue culture are provided, e.g., in Furminger. *Vaccine Production*, in Nicholson *et al.* (eds) Textbook of Influenza pp. 324-332; Merten *et al.* (1996) *Production of influenza virus in cell cultures for vaccine preparation*, in Cohen & Shafferman (eds) Novel Strategies in Design and Production of Vaccines pp. 141-151, and United States patents no. 5,690,937. If

desired, the recovered viruses can be stored at -80°C in the presence of sucrose-phosphate-glutamate (SPG) as a stabilizer.

[0186] In one embodiment, the invention provides compositions comprising viruses (or portions thereof) replicated in the cells of the invention that have been treated with an agent such as benzonase, to eliminate potential oncogenes. Accordingly, an oncogene-free vaccine composition is specifically included within the embodiments of the invention.

5.9 Other Viruses

[0187] In addition to the influenza viruses described above, the cells and methods of the invention can also be used to culture other viruses. In certain embodiments, one or more polypeptides of the virus undergo proteolytic cleavage by a host cell protease at some point during the life-cycle of the virus. In specific embodiments, use of a cell or method of the invention will increase the yield of the virus, the infectivity of the virus, or improve another desirable biological property of the virus known to one skilled in the art.

[0188] Accordingly, in certain embodiments, the cultured virus is a DNA virus. In certain embodiments, the virus is an RNA virus. In certain embodiments, the virus is a single-stranded DNA virus. In certain embodiments, the virus is a double-stranded DNA virus. In certain embodiments, the virus is a positive-sense single-stranded RNA virus. In certain embodiments, the virus is a negative-sense single-stranded RNA virus. In certain embodiments, the virus is a double-stranded RNA virus. In certain embodiments, the virus is a reverse-transcribing virus.

[0189] In certain embodiments, the virus is a member of an order that is selected from the group consisting of Caudovirales, Mononegavirales, and Nidovirales.

[0190] In certain embodiments, the virus is a member of a family or subfamily that is selected from the group consisting of Myoviridae, Siphoviridae, Podoviridae, Rudiviridae, Tectiviridae, Corticoviridae, Lipothrixviridae, Plasmaviridae, Fuselloviridae, Phycodnaviridae, Guttaviridae, Poxviridae, Chordopoxvirinae, Entomopoxvirinae, Iridoviridae, Polydnviridae, Herpesviridae, Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae, Polyomaviridae, Papillomaviridae, Adenoviridae, Ascoviridae, Baculoviridae, Nimaviridae, and Asfarviridae.

[0191] In certain embodiments, the virus is a member of a genus that is selected from the group consisting of T4-like viruses, P1-like viruses, P2-like viruses, Mu-like viruses, SP01-like viruses, ΦH-like viruses, λ-like viruses, T1-like viruses, T5-like viruses, c2-like viruses,

L5-like viruses, ψ M1-like viruses, T7-like viruses, ϕ 29-like viruses, P22-like viruses, Rudivirus, Tectivirus, Corticovirus, Alphalipothrixvirus, Betalipothrixvirus, Gammalipothrixvirus, Plasmavirus, Fusellovirus, Chlorovirus, Prasinovirus, Prymnesiovirus, Phaeovirus, Raphidovirus, Coccolithovirus, Guttavirus, Orthopoxvirus, Parapoxvirus, 5 Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus, Yatapoxvirus, Alphaentomopoxvirus, Betaentomopoxvirus, Gammaentomopoxvirus, Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus, Ichnovirus, Bracovirus, Ictalurivirus, Simplexvirus, Varicellovirus, Mardivirus, Iltovirus, Cytomegalovirus, Muromegalovirus, Roseolovirus, Lymphocryptovirus, Rhadinovirus, Polyomavirus, Papillomavirus, 10 Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus, Ascovirus, Mimivirus, Nucleopolyhedrovirus, Granulovirus, Whispovirus, Asfivirus, and Rhizidiovirus.

[0192] In certain embodiments, the virus is selected from the group consisting of Enterobacteria phage T4, Enterobacteria phage P1, Enterobacteria phage P2, Enterobacteria phage Mu, Bacillus phage SP01, Halobacterium virus Φ H, Enterobacteria phage λ , 15 Enterobacteria phage T1, Enterobacteria phage T5, Lactococcus phage c2, Mycobacterium phage L5, Methanobacterium ψ M1, Enterobacteria phage T7, Bacillus phage ϕ 29, Enterobacteria phage P22, Sulfolobus virus SIRV1, Enterobacteria phage PRD1, Alteromonas phage PM2, Thermoproteus virus 1, Sulfolobus islandicus filamentous virus, Acidianus filamentous virus 1, Acholeplasma phage L2, Sulfolobus virus SSV1, Paramecium 20 bursaria Chlorella virus 1, Micromonas pusilla virus SP'1, Chrysochromulina brevisfilum virus PW1, Ectocarpus siliculosus virus 1, Heterosigma akashiwo virus 01, Emiliana huxleyi virus 86, Sulfolobus neozealandicus droplet-shaped virus, Vaccinia virus, Orf virus, Fowlpox virus, Sheeppox virus, Myxoma virus, Swinepox virus, Molluscum contagiosum virus, Yaba monkey tumor virus, Melolontha melolontha entomopoxvirus, Amsacta moorei 25 entomopoxvirus, Chironomus luridus entomopoxvirus, Invertebrate iridescent virus 6, Invertebrate iridescent virus 3, Frog virus 3, Lymphocystis disease virus 1, Campoletis sonorensis ichnovirus, Cotesia melanoscela bracovirus, Ictalurid herpesvirus 1, Human herpesvirus 1, Human herpesvirus 3, Gallid herpesvirus 2, Gallid herpesvirus 1, Human herpesvirus 5, Murid herpesvirus1, Human herpesvirus 6, Human herpesvirus 4, Saimiriine 30 herpesvirus 2, Simian virus 40, Cottontail rabbit papillomavirus, Human adenovirus C, Fowl adenovirus A, Ovine adenovirus D, Turkey adenovirus B, Spodoptera frugiperda ascovirus, Acanthamoeba polyphaga mimivirus, Autographa californica nucleopolyhedrovirus, Cydia

pomonella granulovirus, White spot syndrome virus 1, African swine fever virus, and Rhizidiomyces virus.

[0193] In certain embodiments, the virus is a member of a family or subfamily that is selected from the group consisting of Inoviridae, Microviridae, Geminiviridae, Circoviridae, Nanoviridae, Parvoviridae, Parvovirinae, and Densovirinae.

[0194] In certain embodiments, the virus is a member of a genus that is selected from the group consisting of Inovirus, Plectrovirus, Microvirus, Spiromicrovirus, Bdellovibrio, Chlamydiamicrovirus, Mastrevirus, Curtovirus, Begomovirus, Circovirus, Gyrovirus, Nanovirus, Babuvirus, Parvovirus, Erythrovirus, Dependovirus, Densovirus, Iteravirus, and Brevidensovirus.

[0195] In certain embodiments, the virus is selected from the group consisting of Enterobacteria phage M13, Acholeplasma phage MV-L51, Enterobacteria phage ϕ X174, Spiroplasma phage 4, Bdellovibrio phage MAC1, Chlamydia phage 1, Maize streak virus, Beet curly top virus, Bean golden mosaic virus - Puerto Rico, Porcine circovirus, Chicken anaemia virus, Subterranean clover stunt virus, Babana bunchy top virus, Minute virus of mice, B19 virus, Adeno-associated virus 2, Junonia coenia densovirus, Bombyx mori densovirus, and Aedes aegypti densovirus.

[0196] In certain embodiments, the virus is a member of a family or subfamily that is selected from the group consisting of Hepadnaviridae and Caulimoviridae.

[0197] In certain embodiments, the virus is a member of a genus that is selected from the group consisting of Orthohepadnavirus, Avihepadnavirus, Badnavirus, Caulimovirus, Tungrovirus, Soymovirus, Cavemovirus, and Petuvirus.

[0198] In certain embodiments, the virus is selected from the group consisting of Hepatitis B virus, Duck hepatitis B virus, Commelina yellow mottle virus, Cauliflower mosaic virus, Rice tungro bacilliform virus, Soybean chlorotic mottle virus, Cassava vein mosaic virus, and Petunia vein clearing virus.

[0199] In certain embodiments, the virus is a member of a family or subfamily that is selected from the group consisting of Pseudoviridae, Metaviridae, and Retroviridae.

[0200] In certain embodiments, the virus is a member of a genus that is selected from the group consisting of Pseudovirus, Hemivirus, Metavirus, Errantivirus, Alpharetrovirus,

Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus, Lentivirus, and Spumavirus.

[0201] In certain embodiments, the virus is selected from the group consisting of Saccharomyces cerevisiae Ty1 virus, Drosophila melanogaster copia virus, Saccharomyces cerevisiae Ty3 virus, Drosophila melanogaster gypsy virus, Avian leucosis virus, Mouse mammary tumour virus, Murine leukaemia virus, Bovine leukaemia virus, Walleye dermal sarcoma virus, Human immunodeficiency virus 1, and Chimpanzee foamy virus.

[0202] In certain embodiments, the virus is a member of a family or subfamily that is selected from the group consisting of Cystoviridae, Reoviridae, Birnaviridae, Totiviridae, Chrysoviridae, Partitiviridae, and Hypoviridae.

[0203] In certain embodiments, the virus is a member of a genus that is selected from the group consisting of Cystovirus, Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Phytoreovirus, Oryzavirus, Aquabirnavirus, Avibirnavirus, Entomobirnavirus, Totivirus, Giardivirus, Leishmanivirus, Chrysovirus, Partivirus, Alphacryptovirus, Betacryptovirus, Hypovirus, and Varicosavirus.

[0204] In certain embodiments, the virus is selected from the group consisting of Pseudomonas phage ψ 6, Mammalian orthoreovirus, Bluetongue virus, Rotavirus A, Colorado tick fever virus, Aquareovirus A, Cypovirus 1, Fiji disease virus, Rice dwarf virus, Rice ragged stunt virus, Infectious pancreatic necrosis virus, Infectious bursal disease virus, Drosophila X virus, Saccharomyces cerevisiae virus L-A, Giardia lamblia virus, Leishmania RNA virus 1-1, Penicillium chrysogenum virus, Atkinsonella hypoxylon virus, White clover cryptic virus 1, White clover cryptic virus 2, Cryphonectria hypovirus 1-EP713, and Lettuce big-vein associated virus.

[0205] In certain embodiments, the viral RNA encodes genomic viral RNA of a virus from the order. In certain embodiments, the virus is a member of a family or subfamily that is selected from the group consisting of Paramyxoviridae, Pneumovirinae, Rhabdoviridae, Filoviridae, Bornaviridae, Orthomyxoviridae, Bunyaviridae, and Arenaviridae.

[0206] In certain embodiments, the virus is a member of a genus that is selected from the group consisting of Respirivirus, Morbillivirus, Rubulavirus, Henipavirus, Avulavirus, Pneumovirus, Metapneumovirus, Vesiculovirus, Lyssavirus, Ephemerovirus, Cytorhabdovirus, Nucleorhabdovirus, Novirhabdovirus, Marburgvirus, Ebolavirus, Bornavirus, Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus, Isavirus,

Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus, Tospovirus, Arenavirus, Ophiovirus, Tenuivirus, or Deltavirus.

[0207] In certain embodiments, the virus is selected from the group consisting of Sendai virus, Measles virus, Mumps virus, Hendra virus, Newcastle disease virus, Human respiratory syncytial virus, Avian pneumovirus, Vesicular stomatitis Indiana virus, Rabies virus, Bovine ephemeral fever virus, Lettuce necrotic yellows virus, Potato yellow dwarf virus, Infectious hematopoietic necrosis virus, Lake Victoria marburgvirus, Zaire ebolavirus, Borna disease virus, Influenza A virus, Influenza B virus, Influenza C virus, Thogoto virus, Infectious salmon anemia virus, Bunyamwera virus, Hantaan virus, Dugbe virus, Rift Valley fever virus, Tomato spotted wilt virus, Lymphocytic choriomeningitis virus, Citrus psorosis virus, Rice stripe virus, and Hepatitis delta virus. In a specific embodiment, the virus is an Influenza A virus or an Influenza B virus. In another specific embodiment, the virus is an attenuated influenza virus, a cold adapted influenza virus, a temperature sensitive influenza virus, or a virus with any combination of these desirable properties. In one embodiment, the influenza virus incorporates an influenza B/Ann Arbor/1/66 strain virus, *e.g.*, a cold adapted, temperature sensitive, attenuated strain of B/Ann Arbor/1/66. In another embodiment, the influenza virus incorporates an influenza A/Ann Arbor/6/60 strain virus, *e.g.*, a cold adapted, temperature sensitive, attenuated strain of A/Ann Arbor/6/60.

[0208] In certain embodiments, the virus is a member of a family or subfamily that is selected from the group consisting of Leviviridae, Dicistroviridae, Picornaviridae, Sequiviridae, Comoviridae, Potyviridae, Caliciviridae, Astroviridae, Nodaviridae, Tetraviridae, Tombusviridae, Coronaviridae, Arteriviridae, Roniviridae, Togaviridae, Flaviviridae, Bromoviridae, Closteroviridae, Barnaviridae, Luteoviridae, Tymoviridae, and Flexiviridae.

[0209] In certain embodiments, the virus is a member of a genus that is selected from the group consisting of Levivirus, Allolevivirus, Cripavirus, Iflavirus, Enterovirus, Rhinovirus, Hepatovirus, Cardiovirus, Aphthovirus, Parechovirus, Sequivirus, Waikavirus, Comovirus, Fabavirus, Nepovirus, Potyvirus, Rymovirus, Bymovirus, Macluravirus, Ipomovirus, Tritimovirus, Vesivirus, Lagovirus, Norovirus, Sapovirus, Hepevirus, Mamastrovirus, Avastrovirus, Alphanodavirus, Betanodavirus, Betatetravirus, Omegatetravirus, Tombusvirus, Carmovirus, Necrovirus, Dianthovirus, Machlomovirus, Avenavirus, Aureusvirus, Panicovirus, Coronavirus, Torovirus, Arterivirus, Okavirus, Alphavirus, Rubivirus, Flavivirus, Pestivirus, Hepacivirus, Alfamovirus, Ilarvirus, Bromovirus,

Cucumovirus, Oleavirus, Closterovirus, Crinivirus, Ampelovirus, Barnavirus, Luteovirus, Polerovirus, Enamovirus, Tobamovirus, Tobravirus, Hordeivirus, Furovirus, Pomovirus, Pecluvirus, Benyvirus, Idaeovirus, Sobemovirus, Umbravirus, Tymovirus, Marafivirus, Maculavirus, Allexivirus, Manadrivirus, Carlavirus, Capillovirus, Foveavirus, Potexvirus, Trichovirus, Vitivirus, and Ourmiavirus.

[0210] In certain embodiments, the virus is selected from the group consisting of Enterobacteria phage MS2, Enterobacteria phage Q β , Cricket paralysis virus, Infectious flacherie virus, Poliovirus, Human rhinovirus A, Hepatitis A virus, Encephalomyocarditis virus, Foot-and-mouth disease virus, Human parechovirus, Parsnip yellow fleck virus, Rice tungro spherical virus, Cowpea mosaic virus, Broad bean wilt virus 1, Tobacco ringspot virus, Potato virus Y, Ryegrass mosaic virus, Barley yellow mosaic virus, Maclura mosaic virus, Sweet potato mild mottle virus, Wheat streak mosaic virus, Swine vesicular exanthema virus, Rabbit hemorrhagic disease virus, Norwalk virus, Sapporo virus, Hepatitis E virus, Human astrovirus, Turkey astrovirus, Nodamura virus, Striped jack nervous necrosis virus, Nudaurelia capensis β virus, Nudaurelia capensis ω virus, Tomato bushy stunt virus, Carnation mottle virus, Tobacco necrosis virus A, Carnation ringspot virus, Maize chlorotic mottle virus, Oat chlorotic stunt virus, Pothos latent virus, Panicum mosaic virus, Infectious bronchitis virus, Equine torovirus, Equine arteritis virus, Gill-associated virus, Sindbis virus, Rubella virus, Yellow fever virus, Bovine viral diarrhea virus, Hepatitis C virus, Alfalfa mosaic virus, Tobacco streak virus, Brome mosaic virus, Cucumber mosaic virus, Olive latent virus 2, Beet eldows virus, Lettuce infectious yellows virus, Grapevine leafroll-associated virus 3, Mushroom bacilliform virus, Barley yellow dwarf virus-PAV, Potato leafroll virus, Pea enation mosaic virus-1, Tobacco mosaic virus, Tobacco rattle virus, Barley stripe mosaic virus, Soil-borne wheat mosaic virus, Potato mop-top virus, Peanut clump virus, Beet necrotic yellow vein virus, Raspberry bushy dwarf virus, Southern bean mosaic virus, Carrot mottle virus, Turnip yellow mosaic virus, Maize rayado fino virus, Grapevine fleck virus, Shallot virus X, Indian citrus ringspot virus, Carnation latent virus, Apple stem grooving virus, Apple stem pitting virus, Potato virus X, Apple chlorotic leaf spot virus, Grape vine virus A, and Ourmia melon virus.

[0211] In certain embodiments, the virus is a member of the family Narnaviridae.

[0212] In certain embodiments, the virus is a member of a genus that is selected from the group consisting of Narnavirus and Mitovirus.

[0213] In certain embodiments, the virus is selected from the group consisting of

[0214] In certain embodiments, the virus is a member of a family or subfamily that is selected from the group consisting of *Saccharomyces cerevisiae* 20SRNA narnavirus and *Cryphonectria parasitica* mitovirus-1 NB631.

6. Specific Embodiments

[0215] 1. A host cell comprising a nucleic acid encoding a protease or pro-protease, wherein the nucleic acid encoding the protease or pro-protease is integrated into the host cell's genome, and wherein the protease or pro-protease is not normally expressed in the cell.

[0216] 2. The host cell of embodiment 1, wherein the cell expresses a protease.

10 [0217] 3. The host cell of embodiment 2, wherein the protease is a serine protease.

[0218] 4. The host cell of embodiment 3, wherein the serine protease is an S1 family protease.

[0219] 5. The host cell of embodiment 4, wherein the protease is trypsin.

[0220] 6. The host cell of embodiment 2, wherein the serine protease is a bacterial subtilisin.

[0221] 7. The host cell of embodiment 6, wherein the protease is SPRT from *Streptomyces griseus*.

[0222] 8. The host cell of embodiment 2, wherein the protease is a protease listed in Table 1.

20 **[0223]** 9. The host cell of embodiment 1, wherein the protease is a pro-protease.

[0224] 10. The host cell of embodiment 9, wherein the pro-protease is trypsinogen or prepro-SPRT protease from *Streptomyces griseus*.

[0225] 11. The host cell of any of the preceding embodiments, wherein expression of the protease or pro-protease is under the control of an inducible promoter.

25 **[0226]** 12. The host cell of embodiment 11, wherein the inducible promoter is induced by interferon or a downstream signaling molecule induced by interferon.

[0227] 13. The host cell of embodiment 11, wherein the inducible promoter is induced by a tetracycline-regulated expression system.

- [0228] 14. The host cell of any one of embodiments 1-10, wherein expression of the protease or pro-protease is under the control of a constitutively active promoter.
- [0229] 15. The host cell of any of the preceding embodiments, wherein the nucleic acid comprises a secretion signal that directs secretion of the protease or pro-protease.
- 5 [0230] 16. The host cell of any of the preceding embodiments, wherein the cell is a mammalian cell.
- [0231] 17. The host cell of embodiment 16, wherein the cell is a canine cell.
- [0232] 18. The host cell of embodiment 17, wherein the cell is an MDCK cell.
- [0233] 19. The host cell of embodiment 16, wherein the cell is a primate cell.
- 10 [0234] 20. The host cell of embodiment 19, wherein the cell is an African green monkey or human cell.
- [0235] 21. The host cell of any of embodiments 1-15, wherein the cell is an avian cell.
- [0236] 22. The host cell of embodiment 21, wherein the cell is a chicken cell.
- [0237] 23. The host cell of any of the preceding embodiments, wherein the cell grows in
15 suspension without being adapted.
- [0238] 24. A method of producing an influenza virus comprising infecting the host cell of any of the preceding embodiments with an influenza virus, culturing the cell under conditions that allow replication of the influenza virus, and collecting influenza virus from the cell culture.
- 20 [0239] 25. A method of producing an influenza virus, comprising transfecting the host cell of any one of embodiments 1 to 23 with nucleic acids encoding an influenza genome, culturing the cell under conditions that allow replication of the influenza virus, and collecting influenza virus from the cell culture.
- [0240] 26. The method of embodiment 24 or 25, wherein the host cell expresses a pro-
25 protease, and wherein the method further comprises adding an exogenous protease to the culture medium.
- [0241] 27. The method of embodiment 26, wherein the exogenous protease is added to a maximum concentration of about 0.1 $\mu\text{g/ml}$.
- [0242] 28. The method of embodiment 26, wherein the exogenous protease is trypsin.

- [0243] 29. A method of replicating an influenza virus in the absence of exogenously added trypsin comprising infecting a host cell with an influenza virus, culturing the cell under conditions that allow replication of the influenza virus, wherein said conditions do not include exogenously added trypsin, and collecting influenza virus from the cell culture.
- 5 [0244] 30. A method of replicating an influenza virus in the absence of exogenously added trypsin comprising transfecting a host cell with nucleic acids encoding an influenza genome, culturing the cell under conditions that allow replication of the influenza virus, wherein said conditions do not include exogenously added trypsin, and collecting influenza virus from the cell culture.
- 10 [0245] 31. The method of embodiment 29 or 30, wherein the host cell expresses an enzymatically active protease or pro-protease not normally expressed by the cell.
- [0246] 32. The method of embodiment 29 or 30, wherein the host cell is a mammalian cell.
- [0247] 33. The method of embodiment 29 or 30, wherein the host cell is an avian cell.
- [0248] 34. The method of embodiment 32, wherein the host cell is a primate cell, canine
15 cell, hamster cell, mouse cell, or rat cell.
- [0249] 35. The method of embodiment 32, wherein the host cell is an MDCK cell.
- [0250] 36. The method of embodiment 34, wherein the host cell is a Vero cell.
- [0251] 37. The method of embodiment 33, wherein the host cell is a chicken cell.
- [0252] 38. A method of increasing the titer of influenza virus grown in cell culture,
20 comprising culturing an influenza virus in a cell culture, wherein the cells in the cell culture stably expresses a protease or pro-protease that i) is heterologous to the cell, and ii) cleaves a hemagglutinin of the influenza virus, thereby increasing the titer of the influenza virus grown in the cell culture relative to the titer obtained by culturing the influenza virus in cells that do not express a heterologous protease or pro-protease.
- 25 [0253] 39. The method of embodiment 38, wherein the cells constitutively express the protease or pro-protease.
- [0254] 40. The method of embodiment 38, wherein the cells inducibly express the protease or pro-protease.
- [0255] 41. The method of embodiment 38, 39 or 40, wherein the cell expresses a protease.

[0256] 42. The method of embodiment 38, 39 or 40, wherein the cell expresses a pro-protease.

[0257] 43. The method of embodiment 41 wherein the protease is trypsin.

[0258] 44. The method of embodiment 41, wherein the protease is SPRT protease from
5 *Streptomyces griseus*.

[0259] 45. The method of embodiment 42, wherein the pro-protease is trypsinogen or prepro-SPRT protease from *Streptomyces griseus*.

10 [0260] 46. A method for producing a heterologous protease or pro-protease in a host cell capable of supporting influenza replication, comprising culturing a cell comprising a nucleic acid encoding a protease or pro-protease not normally expressed in the cell under conditions that permit expression of said protease or pro-protease, thereby producing the protease or pro-protease in the cell.

[0261] 47. The method of embodiment 46, wherein the host cell stably expresses a protease.

15 [0262] 48. The method of embodiment 46, wherein the host cell stably expresses a pro-protease.

[0263] 49. The method of embodiment 46, 47 or 48, wherein the host cell secretes the protease or pro-protease into the cell culture medium.

20 [0264] 50. The method of embodiment 46, 47, 48 or 49, wherein the host cell expresses between about 0.1 ng and about 10 µg of the protease or pro-protease per ml of host cell culture.

[0265] 51. The method of embodiment 46, 47, 48, 49 or 50, wherein the cell expresses an amount of protease or pro-protease sufficient to increase the titer of virus grown in a culture of the host cells expressing the protease or pro-protease.

25 [0266] 52. The method of embodiment 46, 47, 48, 49, 50 or 51, wherein expression of the protease or pro-protease is inducible.

[0267] 53. The method of embodiment 46, 47, 48, 49, 50 or 51, wherein expression of the protease or pro-protease is constitutive.

[0268] 54. An isolated nucleic acid encoding a nucleotide sequence that is at least about 90% identical to SEQ ID NO.:1.

- [0269] 55. The nucleic acid of embodiment 54, wherein the nucleic acid encodes the nucleotide sequence of SEQ ID NO.:1.
- [0270] 56. An isolated polypeptide comprising the amino acid of SEQ ID NO.:2.
- [0271] 57. An isolated nucleic acid encoding the polypeptide of embodiment 56.
- 5 [0272] 58. An expression vector comprising the nucleic acid of embodiment 54, 55 or 57.
- [0273] 59. An isolated cell comprising the expression vector of embodiment 58.
- [0274] 60. A method of making a host cell that stably expresses a protease or pro-protease, comprising introducing a nucleic acid encoding a protease or pro-protease into a host cell, wherein the protease or pro-protease is not normally expressed in the host cell, and isolating
10 the host cell that stably expresses the protease or pro-protease.
- [0275] 61. A method of growing an adherent host cell in suspension comprising introducing a nucleic acid encoding a protease or pro-protease, wherein the nucleic acid encoding the protease or pro-protease is integrated into the host cell's genome, and wherein the protease or pro-protease is not normally expressed in the cell.
- 15 [0276] 62. The method of embodiment 60 or 61, wherein the host cell stably expresses a protease.
- [0277] 63. The method of embodiment 60 or 61, wherein the host cell stably expresses a pro-protease.
- [0278] 64. The method of embodiment 60, 61, 62 or 63, wherein the host cell secretes the
20 protease or pro-protease into the cell culture medium.
- [0279] 65. The method of embodiment 60, 61, 62, 63 or 64, wherein the host cell expresses between about 0.1 ng and about 50 µg of the protease or pro-protease per ml of host cell culture.
- [0280] 66. The method of embodiment 60, 61, 62, 63, 64 or 65, wherein the cell expresses
25 an amount of protease or pro-protease sufficient to increase the titer of virus grown in a culture of the host cells expressing the protease or pro-protease.
- [0281] 67. The method of embodiment 60, 61, 62, 63, 64, 65 or 66, wherein expression of the protease or pro-protease is inducible.
- [0282] 68. The method of embodiment 60, 61, 62, 63, 64, 65 or 66, wherein expression of
30 the protease or pro-protease is constitutive.

[0283] 69. The method of embodiment 60, 61, 62, 63, 64, 65, 66, 67 or 68, wherein the host cell is a mammalian cell.

[0284] 70. The method of embodiment 60, 61, 62, 63, 64, 65, 66, 67 or 68, wherein the host cell is an avian cell.

5 [0285] 71. The method of embodiment 69, wherein the host cell is a primate cell, canine cell, hamster cell, mouse cell, or rat cell.

[0286] 72. The method of embodiment 71, wherein the host cell is an MDCK cell.

[0287] 73. The method of embodiment 71, wherein the host cell is a Vero

[0288] 74. The method of embodiment 70, wherein the host cell is a chicken cell.

10 [0289] 75. The method of embodiment 60, wherein the host cell is a bacterial cell.

[0290] 76. The method of any one of embodiments 60-62 or 64-75, wherein the protease is trypsin.

[0291] 77. The method of any one of embodiments 60-62 or 64-75, wherein the protease is SPRT protease from *Streptomyces griseus*.

15 [0292] 78. The method of any one of embodiments 60-61 or 63-75, wherein the pro-protease is trypsinogen.

7. Examples

[0293] The following examples serve merely to illustrate the invention and are not intended to limit the invention in any way.

20 7.1 **Example 1: Growth of Influenza Strains in MDCK Cells**

[0294] This example describes characterization of several cell lines for culturing influenza. Several different cell lines and primary cells were evaluated for the production of genetic reassortants derived from two laboratory adapted influenza strains, type A and type B, including MRC-5, WI-38, FRhL-2, PerC6, 293, NIH 3T3, CEF, CEK, DF-1, Vero, and
25 MDCK. While many of the cell types supported the replication of some cold-adapted influenza strains to a limited extent, only MDCK consistently produced high titers of both type A and type B viruses. The MDCK cells were also tested for their ability to support replication of a potential pandemic vaccine, ca A/Vietnam/1203/2004. MDCK cells were infected at a low multiplicity of infection with ca A/Vietnam/1203/2004 and virus in the
30 supernatant was quantified at various times post infection. By 48 hours post infection, the

titers of ca A/Vietnam/1203/2004 reached approximately $8 \log_{10}$ TCID₅₀/mL and remained stable for the next 3 to 4 days. See Figure 1.

[0295] In the experiments, MDCK cells obtained from the ATCC (Accession No. CCL-34) were expanded a limited number of times in either media containing 10% fetal bovine serum sourced from the United States or in an appropriate serum free media (*e.g.*, SFMV 100) to produce pre-master cell stocks for initial characterization studies. Appropriate serum free media are described in U.S. Provisional Application No. 60/638,166, filed December 23, 2004, and in U.S. Provisional Application No. 60/641,139, filed January 5, 2005. Cells were readily grown in both types of media and both stocks of cells supported the replication of cold-adapted vaccine strains and pandemic strains as shown in Table 2, below, and in Figure 1, respectively.

Table 2
Comparison of productivity of cold-adapted influenza strains in serum and serum free grown MDCK cells.

Virus strain (6:2 reassortant)	TCID ₅₀ /mL ($\log_{10}$)	
	MDCK with serum	MDCK w/out serum
A/New Caledonia/20/99 (H1N1)	8.1	7.8
A/Panama/20/99 (H3N2)	6.8	6.4
A/Sydney/05/97 (H3N2)	7.0	6.5
B/Brisbane/32/2002	7.2	7.5
B/Hong Kong/330/2001	7.2	7.4
B/Victoria/504/2000	6.9	7.5

7.2 Example 2: Tumorigenicity of MDCK Cell Lines

[0296] The potential tumorigenicity of the two pre-master cell stocks of MDCK cells, one grown in media containing serum and the other in serum free media, were evaluated in the athymic nude mouse model at a stage that would represent 5 cell passages after that expected to be used for vaccine production. To evaluate tumorigenicity, 10^7 cells were injected subcutaneously into groups of 10 mice and after 84 days the animals were sacrificed and examined. Neoplasias were observed in six of the 10 animals inoculated with the cells passaged in serum free media. In contrast, there was no evidence of neoplasia in any of the animals inoculated with cells passaged in media supplemented with 10% fetal bovine serum; although some fibrosarcomas were observed at the site of inoculation, cells passaged in serum were not tumorigenic as shown in Table 3.

Table 3
Tumorigenicity and Karyology of MDCK cells passed in two different media

	Serum free		10% Serum	
	Passage 4	Passage 20	Passage 4	Passage 20
Tumorigenicity	ND	Neoplasias noted	ND	No neoplasias. Fibrosarcomas at injection site
Estimated TP₅₀[*] (no animals with tumors / total animals)	ND	~10 ⁷ (6/10)	ND	Not estimable (>10 ⁷) (0/10)
Karyology Median number; comments	78; Large distribution of cells with chromosome number of 52 to 82	78; Large distribution of cells with chromosome number of 52-82	78; Few cells with anomalous chromosome number (70 to 82)	78; Few cells with anomalous chromosome number (70 to 82)

*TP₅₀: Number of cells required to induce tumors in 50% of animals

5 ND: Not done

[0297] As shown in Table 3, karyotype analyses were also performed on these two premaster cell stocks at both the fourth and twentieth passage in their respective media. The non-tumorigenic cells passaged in 10% FCS had a median number of 78 metaphase chromosomes with relatively limited distribution of cells with other chromosome numbers (70 to 82). While the cells passaged in serum free media also had a median number of 78 metaphase chromosomes, significantly more cells were observed with an aneuploid chromosome number ranging from 52 to 82 metaphase chromosomes. In both cases, the karyology did not change following passage.

15 7.3 Example 3: Adapting MDCK Cells to Grow in Serum Free Media

[0298] MDCK cells from the ATCC are passaged in media containing gamma irradiated FBS. These cells are then passaged a limited number of times in a serum free media formulation chosen to support cell bank production. Serum free media are described in U.S. Provisional Application Nos. 60/638,166 and 60/641,139. These additional passages may be performed at either 37°C or 33°C. Passage of MDCK cells in three media containing plant-derived supplements rather than serum yielded cells with karyotypes similar to that of MDCK cells passaged in FCS containing media (data not shown).

7.4 Example 4: Cloning of MDCK Cells

[0299] Cells were biologically cloned through limiting dilution in order to ensure that the production cells are derived from a unique genetic constellation. Clones were screened for various phenotypic properties including doubling time and relative tumorigenicity, as well as viral production. In an initial proof of concept experiment, fifty-four MDCK clones were obtained in media containing FCS. These clones were passaged and each was infected with a low multiplicity of infection of ca A/New Caledonia/20/99. Several days after infection, the supernatant was removed and the quantity of virus in the supernatant was measured by TCID₅₀. A minority of the clones produced relatively high titers of virus, greater than was produced in the noncloned parental cells. Clones with superior biological and physiological properties are used to establish a Master Cell Bank (MCB) as described below.

7.5 Example 5: Testing and Characterization of a Master Cell Bank

[0300] The MCB is extensively tested to ensure that there is no evidence of adventitious agents. For example, one or more of several PCR and/or antibody-specific tests for available viral agents are conducted, as shown in Table 4, below.

Table 4
Testing regimen for the MCB

General tests	PCR* / Ab specific
Sterility	AAV Types 1&2
Mycoplasma	HCMV
Adventitious agents <i>in vitro</i> (multiple cell lines)	EBV
Adventitious agents <i>in vivo</i>	HSV
PERT	Hepatitis B, C & E
Co-cultivation	HHV 6, 7 & 8
Karyology	HIV 1&2
Electron microscopy	HPV
Tumorigenicity intact cells (TP ₅₀)	HTLV I & II
Oncogenicity of cellular DNA	Polyoma (BK and JC viruses)
Oncogenicity of cellular lysate	Circovirus
Bovine viruses per 9CFR	Canine Parvovirus
Porcine viruses per 9CFR	Canine distemper
	Adenovirus
	SV40

7.6 Example 6: Preclinical Characterization of Cell Culture-Derived Influenza Virus

[0301] This example describes characterization of influenza strains produced from cell culture as well as from eggs and compares the viruses produced from the systems. Generally, the influenza viruses are suitable for use as vaccines in humans, and have biological

properties that make the viruses suitable for such use. In this example, the influenza viruses are cold-adapted (*ca*; have the ability to replicate efficiently at lower temperatures), temperature sensitive (*ts*; have restricted replication in vitro at higher temperatures), and attenuated (*att*; no detectable replication in lung tissues of ferrets), and are referred to herein as *ca ts att* strains. The comparison includes: biochemical, antigenic, and genetic evaluation (sequencing) of viral product; biological and biochemical characterization of the virus following replication in human cells; replication in a permissive animal model; and immunogenicity in a permissive animal model.

7.6.1 Genetic, biochemical and antigenic comparability

[0302] *Ca ts att* strains of type A/H1N1, A/H5N1, A/H3N2 and B replicated to relatively high titers in MDCK cells. In addition, passaging these *ca ts att* strains in MDCK cells did not alter their genomic sequence. Three *ca ts att* strains, *ca* A/Sydney/05/97, *ca* A/Beijing/262/95, and *ca* B/Ann Arbor/1/94 were passaged once or twice in MDCK cells and the entire coding regions of all 6 internal genes were sequenced and compared to the starting material. No nucleotide changes were observed, demonstrating that this passaging through this substrate did not change the genetic composition of these strains. Further sequence characterizations is performed on different vaccine strains produced in MDCK cells under conditions that are expected to mimic the production process including media composition, input dose (moi), temperature of incubation and time of harvest. Based on the preliminary data, it is expected that there will be no changes in the genomic sequence of MDCK-produced virus.

[0303] Because the genome was genetically stable following passage in MDCK cell, the biological traits of the vaccine produced in eggs or MDCK cells are expected to be indistinguishable. However, the primary viral product from cell culture may have some subtle differences compared to the egg based product, particularly with respect to post-translational modification of viral proteins including HA and NA, or composition of lipids in the viral membrane; both of which could potentially change the overall physical properties of the virion. Preliminary preclinical data on the antigenicity of cell culture produced and egg produced vaccine demonstrated that there were no detectable differences in this important parameter. Egg stocks of several vaccine strains were passaged through MDCK cells and the antigenicity of both products was determined by measuring the HAI titers using reference antisera. As show in Table 5, all the HAI titers were within 2-fold of one another, indicating

that replication of the vaccine in cells did not change the antigenicity of the vaccine compared to egg derived material.

Table 5
HAI Titers of strains produced in eggs and MDCK cells

Strain	HAI Titer	
	Egg derived	MDCK derived
A/Panama/20/99	256	256
A/Wuhan/359/95	1024	2048
A/Wyoming/03/2003	512	1024
B/Jilin/20/2003	64	32
B/Hong Kong/330/01	64	64
B/Jiangsu/10/2003	128	128

7.7 Example 7: Infection of Human Epithelial Cells in Culture

[0304] In certain embodiments, to evaluate the biochemical, biological, and structural similarities following replication of the MDCK and egg produced vaccines in cells of human origin, vaccines may be passaged once in relevant diploid human cells, such as normal human bronchial epithelial cells (NHBE). This passage will serve to mimic a single infection event in the human airway and then enable comparison of the progeny virus, the virus that is ultimately responsible for eliciting an effective immune response. Studies of the vaccines' hemagglutinin (binding and fusion) and neuraminidase activities are measured on these materials as well as other biochemical and structural studies including electron microscopy, infectious to total particle ratios, and viral genome equivalents can be evaluated. Overall, these comparisons serve to demonstrate the comparability of the cell-derived vaccine to the effective and safe egg produced vaccine. A summary of analytical studies is summarized in Table 6.

Table 6
Preclinical studies to compare cell and egg produced vaccines

In vivo (ferrets)	In vitro*
Attenuation / Replication Extent of replication in upper airway Kinetics of replication in upper airway	Virus binding Hemagglutination titer Binding of different sialic acids
Immunogenicity Cross-reactivity Kinetics	Physical properties Morphology by EM Infectious : Total particles (genomes)
Infectivity Dose required for detectable replication Dose required for antibody response	Fusion activity pH optimum temperature optimum
	Genomic sequence

	Neuraminidase activity
--	------------------------

*Compare primary products and after one passage in human cells

7.8 Example 8: Preclinical Animal Models

[0305] The ferret is a robust animal model used to evaluate the attenuation and

immunogenicity of attenuated influenza vaccines and component vaccine strains. The performance of cell-derived influenza strains produced from the MCB are compared to the same strains produced in eggs. Head to head comparison of these materials in controlled studies enables a high level of assurance of the comparability of these viral products.

[0306] In order to evaluate the ability of the two vaccines to infect or achieve a “take” in the ferret, animals are lightly anesthetized and inoculated intranasally with either the cell or egg produced viral preparations. Nasal wash material is collected at several time points following inoculation and the quantity of virus is evaluated by one of several available methods in order to evaluate the kinetics and extent of viral replication in the animals’ upper respiratory tract. Experiments are performed with a range of doses and include multiple strains and different trivalent mixtures to generalize the relative infectivity of cell culture grown strains to egg produced strains. These same studies are also used to evaluate the immunogenicity of the influenza strains, a property that is inherently linked to the ability of the virus to initiate infection. Animals are bled and nasal washes are harvested at various points (weeks) post inoculation; these specimens are used to assess the serum antibody and nasal IgA responses to infection. The culmination of these data, infectivity, serum antibody and mucosal antibody responses, will be used to compare and evaluate the relative infectivity of the cell-produced vaccine to the egg produced vaccine. The most likely outcome is predicted to be that the cell and egg produced vaccine strains have similar infectivity and immunogenicity. If the cell derived vaccine appeared to be more infective or more immunogenic than the egg-derived product, further studies evaluating the possibility of lower dosage are performed.

[0307] A number of immunogenicity and replication studies are performed in the ferret model to evaluate the cell culture-derived vaccines with a single unit human dose. Infection with *catsatt* strains generally elicits strong and rapid antibody responses in ferrets. In addition, individual *catsatt* strains are routinely tested and shown to express the attenuated (*att*) phenotype by replicating to relatively high titers in the nasopharynx but to undetectable levels in the lung of these animals. The impact of cell culture growth on these biological traits is also assessed. However, it is unlikely that any differences will be seen, since the *att* phenotype is an integral part of the genetic composition of these strains. The growth kinetics

and crossreactivity of these strains is evaluated following administration of a single human dose in these animals. The selicits serum antibodies that cross-react with multiple strains within a genetic lineage; and it is expected that a cell-derived vaccine will have the same capability.

5 [0308] These comparability evaluations should provide significant insight into potential biochemical and/or biophysical differences of the primary virus product and demonstrate the impact of these epigenetic differences on the performance of the *catsatt* strains measured by first passaging the virus in human cells or animal studies. Based on the sequence information to date, there is no expected impact on the *catsatt* strains immunogenic performance resulting
10 from production on MDCK cells.

[0309] Ferrets are a well document animal model for influenza and are used routinely to evaluate the attenuation phenotype and immunogenicity of *catsatt* strains. In general, 8 – 10 week old animals are used to assess attenuation; typically study designs evaluate n=3-5 animals per test or control group. Immunogenicity studies are evaluated in animals from 8
15 weeks to 6 months of age and generally require n=3-5 animals per test article or control group. These numbers provide sufficient information to obtain statistically valid or observationally important comparisons between groups. During most studies Influenza-like signs may be noticed, but are not likely. Ferrets do not display signs of decrease in appetite or weight, nasal or ocular discharge; observing signs of influenza-like illness is a necessary
20 part of the study and interventions such as analgesics are not warranted. Other signs of discomfort, such as open sores or significant weight loss, would result in appropriate disposition of the animal following discussion with the attending veterinarian.

7.9 Example 9: Master virus seed (MVS) development

[0310] Currently influenza vaccine strains are generated by co-infecting avian cells with a
25 wild type virus and either the type A or type B MDV and isolating and screening the progeny for the desired 6:2 genetic constellation. This process requires several passages of the virus through avian cell cultures and/or SPF eggs. Recently, plasmid rescue has been introduced for producing influenza viral preparation. In this process, Vero (African green monkey) cells from an extensively tested and characterized cell bank are electroporated with 8 DNA
30 plasmids, each containing a cDNA copy of one of the 8 influenza RNA segments. Several days after electroporation the supernatant of these electroporated cells contains influenza virus. The supernatants are then inoculated into SPF eggs to amplify and biologically clone the vaccine strain. Both of these procedures result in a vaccine strain that is inoculated into

SPF eggs to produce the MVS. While plasmid rescue has several advantages including more reliable timing, more genetically accurate gene segments and less potential contamination with adventitious agents from the wild type isolate, individual MVS's generated by these two methods are indistinguishable from one another and can be used to initiate bulk vaccine production.

[0311] Final amplification of the vaccine strains is conducted in cells derived from the MDCK cell banks. This final amplification can be achievable with small-scale cultures (<20L) of MDCK cells. The supernatant from these cells is collected, concentrated and characterized/tested to produce the MVS.

7.10 Example 10: Proteolytic Activation of Infectious Virus with MDCK Cells Expressing a Heterologous Pro-Protease

[0312] The following example describes construction of MDCK cell lines constitutively expressing porcine trypsinogen.

[0313] First, the gene encoding porcine trypsinogen was cloned into the retroviral vector pLNHX (Clontech Inc., Mountain View, CA). To do so, a recombinant nucleotide sequence encoding trypsinogen, the sequence of which is presented as Figure 11 (SEQ ID NO.:3) was synthesized and cloned into a shuttle vector. The trypsinogen gene was then digested out of the shuttle vector with Bgl II and cloned into the Bgl II site of the pLNHX polylinker. The vector thus produced is referred to herein as pTGEN.

[0314] Next, pTGEN was transfected into two different packaging cell lines, Ampho 293 and GP2, using conventional techniques. Retrovirus containing pTGEN (referred to herein as vTGEN) or a luciferase expression vector (referred to herein as vLLRN) for use as a positive control were isolated 48 hours post-infection by lysing the packaging cells using conventional techniques. Infection of MDCK cells with vLLRN indicated that vLLRN produced in GP2 cells could 1) introduce the luciferase gene into the MDCK genome, as luciferase activity could be detected in MDCK cells at 48 hours post infection (Figure 2) and 2) the amount of luciferase expressed by the MDCK cells at 48 hours post infection was proportionate to the concentration of vLLRN used to infect the MDCK cells (Figure 3). Thus, the positive controls demonstrate that the pLNHX system can be used to transfect MDCK cells with heterologous genes, and that such genes are expressed by the MDCK cells.

[0315] To identify clones stably transfected with pTGEN, the following procedure was used. 48 hours post-infection with vTGEN, vLLRN, or vLNHX (vector-only negative control),

G418 was added to the growth medium to select for resistant MDCK clones. Approximately 3 weeks later, individual MDCK clones were isolated and expanded as isogenic cultures.

[0316] Expression of luciferase from stably-transformed MDCK clones is shown in Figure 4. As seen in Figure 4, 12 different individual clones and two different mixtures of 3 clones expressing luciferase at high levels were obtained by this procedure. The 12 individual clones produced between about 10 ng to 300 ng active luciferase per 1000 cells. Thus, these data demonstrate that stably-transformed MDCK clones expressing trans-genes at high levels can be obtained from pLNHX vector systems.

[0317] A summary of the individual clones and clone mixes obtained from the transfection experiments is presented as Figure 5. As shown in Figure 5, 18 MDCK clones expressing trypsinogen each were isolated from retroviral particles produced in Ampho 293 cells and in GP2 cells.

7.11 Example 11: Influenza Infection and Growth in MDCK Cells Expressing Trypsinogen in Culture

[0318] This example describes infection and growth of two different influenza strains in MDCK cells that express trypsinogen in the presence and absence of exogenous trypsin.

[0319] The ability of two MDCK clones expressing trypsinogen (one produced by transfecting MDCK cells with particles produced in Ampho 293 cells, the other with particles produced in GP2 cells) to serve as a viral host was assessed as follows. Cells from the appropriate clone were infected with either strain ca A/Ann Arbor/6/60 MDV-A or ca A/New Caladonia/20/90 at a multiplicity of infection of 0.01 (*i.e.*, 1 influenza virus per 100 cells) at time 0. Cells transfected with pLLRN or pLNHX were used as controls. Viral titer in the cultures was assessed at 12 hours, 24, hours, 48 hours, 72 hours, 96 hours, and 120 hours following infection.

[0320] In one group of experiments, exogenous porcine trypsin was added to the cell culture to a final concentration of 0.1 µg/ml at 24 hours following infection (closed circles). In another group of experiments, the concentration of exogenous porcine trypsin in the culture was maintained at 1 µg/ml from day 1 (24 hours following infection) until day 5 (120 hours following infection). In the last group of experiments, no exogenous trypsin was added.

[0321] The results of the experiments are shown as Figures 6A-6C. Figure 6A present the results of influenza infection of mock-infected MDCK cells with MDV-A (top left) or ca A/NC (top right) and of luciferase-expressing MDCK cells with MDV-A (bottom left) or ca

A/NC (bottom right). As expected, overall viral yield was substantially greater for all experiments when exogenous trypsin was added to 1 $\mu\text{g/ml}$ from day 1 to day 5 (open squares) and yields were lowest when no exogenous trypsin was added (open triangles). Addition of exogenous trypsin at 0.1 $\mu\text{g/ml}$ on day 1 did not significantly increase viral yield (closed circles).

[0322] Figures 6B-6C present the results of influenza infection of two different trypsinogen-expressing MDCK clones with MDV-A (left panels) or ca A/NC (right panels). The MDCK clones used in the experiment shown in Figures 6B were made by transfection with viral particles produced in Ampho 293 cells, while the MDCK clones used in these experiment shown in Figures 6C were made by transfection with viral particles produced in GP2 cells

[0323] As shown in Figures 6B-6C, viral titers were significantly increased when the infected cells expressed trypsinogen. In all cases, viral titers obtained in the absence of exogenous trypsin (open triangles) were less than those obtained in the presence of 1 $\mu\text{g/ml}$ trypsin (open squares), though greater than the viral titers observed for the controls. However, in several cases (*e.g.*, Fig. 6B top left, top right and bottom right; Fig. 6C top left and bottom right), addition of a small amount of trypsin (0.1 $\mu\text{g/ml}$) at day 1 (closed circles) increased resulting viral titers to be comparable to those observed in the presence of exogenous trypsin. Without intending to be bound to any particular theory or mechanism of action, it is believed that the addition of exogenous trypsin was sufficient to cleave the pro-peptide of trypsinogen, thereby activating the proenzyme to its fully active form. Thus, "priming" the culture with a small amount of peptidase can activate the proenzyme produced by the cells in the culture, increasing overall viral yields.

7.12 Example 12: Expression of Tagged Trypsinogen in MDCK Cells

[0324] This example describes production and detection of trypsinogen tagged with six histidine residues in MDCK cells.

[0325] To identify clones expressing trypsinogen at high levels, another set of clones were made by transfecting a gene encoding a 6xHis-labeled trypsinogen fusion protein into MDCK cells. Briefly, the recombinant nucleotide sequence encoding porcine trypsinogen presented as Figure 11 was cloned into the polylinker of pDESTTM26 (Invitrogen Inc., Carlsbad, CA) and the resulting vector transfected into MDCK cells. The pDESTTM26 plasmid encodes the 6x His tag adjacent to the polylinker, such that expression of the artificial trypsinogen gene from pDESTTM26 yields 6x His-tagged porcine trypsinogen. The transfected cells were then

cultured for 48 hours, then transfectants were selected by culturing for about 3 weeks in the presence of G418. 16 G418-resistant clones were then isolated and expanded as single clones.

[0326] Expression of trypsinogen by the 16 clones was then assessed by western blot.

5 Briefly, each clone was cultured for 1-2 days, then protein was isolated from the cells by collecting the cells by centrifugation, washing the cells once with 1x phosphate buffered saline, then lysing the cells in 200 μ l of 1x protein lysis buffer (Promega Corp.; Madison, WI). Next, 10 μ l of each protein lysate was loaded on a 12% denaturing polyacrylimide gel for Western blot analysis. 6xHis-labeled trypsinogen was detected with a rabbit anti-trypsin
10 polyclonal antibody (Chemicon AB1823; Chemicon International; Temecula, CA) specific for trypsin diluted at 1:10,000. Goat anti-rabbit HRP conjugated secondary antibody diluted at 1:3,000 was added, washed twice, and protein visualized by addition of ECL plus substrate (GE Healthcare Bio-Sciences Corp.; Piscataway, NJ). The results of the western blots are presented as Figures 7A and 7B. As shown in these figures, 12 of 16 G418 resistant MDCK
15 clones produced 6xHis-labeled trypsinogen in detectable amounts.

7.13 Example 13: Expression of Trypsin in MDCK Cells Under the Control of an Inducible Promoter

[0327] This example describes the construction of MDCK cell lines that express trypsin under the control of an inducible promoter.

20 [0328] To test another way to provide trypsin to cell cultures at the appropriate time following viral infection, MDCK cells that express trypsin under the control of an inducible promoter were made as follows. First, MDCK cell lines were transfected with pcDNATM6/TR (Invitrogen, Inc., Carlsbad, CA) and transfectants were selected by culturing in the presence of blasticidin. Antibiotic-resistant clones were then isolated and expanded from single
25 clones.

[0329] Next, expression of the tetracycline (tet) repressor protein from the integrated vector in the individual clones was tested with the positive control vector pDEST30Luc. (Invitrogen, Inc., Carlsbad, CA). The goal of this experiment was to identify MDCK clones that express sufficient tet repressor protein to have very low luciferase expression in the
30 absence of doxycycline (dox) and high expression in the presence of dox. To do so, approximately 20,000 cells from each clone to be screened were placed in each well of a 6-well microtiter plate. Each sample was transiently transfected with pDEST30Luc using conventional techniques, then dox was added to 3 of the wells at one day following

transfection. The cells were harvested at two days following transfection, and 20 μ l supernatant was used for each luciferase assay.

[0330] Results of the luciferase assays for 12 clones are presented as Figure 8. As shown in Figure 8, clone 3 exhibited the greatest difference between activities observed in the presence and absence of doxycycline. 36 MDCK-Trex clones were screened with the transient Luciferase assay. Two clones MDCK/R3 and MDCK/R7 were selected for making inducible Trypsin expression MDCK cells (fold induction: +DC/-DC=13.1 and 14.0, respectively). MDCK/R3 or R7 were transfected with pT-Rex-DEST30/Luciferase (Figure 13) as a control. 29 antibiotics resistant clones R3/Luc and R7/Luc were isolated and expanded for Luciferase assay with or without Doxycycline (DC) induction. There is up to a 70-fold difference for R3 and up to a 56-fold difference for R7 after the chemical induction (+DC/-DC= 70 and 56). Our data indicate that R3/Luc and R7/Luc clones stably carried Luciferase gene and that Luciferase expression is inducible by DC.

[0331] To make inducible trypsin expression MDCK cells, MDCK/R3 or R7 were transfected with pT-Rex-DEST30/Trypsin (Figure 13) and antibiotics resistant clones were selected with MDCK growth medium+Blasticidin/G418 for 2-3 weeks. 70 clones expressing trypsin were isolated and expanded for protease activity assays. Genomic DNAs from these clones were isolated and trypsin gene was detected by PCR. No DNA nucleoside mutations were found by DNA sequence analysis.

[0332] Next, the clones exhibiting the greatest difference between activities observed in the presence and absence of doxycycline are evaluated by growth curve studies such as those described in Example 11 to assess growth and titers of influenza viruses cultured on cells inducibly expressing trypsinogen. Approximately 15 clones had high trypsin with DC induction and low trypsin expression without DC. For example, one clone R3/6U7 has baseline Trypsin expression of ~ 10 ng/ml in the absence of DC which increases to ~ 2.5 μ g/ml (~ 250 fold induction) when induced with 3 μ g/ml DC. For the replication of most influenza vaccine strains trypsin is generally used at a concentration of ~ 1.0 μ g/ml. The expression level of trypsin in these cells is induced and controlled with different DC concentrations for flu vaccine production.

[0333] Many inducible trypsin expression MDCK clones are permissive for infection and replication of influenza strains useful for the production of vaccines. The parental MDCK cells generally grow as adherent cells with less than 10% of the cells in suspension.

However, the inducible trypsin MDCK clones are less adherent with about 30% of the cells growing in suspension cells. The majority of the cells in suspension (>90%) are viable by trypan blue staining analysis. By adjusting the endogenous trypsin expression, the percent of cells in suspension may be increased. The use of suspension cells for flu vaccine production can reduce the cost of goods by eliminating the need for carriers.

7.14 Example 14: Cloning of a Bacterial Serine Protease from *Streptomyces griseus*

[0334] This example describes the cloning of a serine protease from the bacterium *Streptomyces griseus*.

[0335] To do so, a primer pair (Spr T forward and reverse) was used to amplify the coding sequence of the SPRT protease from genomic DNA using conventional techniques. The nucleotide sequences of the primer pair are presented as Figure 12. A *Streptomyces griseus* strain deposited with the ATCC (Accession No. 23915) was used as the source for the genomic DNA. The sprT gene was then cloned into pDESTTM 14 using conventional techniques, and the nucleotide sequence of the sprT gene determined using conventional techniques.

[0336] The nucleotide sequence of the sprT gene is presented as Figure 9.

[0337] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above may be used in various combinations. All publications, patents, patent applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.

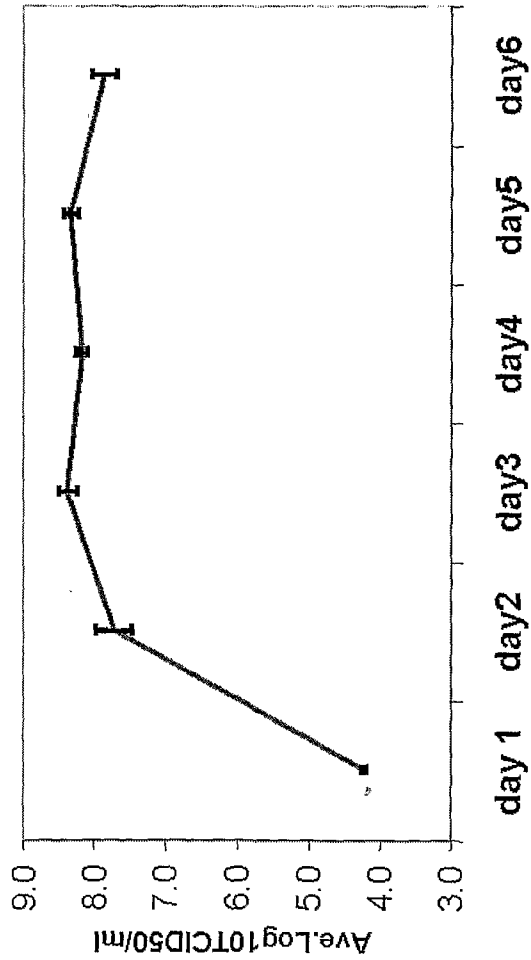
In addition, U.S. Provisional Patent Application Nos.: U.S. 60/ 793,522, filed April 19, 2006; U.S. 60/ 793,525, filed April 19, 2006; U.S. 60/702,006, filed July 22, 2005; U.S. 60/699,556, filed July 15, 2005; U.S. 60/699,555, filed July 15, 2005; U.S. 60/692,965 filed June 21, 2005; and U.S. 60/692,978 filed June 21, 2005, are incorporated by reference in their entirety for all purposes.

What is claimed is:

1. A host cell comprising a nucleic acid encoding a protease or pro-protease, wherein the nucleic acid encoding the protease or pro-protease is integrated into the host cell's
5 genome, and wherein the protease or pro-protease is not normally expressed in the cell.
2. The host cell of claim 1, wherein the cell expresses a protease.
3. The host cell of claim 2, wherein the protease is a serine protease.
4. The host cell of claim 1, wherein the protease is a pro-protease.
- 10 5. The host cell of claim 1, wherein expression of the protease or pro-protease is under the control of an inducible promoter.
6. The host cell of claim 1, wherein expression of the protease or pro-protease is under the control of a constitutively active promoter.
7. The host cell of claim 1, wherein the nucleic acid comprises a secretion signal that
15 directs secretion of the protease or pro-protease.
8. The host cell of claim 1, wherein the cell is a mammalian cell.
9. The host cell of claim 8, wherein the mammalian cell is a human cell, a primate cell, a canine cell, hamster cell, mouse cell, or rat cell.
10. The host cell of claim 1, wherein the cell is an avian cell.
- 20 11. The host cell of claim 1, wherein the host cell grows in suspension without adaptation.
12. A method of producing an influenza virus, comprising:
 - a) introducing an influenza genome into the host cell of claim 1 cell by infecting the
25 host cell with an influenza virus, or transfecting the host cell with nucleic acids encoding an influenza genome;
 - b) culturing the cell under conditions that allow replication of the influenza virus;
and
 - c) collecting influenza virus from the cell culture.

13. The method of claim 12, wherein the host cell expresses a pro-protease, and wherein the method further comprises adding an exogenous protease to the culture medium.
14. The method of claim 13, wherein the exogenous protease is added to a maximum
5 concentration of about 0.1 µg/ml.
15. A method of replicating an influenza virus in the absence of exogenously added protease, comprising:
- a) introducing an influenza genome into the host cell of claim 1 by infecting the host
10 cell with an influenza virus or transfecting the host cell with nucleic acids
encoding an influenza genome;
- b) culturing the cell under conditions that allow replication of the influenza virus,
wherein said conditions do not include exogenously added protease; and
- c) collecting influenza virus from the cell culture.
16. A method of increasing the titer of influenza virus grown in cell culture, comprising
15 culturing the influenza virus in a cell culture, wherein the cells in the cell culture
stably expresses a protease or pro-protease that i) is heterologous to the cell, and ii)
cleaves a hemagglutinin of the influenza virus, thereby increasing the titer of the
influenza virus grown in the cell culture relative to the titer obtained by culturing the
influenza virus in cells that do not express a heterologous protease or pro-protease.
- 20 17. A method for producing a heterologous protease or pro-protease in a host cell capable
of supporting influenza replication, comprising culturing a cell comprising a nucleic
acid encoding a protease or pro-protease not normally expressed in the cell under
conditions that permit expression of said protease or pro-protease, thereby producing
the protease or pro-protease in the cell.
- 25 18. The method of claim 17, wherein the cell expresses an amount of protease or pro-
protease sufficient to increase the titer of virus grown in a culture of the host cells
expressing the protease or pro-protease.
19. An isolated nucleic acid comprising SEQ ID NO.:1.

20. An isolated polypeptide comprising the amino acid of SEQ ID NO.:2.
21. An expression vector comprising the nucleic acid of claim 19.
22. An isolated cell comprising the expression vector of claim 21.

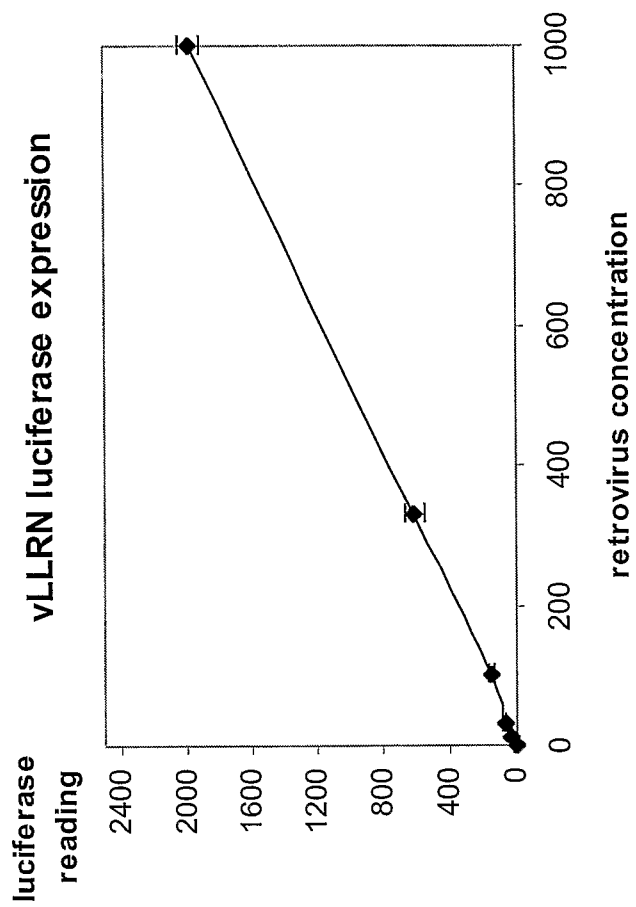


Replication of ca A/Vietnam/1203/2004 (H5N1) in MDCK cells

Fig. 1

Sample	Read 1	Read 2	Read 3	Ave	SD	CV
Mock	31	39	39	36	4.6	0.1
VSVG/Luc	18282	17803	19681	18589	975.8	0.1
VSVG/Vector	148	146	181	158	19.7	0.1
VSVG/Tgen	51	36	67	51	15.5	0.3
Luc 100 pg	698	712	728	713	15.0	0.0

Fig. 2

**Fig. 3**

Sample	Number	Read 1	Read 2	Read 3	Ave	SD	CV
Luc control	4 ng	129993	126327	122143	126154	3927.8	0.0
Luc control	0.02 ng	785	836	766	796	36.2	0.0
GP2V-MDCK	Mix	298	289	247	278	27.2	0.1
GP2L-MDCK	#1	55103	50048	47341	50831	3939.7	0.1
GP2L-MDCK	#2	53638	48254	45491	49128	4143.2	0.1
GP2L-MDCK	#3	60270	54342	51365	55326	4533.3	0.1
GP2L-MDCK	#4	67511	60445	57121	61692	5306.1	0.1
GP2L-MDCK	#5	45429	40880	37881	41397	3800.4	0.1
GP2L-MDCK	#6	29817	26864	25228	27303	2325.8	0.1
GP2L-MDCK	#7	24582	22347	21067	22665	1779.0	0.1
GP2L-MDCK	#8	43642	39165	37032	39946	3373.6	0.1
GP2L-MDCK	#9	46608	41974	38328	42303	4149.8	0.1
GP2L-MDCK	#10	55194	49603	47038	50612	4170.5	0.1
GP2L-MDCK	#11	42621	38085	36421	39042	3209.0	0.1
GP2L-MDCK	#12	7022	7099	7498	7206	255.5	0.0
GP2L-MDCK	#13-15M	71655	63972	60476	65368	5718.7	0.1
GP2L-MDCK	#16-18M	63397	57189	54151	58246	4712.7	0.1

Fig. 4

MDCK cells	Retrovirus	Clones	Clone mix
ATCC P64	Ampho293/trypsinogen	18	1
ATCC P64	Ampho293/vector	6	2
ATCC P64	GP2-293/trypsinogen	18	0
ATCC P64	GP2-293/vector	12	2
ATCC P64	GP2-293/luciferase	12	2
ATCC P64	Mock	0	0
Total		66	7

Fig. 5

Cell controls

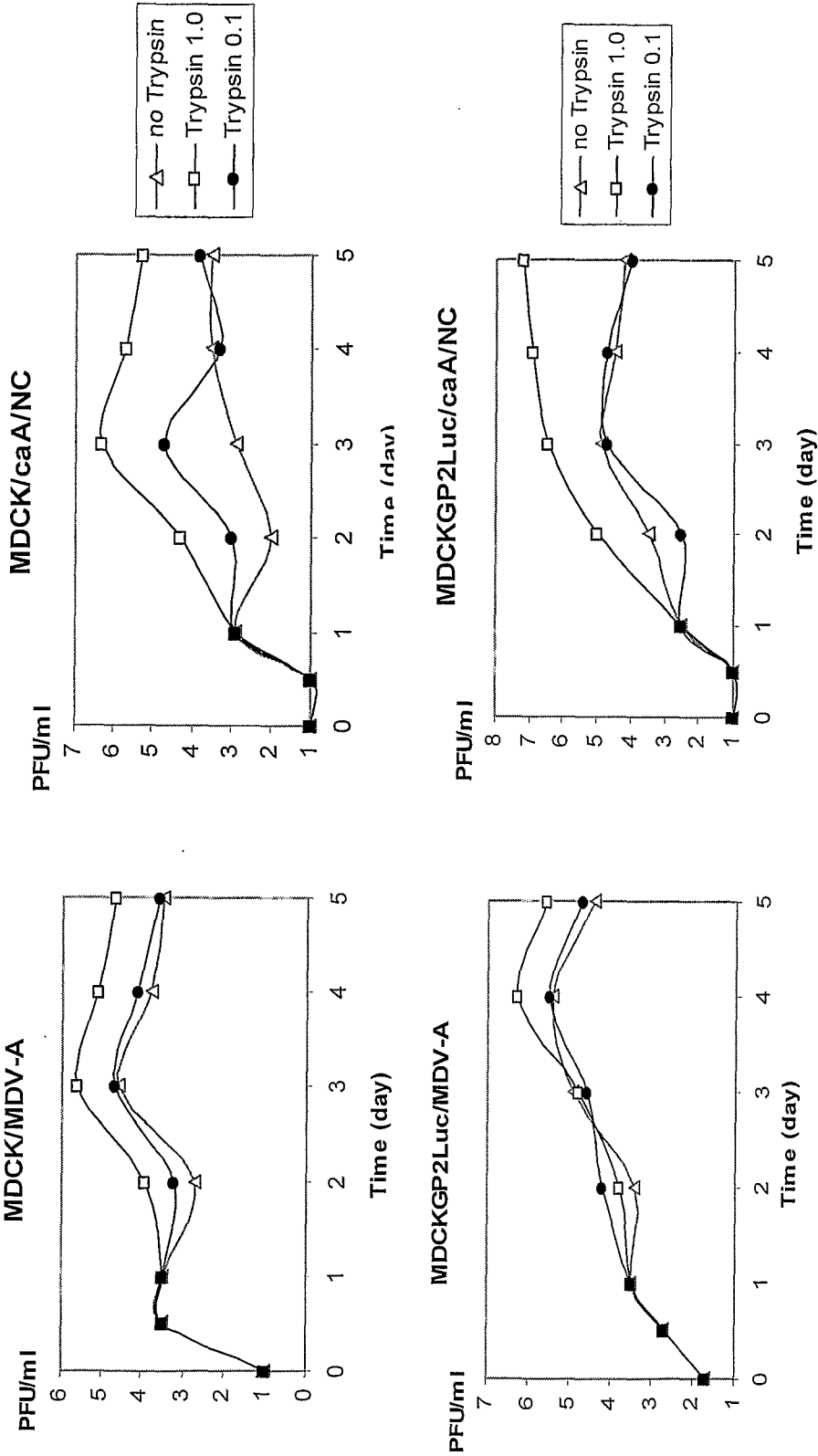


Fig. 6A

MDCKAmphoTrypsinogen

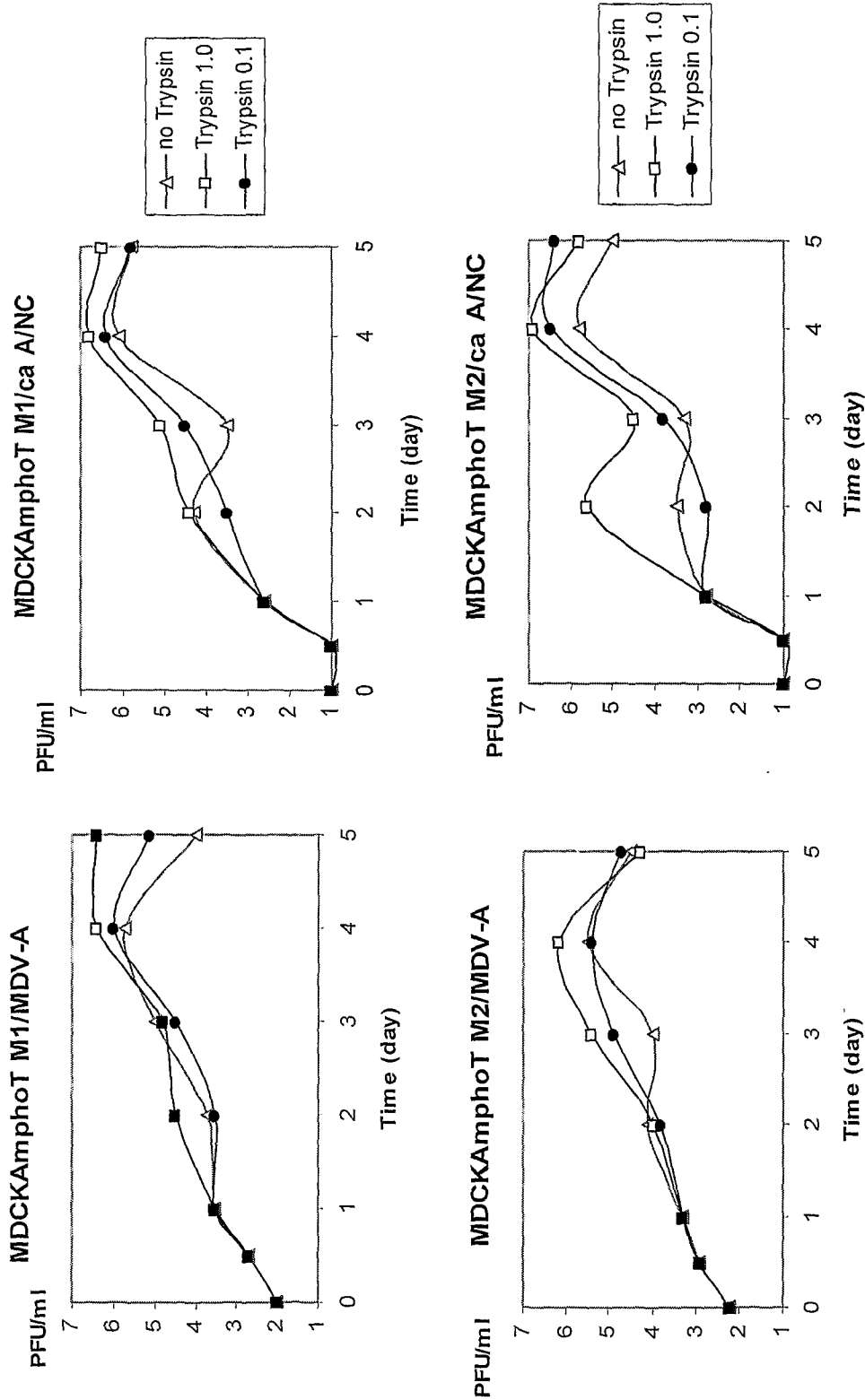


Fig. 6B

MDCK_GP2Trypsinogen

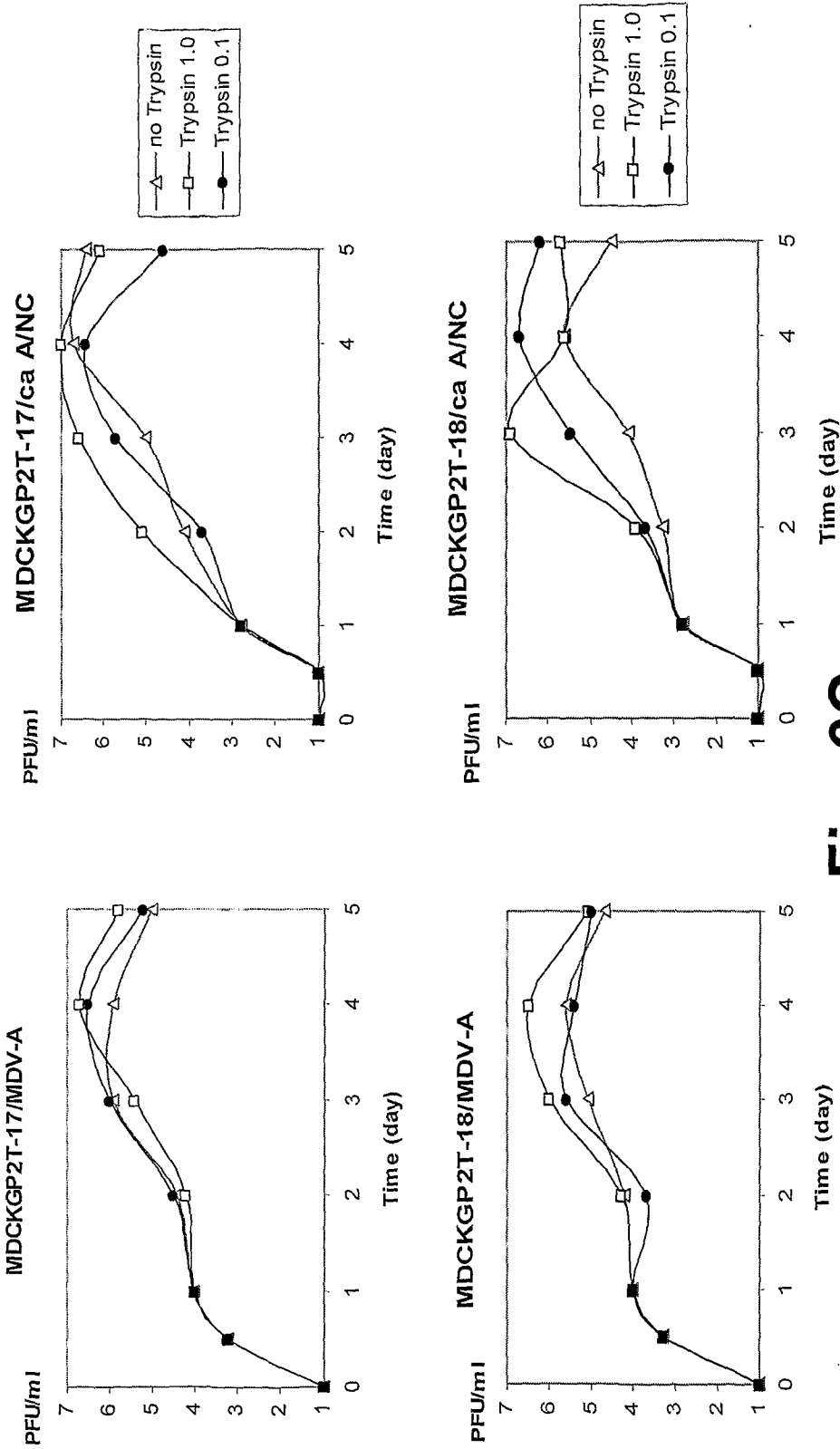


Fig. 6C

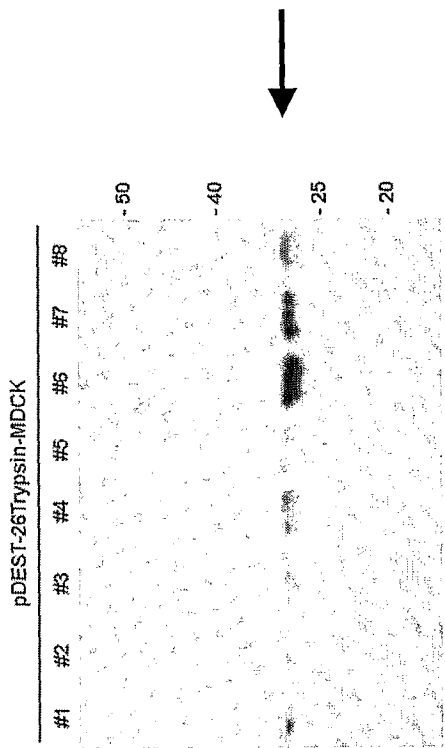


Fig. 7A

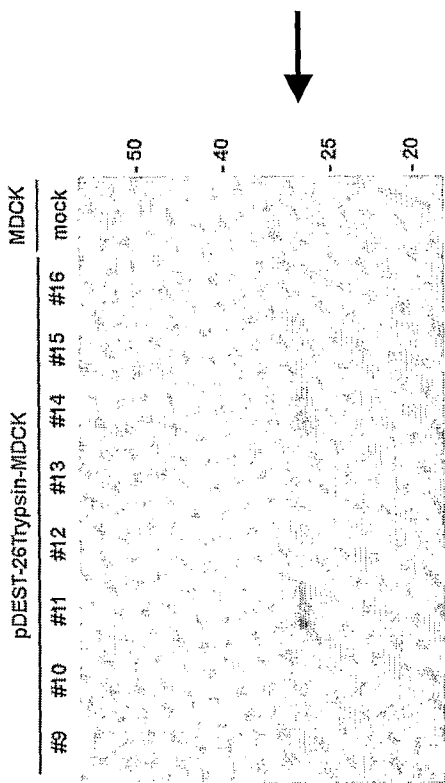


Fig. 7B

Fig. 7

Inducible luciferase expression

Cells	+DC			-DC			Ave	SD	+DC/-DC		
HEK-Trex	226541	215587	246582	4017	3433	4541	3997	554.3	57.4	↓	
MD-Trex1	102	212	113	165	149	274	196	68.0	0.7		
MD-Trex2	373	324	663	173	155	104	144	35.8	3.1		
MD-Trex3	430	637	834	21	15	20	19	3.2	33.9	↓	
MD-Trex4	404	160	322	200	362	323	295	84.6	1.0		
MD-Trex5	527	1139	406	795	1167	872	945	196.4	0.7		
MD-Trex6	1597	795	611	670	452	527	550	110.8	1.8		
MD-Trex7	248	240	102	16	23	33	24	8.5	8.2		
MD-Trex8	450	467	604	65	59	41	55	12.5	9.2		
MD-Trex12	324	397	127	275	383	261	306	66.8	0.9		
MD-Trex13	3079	4659	6043	2015	2868	2513	2465	428.5	1.9		
MD-Trex14	584	904	746	606	456	446	503	89.6	1.5		
MD-Trex15	918	1400	1395	1358	2743	1453	1851	773.7	0.7		

Fig. 8

(SEQ ID NO:1)

AGATCACAGTTTGTACAAAAAAGCAGGCTGCGTTAACGAATTTCGCCCTTCCCCACGAAAGGCAATCC**GTGA**
 AGCAACTCCTGCGTGCCCTCCAGAGATGTTCCGTCGTCCGCCACCGTCGCCATCGCCGTCGTGGCCCTCCAG
 CCCGTACGGCGTCGGCAGCCCCCAACCC**GTCT**CGTCGGCGGAACCCGCGCCGCCAGGGCGAGTTCCCCCTTCAT
 GGTCCGGCTCTCCATGGGCTGGGGCGCGCTGTACGCCAAGGACATCGTCTCACCGCCGCCCACTGTGTGA
 ACGGATCGGGCAACACACCTCGATCACCGCCACCGCGCGTCTCGACCTCCAGTCCAGTCGTCAGCGCATCAAG
 GTCCGCTCCACCAAGTCTCCAGGCCCCCGGTACAACGGCACCGCAAGGACTGGGCGTCAATCAAGCTCGC
 CCAGCCCATCAACAGCCACGCTGAAGATCGCCACCAACCGGTACAAACAGGCACTTACCGTCGCCG
 GCTGGGGCGCCAAACCGGAGGGCGGAGCCAGCGCTACCTGCTCAAGGCCAACGTCCCCTCGTCTCCGAC
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 GCTTCGCCCATCGCCTCGGCCCGCCGACGCTCTGACGGCACGTACCGCACCCATCGATAAACCAGCTTCTT
 GTACAAAGTGNATGATCCGGCTGCTANNAAGCCCGAAAGGAAGCTGAGTGGCTGCTGCACGCTGAGCATACTAG
 CATACCCCTTGGGGCTCTAACG

The Start Codon for PreproSprT is shown in bold.

The first codon of mature SPRT protease is shown in *bold italic*.

Fig. 9

(SEQ ID NO:2)

MKQLLRALQRCVVVATVAIAVVGLQPVTA SAAPNPVVGTRAAQGEFFFMVRLSMGCGGALYAKDIVLTAA
HCVNGSGNNTSITATAGVVDLQSSSAIKVRSTKVLQAPGYNGTGKDWALIKLAQPINQPTLKIATTTAYNQG
TFTVAGWGANREGSQQRYYLLKANVPFVSDAACRSA YGNELVANEEICAGYPDTGGVDTCQGDSDGGPMFRKD
NADEWIQVGIVSWGYGCARPGYPGVYTEVSTFASAIASAARTL

Fig. 10

(SEQ ID NO.:3)

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Fig. 11

Forward SprT primer:	5'	ccccacgaaagaaggcaatccgtg	3'	(SEQ ID NO.: 4)
Reverse SprT primer:	5'	GGTGCCGGTACGTGCCGTCAG	3'	(SEQ ID NO.: 5)

Fig. 12

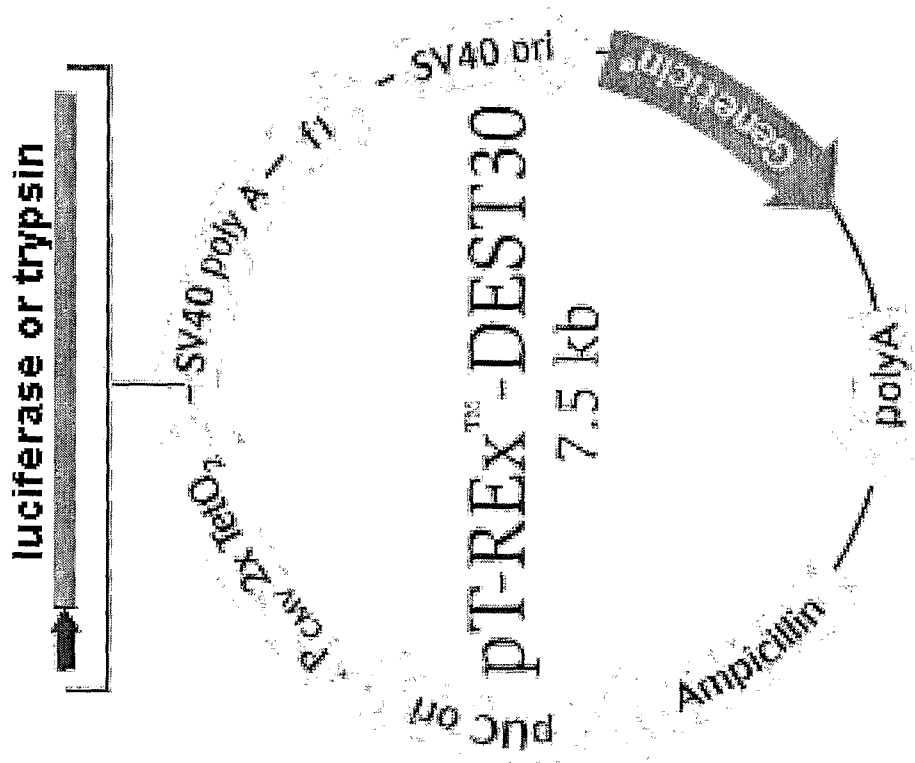


Fig. 13

SEQUENCE LISTING

<110> MedImmune Vaccines, Inc
 Duke, Gregory
 Kemble, George
 Young, James
 Mo, Chengjun
 Hazari, Nisha

<120> METHODS AND COMPOSITIONS FOR EXPRESSING A HETEROLOGOUS PROTEASE

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Pro Phe Met Val Arg Leu Ser Met Gly Cys Gly Gly Ala Leu Tyr Ala
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