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(54) Title: INFLUENZA VIRUS REASSORTMENT

(57) Abstract: New influenza donor strains for the production of reassortant influenza B viruses are provided.

INFLUENZA VIRUS REASSORTMENT

This invention was made in part with Government support under grant no. HHSO10020100061C awarded by the Biomedical Advanced Research and Development Authority (BARDA). The Government has certain rights in the invention.

This application claims the benefit of US provisional application 61/779,888 filed 13th March 2013, the complete contents of which are incorporated herein by reference.

TECHNICAL FIELD

This invention is in the field of influenza B virus reassortment. Furthermore, it relates to manufacturing vaccines for protecting against influenza B viruses.

BACKGROUND ART

The most efficient protection against influenza infection is vaccination against circulating strains and it is important to produce influenza viruses for vaccine production as quickly as possible.

Wild-type influenza viruses often grow to low titres in eggs and cell culture. In order to obtain a better-growing virus strain for vaccine production it is possible to reassort the circulating vaccine strain with a faster-growing high-yield donor strain. This can be achieved by co-infecting a culture host with the circulating influenza strain and the high-yield donor strain and selecting for reassortant viruses which contain the hemagglutinin (HA) and neuraminidase (NA) segments from the vaccine strain and the other viral segments (*i.e.* those encoding PB1, PB2, PA, NP, M_1, M_2, NS_1 and NS_2) from the donor strain. Another approach is to reassort the influenza viruses by reverse genetics (see, for example references 1 and 2).

Whilst it is common practice to use reassortant influenza A strains in vaccine production, reassortant influenza B strains are not usually used because wild-type influenza B viruses usually provide adequate yields in eggs. Furthermore, wild-type influenza B viruses have been reported to have a growth advantage over reassortant influenza B viruses (see, for example, reference 3). Accordingly, high growth influenza B reassortants have been generated only for a small number of recent influenza B viruses. These reassortants typically contain a mixture of backbone gene segments derived from B/Lee/40, B/Brisbane/60/08 and B/Panama/45/90 (4, 5).

To date, only two reassortant influenza B viruses (BX-35 and BX-39) have been used for commercial vaccine manufacturing. BX-35 contains the HA, NA, PA, PB1, and NS segments from the B/Brisbane/60/08 strain, the PB2 and M segments from B/Panama/45/90, and the NP segment from B/Lee/40. BX-39 contains the HA, NA, PBI, and M segments from the circulating B/Hubei-Wujiagang/159/08 strain, the PA and NS segments from B/Panama/45/90, and the PB2 and NP segments from B/Lee/40(6, 7).

There are currently only a limited number of donor strains for reassorting influenza B viruses for vaccine manufacture and the known reassortant influenza B viruses do not always grow better than

the parent strain. Thus, there is a need in the art to provide further and improved donor strains for influenza B virus reassortment.

SUMMARY OF PREFERRED EMBODIMENTS

The invention thus provides reassortant influenza B viruses which can grow at the same speed or faster in a culture host (particularly in cell culture) compared to the corresponding wild-type influenza B virus from which the HA segment is derived. For example, the inventors have surprisingly discovered that a reassortant influenza B virus which comprises the HA segment from a first influenza B virus and the NP and/or PB2 segment from a second influenza B virus which is a B/Victoria/2/87-like strain grows particularly well in cell culture and eggs. The B/Victoria/2/87-like strain may be B/Brisbane/60/08.

The invention also provides reassortant influenza B viruses comprising the HA segment from a first influenza B virus and the NP segment from a second influenza B virus which is not B/Lee/40 or B/Ann Arbor/1/66 or B/Panama/45/90. For example, the reassortant influenza B virus may have a NP segment which does not have the sequence of SEQ ID NOs: 33, 38, 39 or 43. The reassortant influenza B virus may also have a NP segment which does not encode the protein of SEQ ID NOs: 19, 23, 44 or 45. The inventors have discovered that reassortant influenza B viruses which comprise a NP segment from an influenza B virus other than B/Lee/40 or B/Ann Arbor/1/66 or B/Panama/45/90 can grow very well in a culture host. The reassortant influenza B virus may comprise both the NP and PB2 segments from the second influenza B virus. The second influenza B virus is preferably a B/Victoria/2/87-like strain. The B/Victoria/2/87-like strain may be B/Brisbane/60/08.

The inventors have also discovered that a reassortant influenza B virus comprising the HA segment from a B/Yamagata/16/88-like strain and at least one backbone segment from a B/Victoria/2/87-like strain can grow well in a culture host. The reassortant influenza B virus may comprise two, three, four, five or six backbone segments from the B/Victoria/2/87-like strain. In a preferred embodiment, the reassortant influenza B virus comprises all the backbone segments from the B/Victoria/2/87-like strain. The B/Victoria/2/87-like strain may be B/Brisbane/60/08.

The invention also provides a reassortant influenza B virus comprising viral segments from a B/Victoria/2/87-like strain and a B/Yamagata/16/88-like strain, wherein the ratio of segments from the B/Victoria/2/87-like strain and the B/Yamagata/16/88-like strain is 1:7, 2:6, 3:5, 4:4, 5:3, 6:2 or 7:1. A ratio of 7:1, 6:2, 4:4, 3:5 or 1:7, in particular a ratio of 4:4, is preferred because such reassortant influenza B viruses grow particularly well in a culture host. The B/Victoria/2/87-like strain may be B/Brisbane/60/08. The B/Yamagata/16/88-like strain may be B/Panama/45/90. In these embodiments, the reassortant influenza B virus usually does not comprise all backbone segments from the same influenza B donor strain.

Also provided is a reassortant influenza B virus which comprises:

a) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 16 and the M segment of SEQ ID NO: 15; or

b) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34; or

c) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 32, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 16 and the M segment of SEQ ID NO: 15; or

d) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 16 and the M segment of SEQ ID NO: 15, or

e) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

f) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 33, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

g) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 32, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

h) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 33, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

i) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 32, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

j) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

k) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34.

In these reassortant influenza B viruses, the HA and NA segments may be from any influenza B strain.

Reassortant influenza B viruses with the combinations of segments as discussed in section (a) to (k) above are preferred because the inventors have shown that they grow particularly well in culture hosts. The reassortant influenza B strains of sections (a), (b) and (e) grow particularly well in culture hosts and are therefore particularly preferred.

The invention also provides variants of the reassortant influenza B viruses identified in sections (a) to (k) above which comprise viral segments that have at least 97% identity, at least 98% identity, or at least 99% identity to the viral segments identified in these sections. Such variants can preferably grow to a viral titre in a culture host which is within 3% of the viral titre achieved with the reassortant influenza B strain from which the variant is derived in the same time and under the same growth conditions.

The invention provides methods of preparing the reassortant influenza B viruses of the invention. These methods comprise steps of (i) introducing into a culture host one or more expression construct(s) which encode(s) the viral segments required to produce a reassortant influenza B virus of the invention and (ii) culturing the culture host in order to produce the reassortant virus; and optionally (iii) purifying the virus obtained in step (ii).

These methods may further comprise steps of: (iv) infecting a culture host with the virus obtained in step (ii) or step (iii); (v) culturing the culture host from step (iv) to produce further virus; and optionally (vi) purifying the virus obtained in step (v).

Expression constructs which can be used in the methods of the invention are also provided.

For example, the expression construct(s) may encode a reassortant influenza B virus comprising the HA segment from a first influenza B virus and the NP and/or PB2 segment from a second influenza B virus which is a B/Victoria/2/87-like strain. The NP and PB2 segments may both be from the B/Victoria/2/87-like strain. The B/Victoria/2/87-like strain is preferably B/Brisbane/60/08.

The expression construct(s) may also encode a reassortant influenza B virus comprising the HA segment from a first influenza B virus and the NP segment from a second influenza B virus which is not B/Lee/40 or B/Ann Arbor/1/66 or B/Panama/45/90. For example, the expression construct(s) may not encode a NP segment with the sequence of SEQ ID NOs: 19, 23, 44 or 45. The NP and PB2 segments may both be from the second influenza B virus. The second influenza B virus may be a B/Victoria/2/87-like strain and is preferably B/Brisbane/60/08.

The “first influenza virus” and the “second influenza virus” are different to each other.

The expression construct(s) can encode a reassortant influenza B virus comprising viral segments from a B/Victoria/2/87-like strain and a B/Yamagata/16/88-like strain, wherein the ratio of segments from the B/Victoria/2/87-like strain and the B/Yamagata/16/88-like strain is 1:7, 2:6, 3:5, 4:4, 5:3, 6:2 or 7:1. A ratio of 7:1, 6:2, 4:4, 3:5 or 1:7, in particular a ratio of 4:4, is preferred. The B/Victoria/2/87-like strain may be B/Brisbane/60/08. The B/Yamagata/16/88-like strain may be B/Panama/45/90.

Also provided are expression construct(s) which encode(s) a reassortant influenza B virus as described above.

The invention provides an expression system comprising one or more expression construct(s) of the invention. The invention also provides a host cell comprising an expression system of the invention. These host cells can express an influenza B virus from the expression construct(s) in the expression system.

The invention also provides a method for producing influenza viruses comprising steps of (a) infecting a culture host with a reassortant virus of the invention; (b) culturing the host from step (a) to produce the virus; and optionally (c) purifying the virus obtained in step (b).

The invention also provides a method of preparing a vaccine, comprising the steps of (a) preparing a virus by the methods of any one of the embodiments described above and (b) preparing a vaccine from the virus.

Also provided is a method of preparing a vaccine from the reassortant influenza B virus of the invention.

The invention also provides a vaccine which can be obtained by the methods of the invention.

Reassortant viruses

The reassortant influenza B strains of the invention contain viral segments from a vaccine strain and one or more donor strain(s). The vaccine strain is the influenza strain which provides the HA segment of the reassortant influenza B strain. The vaccine strain can be any strain and can vary from season to season.

A donor strain is an influenza B strain which provides one or more of the backbone segments (*i.e.* those encoding PB1, PB2, PA, NP, M_1, M_2, NS_1 and NS_2) of the influenza B strain. The NA segment may also be provided by a donor strain or it may be provided by the vaccine strain. The reassortant influenza B viruses of the invention may also comprise one or more, but not all, of the backbone segments from the vaccine strain. As the reassortant influenza B virus contains a total of eight segments, it will therefore contain *x* (wherein *x* is from 1-7) viral segments from the vaccine strain and 8-*x* viral segments from the one or more donor strain(s).

As mentioned above, the purpose of the invention is to provide reassortant influenza B strains which, once rescued, can grow to higher or similar viral titres in a culture host. Thus, the reassortant

influenza B virus strains of the invention can grow to higher or similar viral titres in cell culture and/or in eggs in the same time (for example 12 hours, 24 hours, 48 hours or 72 hours) and under the same growth conditions compared to the wild-type vaccine strain. In particular, they can grow to higher or similar viral titres in MDCK cells (such as MDCK 33016) in the same time and under the same growth conditions compared to the wild-type vaccine strain. The viral titre can be determined by standard methods known to those of skill in the art. Usefully, the reassortant influenza B viruses of the invention may achieve a viral titre which is at least 5% higher, at least 10% higher, at least 20% higher, at least 50% higher, at least 100% higher, at least 200% higher, or at least 500% higher than the viral titre of the wild-type vaccine strain in the same time frame and under the same conditions. The reassortant influenza B viruses may also grow to similar viral titres in the same time and under the same growth conditions compared to the wild-type vaccine strain. A similar titre in this context means that the reassortant influenza B viruses grow to a titre which is within 3% of the viral titre achieved with the wild-type vaccine strain in the same time and under the same growth conditions (*i.e.* wild-type titre ± 3%).

Influenza B viruses currently do not display different HA subtypes, but influenza B virus strains do fall into two distinct lineages. These lineages emerged in the late 1980s and have HAs which can be antigenically and/or genetically distinguished from each other [8]. Current influenza B virus strains are either B/Victoria/2/87-like or B/Yamagata/16/88-like. These strains are usually distinguished antigenically, but differences in amino acid sequences have also been described for distinguishing the two lineages *e.g.* B/Yamagata/16/88-like strains often (but not always) have HA proteins with deletions at amino acid residue 164, numbered relative to the 'Lee40' HA sequence [9]. In some embodiments, the reassortant influenza B viruses of the invention may comprise viral segments from a B/Victoria/2/87-like strain. They may comprise viral segments from a B/Yamagata/16/88-like strain. Alternatively, they may comprise viral segments from a B/Victoria/2/87-like strain and a B/Yamagata/16/88-like strain.

Where the reassortant influenza B virus comprises viral segments from two or more influenza B virus strains, these viral segments may be derived from influenza B strains which have related neuraminidases. For instance, the influenza B strains which provide the viral segments may both have a B/Victoria/2/87-like neuraminidase [10] or may both have a B/Yamagata/16/88-like neuraminidase. For example, two B/Victoria/2/87-like neuraminidases may both have one or more of the following sequence characteristics: (1) not a serine at residue 27, but preferably a leucine; (2) not a glutamate at residue 44, but preferably a lysine; (3) not a threonine at residue 46, but preferably an isoleucine; (4) not a proline at residue 51, but preferably a serine; (5) not an arginine at residue 65, but preferably a histidine; (6) not a glycine at residue 70, but preferably a glutamate; (7) not a leucine at residue 73, but preferably a phenylalanine; and/or (8) not a proline at residue 88, but preferably a glutamine. Similarly, in some embodiments the neuraminidase may have a deletion at residue 43, or it may have a threonine; a deletion at residue 43, arising from a trinucleotide deletion in the NA gene,

which has been reported as a characteristic of B/Victoria/2/87-like strains, although recent strains have regained Thr-43 [10]. Conversely, of course, the opposite characteristics may be shared by two B/Yamagata/16/88-like neuraminidases *e.g.* S27, E44, T46, P51, R65, G70, L73, and/or P88. These amino acids are numbered relative to the 'Lee40' neuraminidase sequence [11]. The reassortant influenza B virus may comprise a NA segment with the characteristics described above. Alternatively, or in addition, the reassortant influenza B virus may comprise a viral segment (other than NA) from an influenza B strain with a NA segment with the characteristics described above.

The backbone viral segments of an influenza B virus which is a B/Victoria/2/87-like strain can have a higher level of identity to the corresponding viral segment from B/Victoria/2/87 than it does to the corresponding viral segment of B/Yamagata/16/88 and *vice versa*. For example, the NP segment of B/Panama/45/90 (which is a B/Yamagata/16/88-like strain) has 99% identity to the NP segment of B/Yamagata/16/88 and only 96% identity to the NP segment of B/Victoria/2/87.

Where the reassortant influenza B virus of the invention comprises a backbone viral segment from a B/Victoria/2/87-like strain, the viral segments may encode proteins with the following sequences. The PA protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 1. The PB1 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 2. The PB2 protein may have at least 97%, at least 98%, at least 99% or 100% identity to the sequence of SEQ ID NO: 3. The NP protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 4. The M_1 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 5. The M_2 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 6. The NS_1 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 7. The NS_2 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 8. In some embodiments, the reassortant influenza B virus may also comprise all of these backbone segments.

Where the reassortant influenza B viruses of the invention comprise a backbone viral segment from a B/Yamagata/16/88-like strain, the viral segment may encode proteins with the following sequences. The PA protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 20. The PB1 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 21. The PB2 protein may have at least 97%, at least 98%, at least 99% or 100% identity to the sequence of SEQ ID NO: 22. The NP protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 23. The M_1 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 24. The M_2 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 25. The NS_1 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity

to the sequence of SEQ ID NO: 26. The NS_2 protein may have at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 27.

The invention can be practised with donor strains having a viral segment that has at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least about 99%, or 100% identity to a sequence of SEQ ID NOs 11-16 or 30-35. Due to the degeneracy of the genetic code, it is possible to have the same polypeptide encoded by several nucleic acids with different sequences. For example, the nucleic acid sequences of SEQ ID NOs: 40 and 41 have only 73% identity even though they encode the same viral protein. Thus, the invention may be practised with viral segments that encode the same polypeptides as the sequences of SEQ ID NOs 11-16 or 30-35.

In general a reassortant influenza virus will contain only one of each backbone segment. For example, when the influenza virus comprises the NP segment from B/Brisbane/60/08 it will not at the same time comprise the NP segment from another influenza strain.

In some embodiments, the reassortant influenza B virus of the invention may comprise all backbone segments from the same influenza B donor strain. It may alternatively comprise backbone segments from more than one influenza donor strain, for example from two, three, four or five donor strains. Where the reassortant influenza B virus comprises backbone segments from two or three donor strains, each donor strain may provide more than one of the backbone segments of the reassortant influenza B virus, but one or two of the donor strains can also provide only a single backbone segment. It is preferred that at least one of the backbone segments is from a B/Yamagata/16/88-like strain as the inventors have found that such reassortant influenza viruses grow well in cell culture. A preferred B/Yamagata/16/88-like strain in the context of the invention is B/Panama/45/90. In general the reassortant influenza B virus cannot comprise more than six backbone segments. Accordingly, for example, if one of the donor strains provides five of the viral segments, the reassortant influenza B virus can only comprise backbone segments from a total of two different influenza strains (for example, two donor strains or a donor strain and a vaccine strain).

When the reassortant influenza B virus comprises the backbone segments from a single donor strain, the reassortant viruses will generally include segments from the donor strain and the vaccine strain in a ratio of 1:7, 2:6, 3:5, 4:4, 5:3, 6:2 or 7:1. When the reassortant viruses comprise backbone segments from two donor strains, the reassortant virus can include segments from the first donor strain, the second donor strain and the vaccine strain in a ratio of 1:1:6, 1:2:5, 1:3:4, 1:4:3, 1:5:2, 1:6:1, 2:1:5, 2:2:4, 2:3:3, 2:4:2, 2:5:1, 3:1:4, 3:2:3, 3:3:2, 3:4:1, 4:1:3, 4:2:2, 4:3:1, 5:1:2, 5:2:1 or 6:1:1.

The reassortant influenza B viruses contain the HA segment from the vaccine strain as this encodes the main vaccine antigens of the influenza virus and therefore comes from the vaccine strain. The reassortant viruses of the invention preferably also have the NA segment from the vaccine strain, but

the invention also encompasses reassortants which comprise the HA and NA segments from different strains.

Strains which can be used as vaccine strains include strains which are resistant to antiviral therapy (*e.g.* resistant to oseltamivir [12] and/or zanamivir), including resistant pandemic strains [13].

Reassortant viruses which contain an NS segment that does not encode a functional NS protein are also within the scope of the present invention. NS1 knockout mutants are described in reference 14. These NS1-mutant virus strains are particularly suitable for preparing live attenuated influenza vaccines.

Variations in the DNA and the amino acid sequence may stem from spontaneous mutations which can occur during passaging of the viruses. Such variant influenza strains can also be used in the invention.

Reverse genetics

The invention is particularly suitable for producing the reassortant influenza B virus strains through reverse genetics techniques. In these techniques, the viruses are produced in culture hosts using one or more expression construct(s). The expression construct(s) may encode all the segments which are necessary to produce the reassortant influenza B viruses of the invention.

Reverse genetics for influenza viruses can be practised with 12 plasmids to express the four proteins required to initiate replication and transcription (PB1, PB2, PA and NP) and all eight viral genome segments. To reduce the number of constructs, however, a plurality of RNA polymerase I transcription cassettes (for viral RNA synthesis) can be included on a single plasmid (*e.g.* sequences encoding 1, 2, 3, 4, 5, 6, 7 or all 8 influenza vRNA segments), and a plurality of protein-coding regions with RNA polymerase II promoters on another plasmid (*e.g.* sequences encoding 1, 2, 3, 4, 5, 6, 7 or 8 influenza mRNA transcripts) [15]. It is also possible to include one or more influenza vRNA segments under control of a pol I promoter and one or more influenza protein coding regions under control of another promoter, in particular a pol II promoter, on the same plasmid. This is preferably done by using bi-directional plasmids.

Preferred aspects of the reference 15 method involve: (a) PB1, PB2, NP and PA mRNA-encoding regions on a single expression construct; and (b) all 8 vRNA encoding segments on a single expression construct. Including the neuraminidase (NA) and hemagglutinin (HA) segments on one expression construct and the six other viral segments on another expression construct is particularly preferred as newly emerging influenza virus strains usually have mutations in the NA and/or HA segments. Therefore, the advantage of having the HA and/or NA segments on a separate expression construct is that only the vector comprising the HA and NA sequence needs to be replaced. Thus, in one aspect of the invention the NA and/or HA segments of the vaccine strain may be included on one expression construct and the vRNA encoding segments from the donor strain(s) of the invention,

excluding the HA and/or NA segment(s), are included on a different expression construct. The invention thus provides an expression construct comprising one, two, three, four, five or six vRNA encoding backbone viral segments of a donor strain of the invention. The expression construct may not comprise HA and/or NA viral segments that produce a functional HA and/or NA protein.

Known reverse genetics systems involve expressing DNA molecules which encode desired viral RNA (vRNA) molecules from pol I promoters, bacterial RNA polymerase promoters, bacteriophage polymerase promoters, *etc.* As influenza viruses require the presence of viral polymerase to initiate the life cycle, systems may also provide these proteins *e.g.* the system further comprises DNA molecules that encode viral polymerase proteins such that expression of both types of DNA leads to assembly of a complete infectious virus. It is also possible to supply the viral polymerase as a protein.

Where reverse genetics is used for the expression of influenza vRNA, it will be evident to the person skilled in the art that precise spacing of the sequence elements with reference to each other is important for the polymerase to initiate replication. It is therefore important that the DNA molecule encoding the viral RNA is positioned correctly between the pol I promoter and the termination sequence, but this positioning is well within the capabilities of those who work with reverse genetics systems.

In order to produce a recombinant virus, a cell must express all segments of the viral genome which are necessary to assemble a virion. DNA cloned into the expression constructs of the present invention preferably provides all of the viral RNA and proteins, but it is also possible to use a helper virus to provide some of the RNA and proteins, although systems which do not use a helper virus are preferred. As the influenza virus is a segmented virus, the viral genome will usually be expressed using more than one expression construct in the methods of the invention. It is also envisioned, however, to combine one or more segments or even all segments of the viral genome on a single expression construct.

In some embodiments an expression construct will also be included which leads to expression of an accessory protein in the host cell. For instance, it can be advantageous to express a non-viral serine protease (*e.g.* trypsin) as part of a reverse genetics system.

Expression constructs

Expression constructs used in the expression systems of the invention may be uni-directional or bi-directional expression constructs. Where more than one transgene is used in the methods (whether on the same or different expression constructs) it is possible to use uni-directional and/or bi-directional expression.

As influenza viruses require a protein for infectivity, it is generally preferred to use bi-directional expression constructs as this reduces the total number of expression constructs required by the host cell. Thus, the method of the invention may utilise at least one bi-directional expression construct

wherein a gene or cDNA is located between an upstream pol II promoter and a downstream non-endogenous pol I promoter. Transcription of the gene or cDNA from the pol II promoter produces capped positive-sense viral mRNA which can be translated into a protein, while transcription from the non-endogenous pol I promoter produces negative-sense vRNA. The bi-directional expression construct may be a bi-directional expression vector.

Bi-directional expression constructs contain at least two promoters which drive expression in different directions (*i.e.* both 5' to 3' and 3' to 5') from the same construct. The two promoters can be operably linked to different strands of the same double stranded DNA. Preferably, one of the promoters is a pol I promoter and at least one of the other promoters is a pol II promoter. This is useful as the pol I promoter can be used to express uncapped vRNAs while the pol II promoter can be used to transcribe mRNAs which can subsequently be translated into proteins, thus allowing simultaneous expression of RNA and protein from the same construct. Where more than one expression construct is used within an expression system, the promoters may be a mixture of endogenous and non-endogenous promoters.

The pol I and pol II promoters used in the expression constructs may be endogenous to an organism from the same taxonomic order from which the host cell is derived. Alternatively, the promoters can be derived from an organism in a different taxonomic order than the host cell. The term "order" refers to conventional taxonomic ranking, and examples of orders are primates, rodentia, carnivora, marsupialia, cetacean, *etc*. Humans and chimpanzees are in the same taxonomic order (primates), but humans and dogs are in different orders (primates *vs.* carnivora). For example, the human pol I promoter can be used to express viral segments in canine cells (*e.g.* MDCK cells) [16].

The expression construct will typically include an RNA transcription termination sequence. The termination sequence may be an endogenous termination sequence or a termination sequence which is not endogenous to the host cell. Suitable termination sequences will be evident to those of skill in the art and include, but are not limited to, RNA polymerase I transcription termination sequence, RNA polymerase II transcription termination sequence, and ribozymes. Furthermore, the expression constructs may contain one or more polyadenylation signals for mRNAs, particularly at the end of a gene whose expression is controlled by a pol II promoter.

An expression system may contain at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven or at least twelve expression constructs.

An expression construct may be a vector, such as a plasmid or other episomal construct. Such vectors will typically comprise at least one bacterial and/or eukaryotic origin of replication. Furthermore, the vector may comprise a selectable marker which allows for selection in prokaryotic and/or eukaryotic cells. Examples of such selectable markers are genes conferring resistance to antibiotics, such as

ampicillin or kanamycin. The vector may further comprise one or more multiple cloning sites to facilitate cloning of a DNA sequence.

As an alternative, an expression construct may be a linear expression construct. Such linear expression constructs will typically not contain any amplification and/or selection sequences. However, linear constructs comprising such amplification and/or selection sequences are also within the scope of the present invention. Reference 17 describes individual linear expression constructs for each viral segment. It is also possible to include more than one, for example two, three four, five or six viral segments on the same linear expression construct. Such a system has been described, for example, in reference 18. It is also possible to use an expression system in which some viral segments (for example the HA and/or NA segment) are encoded on a linear construct and the remaining viral segments (for example the backbone segments) are encoded on a non-linear construct, such as a vector, a plasmid or other episomal construct.

Expression constructs can be generated using methods known in the art. Such methods were described, for example, in reference 19. Where the expression construct is a linear expression construct, it is possible to linearise it before introduction into the host cell utilising a single restriction enzyme site. Alternatively, it is possible to excise the expression construct from a vector using at least two restriction enzyme sites. Furthermore, it is also possible to obtain a linear expression construct by amplifying it using a nucleic acid amplification technique (*e.g.* by PCR).

The expression constructs used in the systems of the invention may be non-bacterial expression constructs. This means that the construct can drive expression in a eukaryotic cell of viral RNA segments encoded therein, but it does not include components which would be required for propagation of the construct in bacteria. Thus the construct will not include a bacterial origin of replication (ori), and usually will not include a bacterial selection marker (e.g. an antibiotic resistance marker). Such expression constructs are described in reference 20.

The expression constructs may be prepared by chemical synthesis. The expression constructs may either be prepared entirely by chemical synthesis or in part. Suitable methods for preparing expression constructs by chemical synthesis are described, for example, in reference 20.

The expression constructs of the invention can be introduced into host cells using any technique known to those of skill in the art. For example, expression constructs can be introduced into host cells by employing electroporation, DEAE-dextran, calcium phosphate precipitation, liposomes, microinjection, or microparticle-bombardment. The expression construct(s) can be introduced into the same cell type which is subsequently used for the propagation of the reassortant influenza B viruses. Alternatively, the cells into which the expression constructs are introduced and the cells used for propagation of the reassortant influenza B viruses may be different. In some embodiments, untransfected cells of the same or a different cell type may be added to the host cells following transfection of the host cells with the expression construct(s), as described in reference 21.

Conventional reassortment

Traditionally, influenza viruses are reassorted by co-infecting a culture host, usually eggs, with a donor strain and a vaccine strain. Reassortant viruses are selected by adding antibodies with specificity for the HA and/or NA proteins of the donor strain in order to select for reassortant viruses that contain the vaccine strain's HA and/or NA proteins. Over several passages of this treatment one can select for fast growing reassortant viruses containing the vaccine strain's HA and/or NA segments.

The reassortant influenza viruses can also be selected by adding an inhibitory agent which preferentially reduces the transcription and/or translation of the viral segments are not present in the desired reassortant influenza virus, as taught in WO2011/145081.

The invention is suitable for use in these methods. It can be easier to use a vaccine strain from a different influenza B lineage compared to the donor strain(s) as this facilitates selection for reassortant viruses. It is also possible, however, to use a vaccine strain from the same influenza B lineage as the donor strain(s) and in some aspects of the invention this preferred. In this case, antibodies or inhibitory agents with preferential specificity for the HA and/or NA proteins of the donor strain(s) should be available.

Culture host

The culture host for use in the invention, can be any eukaryotic cell that can produce the virus of interest. The invention will typically use a cell line although, for example, primary cells may be used as an alternative. The cell will typically be mammalian or avian. Suitable mammalian cells include, but are not limited to, hamster, cattle, primate (including humans and monkeys) and dog cells. Various cell types may be used, such as kidney cells, fibroblasts, retinal cells, lung cells, *etc.* Examples of suitable hamster cells are the cell lines having the names BHK21 or HKCC. Suitable monkey cells are *e.g.* African green monkey cells, such as kidney cells as in the Vero cell line [22-24]. Suitable dog cells are *e.g.* kidney cells, as in the CLDK and MDCK cell lines. Suitable avian embryonic stem cells, include the EBx cell line derived from chicken embryonic stem cells, EB45, EB14, and EB14-074 [25]. Chicken embryo fibroblasts (CEF) may also be used.

Further suitable cells include, but are not limited to: CHO; 293T; BHK; MRC 5; PER.C6 [26]; FRhL2; WI-38; etc. Suitable cells are widely available *e.g.* from the American Type Cell Culture (ATCC) collection [27], from the Coriell Cell Repositories [28], or from the European Collection of Cell Cultures (ECACC). For example, the ATCC supplies various different Vero cells under catalogue numbers CCL 81, CCL 81.2, CRL 1586 and CRL-1587, and it supplies MDCK cells under catalogue number CCL 34. PER.C6 is available from the ECACC under deposit number 96022940.

Preferred cells for use in the invention are MDCK cells [29-31], derived from Madin Darby canine kidney. The original MDCK cells are available from the ATCC as CCL 34. It is preferred that derivatives of MDCK cells are used. Such derivatives were described, for instance, in reference 29

which discloses MDCK cells that were adapted for growth in suspension culture ('MDCK 33016' or '33016-PF', deposited as DSM ACC 2219). Furthermore, reference 32 discloses MDCK-derived cells that grow in suspension in serum free culture ('B-702', deposited as FERM BP-7449). In some embodiments, the MDCK cell line used may be tumorigenic. It is also envisioned to use non-tumorigenic MDCK cells. For example, reference 33 discloses non tumorigenic MDCK cells, including 'MDCK-S' (ATCC PTA-6500), 'MDCK-SF101' (ATCC PTA-6501), 'MDCK-SF102' (ATCC PTA-6502) and 'MDCK-SF103' (ATCC PTA-6503). Reference 34 discloses MDCK cells with high susceptibility to infection, including 'MDCK.5F1' cells (ATCC CRL 12042).

It is possible to use a mixture of more than one cell type to practise the methods of the invention. However, it is preferred that the methods of the invention are practised with a single cell type *e.g.* with monoclonal cells. Preferably, the cells used in the methods of the present invention are from a single cell line. Furthermore, the same cell line may be used for reassorting the virus and for any subsequent propagation of the virus.

Preferably, the cells are cultured in the absence of serum, to avoid a common source of contaminants. Various serum-free media for eukaryotic cell culture are known to the person skilled in the art (e.g. Iscove's medium, ultra CHO medium (BioWhittaker), EX-CELL (JRH Biosciences)). Furthermore, protein-free media may be used (e.g. PF-CHO (JRH Biosciences)). Otherwise, the cells for replication can also be cultured in the customary serum-containing media (e.g. MEM or DMEM medium with 0.5% to 10% of fetal calf serum).

The cells may be in adherent culture or in suspension.

The reassortant influenza B viruses of the invention may also be propagated using eggs as the culture host. The current standard method for influenza virus growth for vaccines uses embryonated hen eggs, with virus being purified from the egg contents (allantoic fluid). It is also possible to passage a virus through eggs and subsequently propagate it in cell culture and *vice versa*.

Virus preparation

In one embodiment, the invention provides a method for producing influenza viruses comprising steps of (a) infecting a culture host with a reassortant virus of the invention; (b) culturing the host from step (a) to produce the virus; and optionally (c) purifying the virus produced in step (b).

The culture host in step (b) may be cells (as described above) or embryonated hen eggs. Where cells are used as a culture host in this aspect of the invention, it is known that cell culture conditions (*e.g.* temperature, cell density, pH value, *etc.*) are variable over a wide range subject to the cell line and the virus employed and can be adapted to the requirements of the application. The following information therefore merely represents guidelines.

Cells are preferably cultured in serum-free or protein-free media.

Multiplication of the cells can be conducted in accordance with methods known to those of skill in the art. For example, the cells can be cultivated in a perfusion system using ordinary support methods like centrifugation or filtration. Moreover, the cells can be multiplied according to the invention in a fed-batch system before infection. In the context of the present invention, a culture system is referred to as a fed-batch system in which the cells are initially cultured in a batch system and depletion of nutrients (or part of the nutrients) in the medium is compensated by controlled feeding of concentrated nutrients. It can be advantageous to adjust the pH value of the medium during multiplication of cells before infection to a value between pH 6.6 and pH 7.8 and especially between a value between pH 7.2 and pH 7.3. Culturing of cells preferably occurs at a temperature between 30 and 40°C. When culturing the infected cells (step b), the cells are preferably cultured at a temperature of between 30 °C and 36°C or between 32 °C and 34 °C or at 33°C. This is particularly preferred, as it has been shown that incubation of infected cells in this temperature range results in production of a virus that results in improved efficacy when formulated into a vaccine [35].

Oxygen partial pressure can be adjusted during culturing before infection preferably at a value between 25% and 95% and especially at a value between 35% and 60%. The values for the oxygen partial pressure stated in the context of the invention are based on saturation of air. Infection of cells occurs at a cell density of preferably about 8-25x10^5 cells/mL in the batch system or preferably about 5-20x10^6 cells/mL in the perfusion system. The cells can be infected with a viral dose (MOI value, "multiplicity of infection"; corresponds to the number of virus units per cell at the time of infection) between 10^{-8} and 10, preferably between 0.0001 and 0.5.

Virus may be grown on cells in adherent culture or in suspension. Microcarrier cultures can be used. In some embodiments, the cells may be adapted for growth in suspension.

The methods according to the invention also include harvesting and isolation of viruses or the proteins generated by them. During isolation of viruses or proteins, the cells are separated from the culture medium by standard methods like separation, filtration or ultrafiltration. The viruses or the proteins are then concentrated according to methods sufficiently known to those skilled in the art, like gradient centrifugation, filtration, precipitation, chromatography, etc., and then purified. It is also preferred according to the invention that the viruses are inactivated during or after purification. Virus inactivation can occur, for example, by β-propiolactone or formaldehyde at any point within the purification process.

Vaccine

Vaccines (particularly for influenza virus) are generally based either on live virus or on inactivated virus. Inactivated vaccines may be based on whole virions, 'split' virions, or on purified surface antigens. Antigens can also be presented in the form of virosomes. The invention can be used for manufacturing any of these types of vaccine but inactivated vaccines are preferred.

Where an inactivated virus is used, the vaccine may comprise whole virion, split virion, or purified surface antigens (for influenza, including hemagglutinin and, usually, also including neuraminidase). Chemical means for inactivating a virus include treatment with an effective amount of one or more of the following agents: detergents, formaldehyde, β-propiolactone, methylene blue, psoralen, carboxyfullerene (C60), binary ethylamine, acetyl ethyleneimine, or combinations thereof. Non-chemical methods of viral inactivation are known in the art, such as for example UV light or gamma irradiation.

Virions can be harvested from virus-containing fluids, *e.g.* allantoic fluid or cell culture supernatant, by various methods. For example, a purification process may involve zonal centrifugation using a linear sucrose gradient solution that includes detergent to disrupt the virions. Antigens may then be purified, after optional dilution, by diafiltration.

Split virions are obtained by treating purified virions with detergents (*e.g.* ethyl ether, polysorbate 80, deoxycholate, tri-*N*-butyl phosphate, Triton X-100, Triton N101, cetyltrimethylammonium bromide, Tergitol NP9, *etc.*) to produce subvirion preparations, including the 'Tween-ether' splitting process. Methods of splitting influenza viruses, for example are well known in the art *e.g.* see refs. 36-41, *etc.* Splitting of the virus is typically carried out by disrupting or fragmenting whole virus, whether infectious or non-infectious with a disrupting concentration of a splitting agent. The disruption results in a full or partial solubilisation of the virus proteins, altering the integrity of the virus. Preferred splitting agents are non-ionic and ionic (*e.g.* cationic) surfactants *e.g.* alkylglycosides, alkylthioglycosides, acyl sugars, sulphobetaines, betains, polyoxyethylenealkylethers, N,N-dialkyl-Glucamides, Hecameg, alkylphenoxy-polyethoxyethanols, NP9, quaternary ammonium compounds, sarcosyl, CTABs (cetyl trimethyl ammonium bromides), tri-*N*-butyl phosphate, Cetavlon, myristyltrimethylammonium salts, lipofectin, lipofectamine, and DOT-MA, the octyl- or nonylphenoxy polyoxyethanols (*e.g.* the Triton surfactants, such as Triton X-100 or Triton N101), polyoxyethylene sorbitan esters (the Tween surfactants), polyoxyethylene ethers, polyoxyethlene esters, *etc.* One useful splitting procedure uses the consecutive effects of sodium deoxycholate and formaldehyde, and splitting can take place during initial virion purification (e.g. in a sucrose density gradient solution). Thus a splitting process can involve clarification of the virion-containing material (to remove non-virion material), concentration of the harvested virions (*e.g.* using an adsorption method, such as $CaHPO_4$ adsorption), separation of whole virions from non-virion material, splitting of virions using a splitting agent in a density gradient centrifugation step (*e.g.* using a sucrose gradient that contains a splitting agent such as sodium deoxycholate), and then filtration (*e.g.* ultrafiltration) to remove undesired materials. Split virions can usefully be resuspended in sodium phosphate-buffered isotonic sodium chloride solution. Examples of split influenza vaccines are the BEGRIVAC™, FLUARIX™, FLUZONE™ and FLUSHIELD™ products.

Purified influenza virus surface antigen vaccines comprise the surface antigens hemagglutinin and, typically, also neuraminidase. Processes for preparing these proteins in purified form are well known

in the art. The FLUVIRIN™, AGRIPPAL™ and INFLUVAC™ products are influenza subunit vaccines.

Another form of inactivated antigen is the virosome [42] (nucleic acid free viral-like liposomal particles). Virosomes can be prepared by solubilization of virus with a detergent followed by removal of the nucleocapsid and reconstitution of the membrane containing the viral glycoproteins. An alternative method for preparing virosomes involves adding viral membrane glycoproteins to excess amounts of phospholipids, to give liposomes with viral proteins in their membrane.

The methods of the invention may also be used to produce live vaccines. Such vaccines are usually prepared by purifying virions from virion-containing fluids. For example, the fluids may be clarified by centrifugation, and stabilized with buffer (*e.g.* containing sucrose, potassium phosphate, and monosodium glutamate). Various forms of influenza virus vaccine are currently available (e.g. see chapters 17 & 18 of reference 43). Live virus vaccines include MedImmune's FLUMIST™ product.

The virus may be attenuated. The virus may be temperature-sensitive. The virus may be cold-adapted. These three features are particularly useful when using live virus as an antigen.

HA is the main immunogen in current inactivated influenza vaccines, and vaccine doses are standardised by reference to HA levels, typically measured by SRID. Existing vaccines typically contain about 15μg of HA per strain, although lower doses can be used *e.g.* for children, or in pandemic situations, or when using an adjuvant. Fractional doses such as ½ (*i.e.* 7.5μg HA per strain), ¼ and $^1/_8$ have been used, as have higher doses (*e.g.* 3x or 9x doses [44,45]). Thus vaccines may include between 0.1 and 150μg of HA per influenza strain, preferably between 0.1 and 50μg *e.g.* 0.1-20μg, 0.1-15μg, 0.1-10μg, 0.1-7.5μg, 0.5-5μg, *etc.* Particular doses include *e.g.* about 45, about 30, about 15, about 10, about 7.5, about 5, about 3.8, about 3.75, about 1.9, about 1.5, *etc.* per strain.

For live vaccines, dosing is measured by median tissue culture infectious dose ($TCID_{50}$) rather than HA content, and a $TCID_{50}$ of between 10^6 and 10^8 (preferably between $10^{6.5}$-$10^{7.5}$) per strain is typical.

Influenza strains used with the invention may have a natural HA as found in a wild-type virus, or a modified HA. For instance, it is known to modify HA to remove determinants (*e.g.* hyper-basic regions around the HA1/HA2 cleavage site) that cause a virus to be highly pathogenic in avian species. The use of reverse genetics facilitates such modifications.

As well as being suitable for immunizing against inter-pandemic strains, the vaccines of the invention are particularly useful for immunizing against pandemic or potentially-pandemic strains. The invention is suitable for vaccinating humans as well as non-human animals.

Vaccines of the invention may include antigen(s) from one or more (*e.g.* 1, 2, 3, 4 or more) influenza virus strains, including influenza A virus and/or influenza B virus provided that at least one influenza strain is a reassortant influenza strain of the invention. Vaccines wherein two antigens are from a

reassortant influenza strain of the invention are also envisioned. Where a vaccine includes more than one strain of influenza, the different strains are typically grown separately and are mixed after the viruses have been harvested and antigens have been prepared. Thus a process of the invention may include the step of mixing antigens from more than one influenza strain. A trivalent vaccine is typical, including antigens from two influenza A virus strains and one influenza B virus strain. A tetravalent vaccine is also useful [46], including antigens from two influenza A virus strains and two influenza B virus strains (preferably two influenza B strains of different lineages), or three influenza A virus strains and one influenza B virus strain. Where the influenza vaccine includes antigens from more than one influenza B strain one or more of these may be derived from a reassortant influenza B virus of the invention.

Vaccines of the invention are pharmaceutically acceptable. They usually include components in addition to the antigens *e.g.* they typically include one or more pharmaceutical carrier(s) and/or excipient(s). As described below, adjuvants may also be included. A thorough discussion of such components is available in reference 47.

Vaccines will generally be in aqueous form. However, some vaccines may be in dry form, *e.g.* in the form of injectable solids or dried or polymerized preparations on a patch.

Vaccines may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the vaccine should be substantially free from (*i.e.* less than 5μg/ml) mercurial material *e.g.* thiomersal-free [40,48]. Vaccines containing no mercury are more preferred. An α-tocopherol succinate can be included as an alternative to mercurial compounds [40]. Preservative-free vaccines are particularly preferred.

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

Vaccines will generally have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, preferably between 240-360 mOsm/kg, and will more preferably fall within the range of 290-310 mOsm/kg. Osmolality has previously been reported not to have an impact on pain caused by vaccination [49], but keeping osmolality in this range is nevertheless preferred.

Vaccines may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer (particularly with an aluminum hydroxide adjuvant); or a citrate buffer. Buffers will typically be included in the 5-20mM range.

The pH of a vaccine will generally be between 5.0 and 8.1, and more typically between 6.0 and 8.0 *e.g.* 6.5 and 7.5, or between 7.0 and 7.8. A process of the invention may therefore include a step of adjusting the pH of the bulk vaccine prior to packaging.

The vaccine is preferably sterile. The vaccine is preferably non-pyrogenic *e.g.* containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. The vaccine is preferably gluten-free.

Vaccines of the invention may include detergent *e.g.* a polyoxyethylene sorbitan ester surfactant (known as 'Tweens'), an octoxynol (such as octoxynol-9 (Triton X-100) or t-octylphenoxypolyethoxyethanol), a cetyl trimethyl ammonium bromide ('CTAB'), or sodium deoxycholate, particularly for a split or surface antigen vaccine. The detergent may be present only at trace amounts. Thus the vaccine may include less than 1mg/ml of each of octoxynol-10 and polysorbate 80. Other residual components in trace amounts could be antibiotics (*e.g.* neomycin, kanamycin, polymyxin B).

A vaccine may include material for a single immunisation, or may include material for multiple immunisations (*i.e.* a 'multidose' kit). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to including a preservative in multidose vaccines, the vaccines may be contained in a container having an aseptic adaptor for removal of material.

Influenza vaccines are typically administered in a dosage volume of about 0.5ml, although a half dose (*i.e.* about 0.25ml) may be administered to children.

Vaccines and kits are preferably stored at between 2°C and 8°C. They should not be frozen. They should ideally be kept out of direct light.

Host cell DNA

Where virus has been isolated and/or grown on a cell line, it is standard practice to minimize the amount of residual cell line DNA in the final vaccine, in order to minimize any potential oncogenic activity of the DNA.

Thus a vaccine of the invention preferably contains less than 10ng (preferably less than 1ng, and more preferably less than 100pg) of residual host cell DNA per dose, although trace amounts of host cell DNA may be present.

It is preferred that the average length of any residual host cell DNA is less than 500bp *e.g.* less than 400bp, less than 300bp, less than 200bp, less than 100bp, *etc.*

Contaminating DNA can be removed during vaccine preparation using standard purification procedures *e.g.* chromatography, *etc.* Removal of residual host cell DNA can be enhanced by nuclease treatment *e.g.* by using a DNase. A convenient method for reducing host cell DNA contamination is disclosed in references 50 & 51, involving a two-step treatment, first using a DNase (*e.g.* Benzonase), which may be used during viral growth, and then a cationic detergent (*e.g.* CTAB), which may be used during virion disruption. Treatment with an alkylating agent, such as β-propiolactone, can also be used to remove host cell DNA, and advantageously may also be used to inactivate virions [52].

Adjuvants

Vaccines of the invention may advantageously include an adjuvant, which can function to enhance the immune responses (humoral and/or cellular) elicited in a subject who receives the vaccine. Preferred adjuvants comprise oil-in-water emulsions. Various such adjuvants are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolisable) and biocompatible. The oil droplets in the emulsion are generally less than 5μm in diameter, and ideally have a sub-micron diameter, with these small sizes being achieved with a microfluidiser to provide stable emulsions. Droplets with a size less than 220nm are preferred as they can be subjected to filter sterilization.

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

Mixtures of oils can be used.

Surfactants can be classified by their 'HLB' (hydrophile/lipophile balance). Preferred surfactants of the invention have a HLB of at least 10, preferably at least 15, and more preferably at least 16. The invention can be used with surfactants including, but not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol

(IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the Tergitol™ NP series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Non-ionic surfactants are preferred. Preferred surfactants for including in the emulsion are Tween 80 (polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

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

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

Where the vaccine contains a split virus, it is preferred that it contains free surfactant in the aqueous phase. This is advantageous as the free surfactant can exert a 'splitting effect' on the antigen, thereby disrupting any unsplit virions and/or virion aggregates that might otherwise be present. This can improve the safety of split virus vaccines [53].

Preferred emulsions have an average droplets size of <1μm *e.g.* ≤750nm, ≤500nm, ≤400nm, ≤300nm, ≤250nm, ≤220nm, ≤200nm, or smaller. These droplet sizes can conveniently be achieved by techniques such as microfluidisation.

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

- A submicron emulsion of squalene, Tween 80, and Span 85. The composition of the emulsion by volume can be about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85. In weight terms, these ratios become 4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85. This adjuvant is known as 'MF59' [54-56], as described in more detail in Chapter 10 of ref. 57 and chapter 12 of ref. 58. The MF59 emulsion advantageously includes citrate ions *e.g.* 10mM sodium citrate buffer.
- An emulsion comprising squalene, a tocopherol, and polysorbate 80. The emulsion may include phosphate buffered saline. These emulsions may have by volume from 2 to 10% squalene, from 2 to 10% tocopherol and from 0.3 to 3% polysorbate 80, and the weight ratio of squalene:tocopherol is preferably <1 (e.g. 0.90) as this can provide a more stable emulsion. Squalene and polysorbate 80 may be present in a volume ratio of about 5:2 or at a weight ratio of about 11:5. Thus the three components (squalene, tocopherol, polysorbate 80) may be

present at a weight ratio of 1068:1186:485 or around 55:61:25. One such emulsion ('AS03') can be made by dissolving Tween 80 in PBS to give a 2% solution, then mixing 90ml of this solution with a mixture of (5g of DL α tocopherol and 5ml squalene), then microfluidising the mixture. The resulting emulsion may have submicron oil droplets e.g. with an average diameter of between 100 and 250nm, preferably about 180nm. The emulsion may also include a 3-de-O-acylated monophosphoryl lipid A (3d MPL). Another useful emulsion of this type may comprise, per human dose, 0.5-10 mg squalene, 0.5-11 mg tocopherol, and 0.1-4 mg polysorbate 80 [59] *e.g.* in the ratios discussed above.

- An emulsion of squalene, a tocopherol, and a Triton detergent (*e.g.* Triton X-100). The emulsion may also include a 3d-MPL (see below). The emulsion may contain a phosphate buffer.

- An emulsion comprising a polysorbate (*e.g.* polysorbate 80), a Triton detergent (*e.g.* Triton X-100) and a tocopherol (*e.g.* an α-tocopherol succinate). The emulsion may include these three components at a mass ratio of about 75:11:10 (*e.g.* 750μg/ml polysorbate 80, 110μg/ml Triton X-100 and 100μg/ml α-tocopherol succinate), and these concentrations should include any contribution of these components from antigens. The emulsion may also include squalene. The emulsion may also include a 3d-MPL (see below). The aqueous phase may contain a phosphate buffer.

- An emulsion of squalane, polysorbate 80 and poloxamer 401 ("Pluronic™ L121"). The emulsion can be formulated in phosphate buffered saline, pH 7.4. This emulsion is a useful delivery vehicle for muramyl dipeptides, and has been used with threonyl-MDP in the "SAF-1" adjuvant [60] (0.05-1% Thr-MDP, 5% squalane, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the "AF" adjuvant [61] (5% squalane, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidisation is preferred.

- An emulsion comprising squalene, an aqueous solvent, a polyoxyethylene alkyl ether hydrophilic nonionic surfactant (*e.g.* polyoxyethylene (12) cetostearyl ether) and a hydrophobic nonionic surfactant (*e.g.* a sorbitan ester or mannide ester, such as sorbitan monoleate or 'Span 80'). The emulsion is preferably thermoreversible and/or has at least 90% of the oil droplets (by volume) with a size less than 200 nm [62]. The emulsion may also include one or more of: alditol; a cryoprotective agent (*e.g.* a sugar, such as dodecylmaltoside and/or sucrose); and/or an alkylpolyglycoside. The emulsion may include a TLR4 agonist [63]. Such emulsions may be lyophilized.

- An emulsion of squalene, poloxamer 105 and Abil-Care [64]. The final concentration (weight) of these components in adjuvanted vaccines are 5% squalene, 4% poloxamer 105 (pluronic polyol) and 2% Abil-Care 85 (Bis-PEG/PPG-16/16 PEG/PPG-16/16 dimethicone; caprylic/capric triglyceride).

- An emulsion having from 0.5-50% of an oil, 0.1-10% of a phospholipid, and 0.05-5% of a non-ionic surfactant. As described in reference 65, preferred phospholipid components are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, sphingomyelin and cardiolipin. Submicron droplet sizes are advantageous.

- A submicron oil-in-water emulsion of a non-metabolisable oil (such as light mineral oil) and at least one surfactant (such as lecithin, Tween 80 or Span 80). Additives may be included, such as QuilA saponin, cholesterol, a saponin-lipophile conjugate (such as GPI-0100, described in reference 66, produced by addition of aliphatic amine to desacylsaponin via the carboxyl group of glucuronic acid), dimethyidioctadecylammonium bromide and/or N,N-dioctadecyl-N,N-bis (2-hydroxyethyl)propanediamine.

- An emulsion in which a saponin (*e.g.* QuilA or QS21) and a sterol (*e.g.* a cholesterol) are associated as helical micelles [67].

- An emulsion comprising a mineral oil, a non-ionic lipophilic ethoxylated fatty alcohol, and a non-ionic hydrophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [68].

- An emulsion comprising a mineral oil, a non-ionic hydrophilic ethoxylated fatty alcohol, and a non-ionic lipophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [68].

In some embodiments an emulsion may be mixed with antigen extemporaneously, at the time of delivery, and thus the adjuvant and antigen may be kept separately in a packaged or distributed vaccine, ready for final formulation at the time of use. In other embodiments an emulsion is mixed with antigen during manufacture, and thus the composition is packaged in a liquid adjuvanted form. The antigen will generally be in an aqueous form, such that the vaccine is finally prepared by mixing two liquids. The volume ratio of the two liquids for mixing can vary (*e.g.* between 5:1 and 1:5) but is generally about 1:1. Where concentrations of components are given in the above descriptions of specific emulsions, these concentrations are typically for an undiluted composition, and the concentration after mixing with an antigen solution will thus decrease.

Packaging of vaccines

Suitable containers for vaccines of the invention (or kit components) include vials, syringes (*e.g.* disposable syringes), nasal sprays, *etc*. These containers should be sterile.

Where a composition/component is located in a vial, the vial is preferably made of a glass or plastic material. The vial is preferably sterilized before the composition is added to it. To avoid problems with latex-sensitive patients, vials are preferably sealed with a latex-free stopper, and the absence of latex in all packaging material is preferred. The vial may include a single dose of vaccine, or it may

include more than one dose (a 'multidose' vial) *e.g.* 10 doses. Preferred vials are made of colourless glass. A vial may have a cap that permits aseptic removal of its contents, particularly for multidose vials.

Where a component is packaged into a syringe, the syringe may have a needle attached to it. If a needle is not attached, a separate needle may be supplied with the syringe for assembly and use. Such a needle may be sheathed. Safety needles are preferred. 1-inch 23-gauge, 1-inch 25-gauge and 5/8-inch 25-gauge needles are typical. Syringes may be provided with peel-off labels on which the lot number, influenza season and expiration date of the contents may be printed, to facilitate record keeping. The plunger in the syringe preferably has a stopper to prevent the plunger from being accidentally removed during aspiration. The syringes may have a latex rubber cap and/or plunger. Disposable syringes contain a single dose of vaccine. The syringe will generally have a tip cap to seal the tip prior to attachment of a needle, and the tip cap is preferably made of a butyl rubber. If the syringe and needle are packaged separately then the needle is preferably fitted with a butyl rubber shield. Preferred syringes are those marketed under the trade name "Tip-Lok"™.

Containers may be marked to show a half-dose volume *e.g.* to facilitate delivery to children. For instance, a syringe containing a 0.5ml dose may have a mark showing a 0.25ml volume.

Where a glass container (*e.g.* a syringe or a vial) is used, then it is preferred to use a container made from a borosilicate glass rather than from a soda lime glass.

A kit or vaccine may be packaged (*e.g.* in the same box) with a leaflet including details of the vaccine *e.g.* instructions for administration, details of the antigens within the vaccine, *etc.* The instructions may also contain warnings *e.g.* to keep a solution of adrenaline readily available in case of anaphylactic reaction following vaccination, *etc.*

Methods of treatment, and administration of the vaccine

The invention provides a vaccine manufactured according to the invention. These vaccines are suitable for administration to human or non-human animal subjects, such as pigs or birds, and the invention provides a method of raising an immune response in a subject, comprising the step of administering a vaccine of the invention to the subject. The invention also provides a vaccine of the invention for use as a medicament, and provides the use of a vaccine of the invention for the manufacture of a medicament for raising an immune response in a subject.

The immune response raised by these methods and uses will generally include an antibody response, preferably a protective antibody response. Methods for assessing antibody responses, neutralising capability and protection after influenza virus vaccination are well known in the art. Human studies have shown that antibody titers against hemagglutinin of human influenza virus are correlated with protection (a serum sample hemagglutination-inhibition titer of about 30–40 gives around 50% protection from infection by a homologous virus) [69]. Antibody responses are typically measured by

hemagglutination inhibition, by microneutralisation, by single radial immunodiffusion (SRID), and/or by single radial hemolysis (SRH). These assay techniques are well known in the art.

Vaccines of the invention can be administered in various ways. The most preferred immunisation route is by intramuscular injection (*e.g.* into the arm or leg), but other available routes include subcutaneous injection, intranasal [70-72], oral [73], intradermal [74,75], transcutaneous, transdermal [76], *etc.*

Vaccines prepared according to the invention may be used to treat both children and adults. Influenza vaccines are currently recommended for use in pediatric and adult immunisation, from the age of 6 months. Thus a human subject may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred subjects for receiving the vaccines are the elderly (*e.g.* ≥50 years old, ≥60 years old, and preferably ≥65 years), the young (*e.g.* ≤5 years old), hospitalised subjects, healthcare workers, armed service and military personnel, pregnant women, the chronically ill, immunodeficient subjects, subjects who have taken an antiviral compound (*e.g.* an oseltamivir or zanamivir compound; see below) in the 7 days prior to receiving the vaccine, people with egg allergies and people travelling abroad. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population. For pandemic strains, administration to all age groups is preferred.

Preferred vaccines of the invention satisfy 1, 2 or 3 of the CPMP criteria for efficacy. In adults (18-60 years), these criteria are: (1) ≥70% seroprotection; (2) ≥40% seroconversion; and/or (3) a GMT increase of ≥2.5-fold. In elderly (>60 years), these criteria are: (1) ≥60% seroprotection; (2) ≥30% seroconversion; and/or (3) a GMT increase of ≥2-fold. These criteria are based on open label studies with at least 50 patients.

Treatment can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes *e.g.* a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, *etc.* Administration of more than one dose (typically two doses) is particularly useful in immunologically naïve patients *e.g.* for people who have never received an influenza vaccine before, or for vaccinating against a new HA subtype (as in a pandemic outbreak). Multiple doses will typically be administered at least 1 week apart (*e.g.* about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, *etc.*).

Vaccines produced according to the invention may be administered to patients at substantially the same time as (*e.g.* during the same medical consultation or visit to a healthcare professional or vaccination centre) other vaccines *e.g.* at substantially the same time as a measles vaccine, a mumps vaccine, a rubella vaccine, a MMR vaccine, a varicella vaccine, a MMRV vaccine, a diphtheria vaccine, a tetanus vaccine, a pertussis vaccine, a DTP vaccine, a conjugated *H.influenzae* type b

vaccine, an inactivated poliovirus vaccine, a hepatitis B virus vaccine, a meningococcal conjugate vaccine (such as a tetravalent A-C-W135-Y vaccine), a respiratory syncytial virus vaccine, a pneumococcal conjugate vaccine, *etc.* Administration at substantially the same time as a pneumococcal vaccine and/or a meningococcal vaccine is particularly useful in elderly patients.

Similarly, vaccines of the invention may be administered to patients at substantially the same time as (*e.g.* during the same medical consultation or visit to a healthcare professional) an antiviral compound, and in particular an antiviral compound active against influenza virus (*e.g.* oseltamivir and/or zanamivir). These antivirals include neuraminidase inhibitors, such as a (3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid or 5-(acetylamino)-4-[(aminoiminomethyl)-amino]-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galactonon-2-enonic acid, including esters thereof (*e.g.* the ethyl esters) and salts thereof (*e.g.* the phosphate salts). A preferred antiviral is (3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate (1:1), also known as oseltamivir phosphate (TAMIFLU™).

General

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

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

The term "about" in relation to a numerical value *x* is optional and means, for example, $x \pm 10\%$.

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

The various steps of the methods may be carried out at the same or different times, in the same or different geographical locations, *e.g.* countries, and by the same or different people or entities.

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

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

References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in

the art, for example those described in section 7.7.18 of reference 77. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is taught in reference 78.

References to a percentage sequence identity between two nucleic acid sequences mean that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of reference 77. A preferred alignment program is GCG Gap (Genetics Computer Group, Wisconsin, Suite Version 10.1), preferably using default parameters, which are as follows: open gap = 3; extend gap = 1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 compares the HA yield of different reassortant influenza B strains in MDCK cells relative to the wild-type (WT) or reverse genetics-derived (RG) B/Brisbane/60/08 strain. The viral segments of the tested influenza B viruses are shown in Table 1. The y-axis indicates the HA yield in µg/mL.

Figure 2 compares the HA yield of different reassortant influenza B strains in MDCK cells relative to the wild-type (WT) or reverse genetics-derived (RG) B/Panama/45/90 strain. The viral segments of the tested influenza B viruses are shown in Table 1. The y-axis indicates the HA yield in µg/mL.

Figure 3 compares the HA yield of reassortant influenza B viruses comprising the B/Panama/45/90 or B/Brisbane/60/08 backbone with the HA yield obtained with the corresponding wild-type strain. The different experiments were performed using the B/Brisbane/60/08 HA and NA segments (A), the B/Panama/45/90 HA and NA segments (B), the B/Florida/4/06 HA and NA segments (C) or the B/Lee/40 HA and NA segments (D). The white bar shows the results with wild-type-strain, the cross-hatched bar indicates the results with the B/Panama/45/90 backbone and the checked bar shows the results with the B/Brisbane/60/08 backbone. The y-axis in Figure 3(A), 3(B) and 3(C) indicates the HA yield in µg/mL as determined by ELISA and the y-axis in Figure 3(D) shows the HA titre as determined by HA assay.

Figure 4 compares the HA yield of reassortant influenza B viruses comprising the #2, #9, #32, or #34 hybrid backbone (as shown in Table 1) with the HA yield obtained with the BX-35, B/Panama/45/90, or B/Brisbane/60/08 backbone or the corresponding wild-type virus. The different experiments were performed using the B/Brisbane/60/08 HA and NA segments (A), the B/Panama/45/90 HA and NA segments (B), the BX-35 HA and NA segments (C) or the B/Florida/4/06 HA and NA segments (D). The y-axis indicates the HA yield in µg/mL.

Figure 5 compares the HA yield of reassortant influenza B viruses comprising the #34 or B/Brisbane/60/08 backbones with the HA yield obtained with the corresponding wild-type strain. The different experiments were performed using the B/Panama/45/90 HA and NA segments (A), the

B/Brisbane/60/08 HA and NA segments (B), the B/Florida/4/06 HA and NA segments (C), the B/Brisbane/03/07 HA and NA segments (D), the B/Brisbane/32/02 HA and NA segments (E), the BX-35 HA and NA segments (F), the B/Malaysia/2506/04 HA and NA segments (G), or the B/Hubei-Wujiagang/159/08 HA and NA segments (H). The white bar shows the results with the B/Brisbane/60/08 backbone, the cross-hatched bar indicates the results with the #34 backbone and the checked bar shows the results with the wild-type strain. The y-axis indicates the HA yield in µg/mL.

Figure 6 compares the HA titre (A) and the viral growth (B) of (a) a reverse genetics-derived reassortant influenza B virus comprising all backbone segments from B/Brisbane/60/08 (#35) and the HA and NA segments from B/Wisconsin/1/10, (b) a reassortant influenza B virus which comprises the PB2, NP and M segments from B/Lee/40 and all other genes from B/Wisconsin/1/10 (#41), and (c) the wild-type B/Wisconsin/1/10 strain (WT) following growth in embryonated chicken eggs. Each triangle represents an individual egg and the bars represent mean values. "280" and "2800" indicate the infectious doses (IU) with which the eggs were inoculated. The y-axis in (A) represents the HA yield and the y-axis in (B) represents IU/mL.

MODES FOR CARRYING OUT THE INVENTION

Development of new donor strains

In order to provide high-growth donor strains, the inventors found that reassortant influenza B viruses comprising backbone segments from B/Brisbane/60/08 and B/Panama/45/90 grow particularly well in eggs and in cells, such as MDCK cells. To this end, reassortant influenza B viruses comprising backbone segments from these viruses are produced and the resulting influenza B viruses are grown in MDCK cells. The viral yield is determined by ELISA (as described in PCT/IB2012/057235) or a HA assay as known in the art.

Growth characteristics of reassortant influenza B viruses

Reassortant influenza B viruses are produced by reverse genetics which contain the HA and NA proteins from various influenza strains and the other viral segments from B/Brisbane/60/08 and/or B/Panama/45/90. As a control the corresponding wild-type influenza B strain is used. These viruses are cultured either in embyronated chicken eggs or in MDCK cells. The following influenza B strains are used:

Table 1

	Backbone segments						Antigenic determinants	
combo #	**PA**	**PB1**	**PB2**	**NP**	**NS**	**M**	**HA**	**NA**
1 (WT)	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane
2	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane
3	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane

4	Brisbane	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane
5	Brisbane	Brisbane	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane
6	Panama	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane
7	Panama	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane
8	Panama	Brisbane	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane
9	Brisbane	Panama	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane
10	Brisbane	Panama	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane
11	Brisbane	Brisbane	Panama	Panama	Brisbane	Brisbane	Brisbane	Brisbane
12	Panama	Panama	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane
13	Panama	Panama	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane
14	Panama	Brisbane	Panama	Panama	Brisbane	Brisbane	Brisbane	Brisbane
15	Brisbane	Panama	Panama	Panama	Brisbane	Brisbane	Brisbane	Brisbane
16	Panama	Panama	Panama	Panama	Brisbane	Brisbane	Brisbane	Brisbane
17	Panama	Panama	Panama	Panama	Panama	Panama	Brisbane	Brisbane
20	Brisbane	Panama	Panama	Panama	Panama	Panama	Panama	Panama
21	Panama	Brisbane	Panama	Panama	Panama	Panama	Panama	Panama
22	Panama	Panama	Brisbane	Panama	Panama	Panama	Panama	Panama
23	Panama	Panama	Panama	Brisbane	Panama	Panama	Panama	Panama
24	Brisbane	Brisbane	Panama	Panama	Panama	Panama	Panama	Panama
25	Brisbane	Panama	Brisbane	Panama	Panama	Panama	Panama	Panama
26	Brisbane	Panama	Panama	Brisbane	Panama	Panama	Panama	Panama
27	Panama	Brisbane	Brisbane	Panama	Panama	Panama	Panama	Panama
28	Panama	Brisbane	Panama	Brisbane	Panama	Panama	Panama	Panama
29	Panama	Panama	Brisbane	Brisbane	Panama	Panama	Panama	Panama
30	Brisbane	Brisbane	Brisbane	Panama	Panama	Panama	Panama	Panama
31	Brisbane	Brisbane	Panama	Brisbane	Panama	Panama	Panama	Panama
32	Brisbane	Panama	Brisbane	Brisbane	Panama	Panama	Panama	Panama
33	Panama	Brisbane	Brisbane	Brisbane	Panama	Panama	Panama	Panama
34	Brisbane	Brisbane	Brisbane	Brisbane	Panama	Panama	Panama	Panama
35	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Panama	Panama

The results indicate that reassortant viruses #2, #9, #22, #23, #29, #30, #31, #32, #33, #34 and #35 grow equally well or even better in the culture host (see Figures 1 and 2) than the corresponding wild-type strain. Most of these strains comprise the NP segment from B/Brisbane/60/08 and some (in particular those which grew best) further contain the PB2 segment from B/Brisbane/60/08. All of these viruses also contain viral segments from the B/Victoria/2/87-like strain and the B/Yamagata/16/88-like strain at a ratio 7:1, 6:2, 4:4, 3:4 or 1:7.

Growth characteristics of reassortant influenza B viruses comprising backbone segments from B/Brisbane/60/08

In order to test whether the reassortant influenza B viruses of the invention can be used with HA and NA segments from different strains, reassortant influenza B viruses comprising the HA and NA segments from B/Panama/45/90, B/Lee/40 or B/Florida/04/06 and the backbone segments from B/Brisbane/60/08 are produced. The reassortant influenza viruses are grown in MDCK cells for 60 hours and the HA yield is determined by ELISA or a HA assay. The data (see Figure 3) show that all of the reassortant influenza B viruses grew to higher titres compared to the wild-type influenza B viruses which indicates that reassortant influenza B viruses of the invention are useful for a variety of different HA and NA segments.

Growth characteristics of reassortant influenza B viruses comprising hybrid backbone segments

The growth characteristics of reassortant influenza B viruses comprising the backbone segments of the invention are also determined relative to the wild-type influenza B virus and the known influenza B reassortant BX35 which comprises the HA, NA, PA, PBI, and NS segments from B/Brisbane/60/08, the PB2 and M segments from B/Panama/45/90, and the NP segment from B/Lee/40. The backbones are tested with the HA and NA proteins of B/Brisbane/60/08, B/Panama/45/90, BX-35 and B/Florida/04/06. The reassortant influenza viruses are grown in MDCK cells for 60 hours and the HA yield is determined by ELISA or RP-HPLC. The data (see Figure 4) show that reassortant influenza B viruses comprising the backbones of the invention can grow to higher titres compared to the wild-type and to reassortant influenza B viruses comprising the known BX35 backbone.

The HA yield obtained with the #34 and #35 is further tested using a number of different HA and NA segments. MDCK cells are infected with these reassortant influenza B viruses and the corresponding wild-type influenza B virus. The data (see Figure 5) show that all of the reassortant influenza B viruses comprising a backbone according to the invention can grow equally well or better than the corresponding wild-type strain.

Growth characteristics of reassortant influenza B viruses in eggs

Embryonated chicken eggs were inoculated with either 280 or 2800 infectious doses (IU) of (a) a reverse genetics-derived reassortant influenza B virus comprising all backbone segments from B/Brisbane/60/08 (#35 backbone) and the HA and NA segments from B/Wisconsin/1/10, (b) a reassortant influenza B virus which comprises PB2, NP and M segments from B/Lee/40 and all other genes from B/Wisconsin/1/10 (BX-41), and (c) the wild-type B/Wisconsin/1/10 strain. Egg allantoic fluid was harvested 72h post-infection, and assayed for HA titer by HA Assay or virus growth by a focus-formation assay. The data (see Figure 6) show that the reassortant influenza B virus according to the invention can grow equally well or better than the control strains.

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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SEQUENCES

SEQ ID NO: 1 (PA, B/Brisbane/60/08)

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SEQ ID NO: 2 (PB1, B/Brisbane/60/08)

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SEQ ID NO: 5 (M_1, B/Brisbane/60/08)

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SEQ ID NO: 6 (M_2, B/Brisbane/60/08)

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SEQ ID NO: 7 (NS_1, B/Brisbane/60/08)

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SEQ ID NO: 8 (NS_2, B/Brisbane/60/08)

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SEQ ID NO: 9 (HA, B/Brisbane/60/08)

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SEQ ID NO: 10 (NA, B/Brisbane/60/08)

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SEQ ID NO: 11 (PA, B/Brisbane/60/08)

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SEQ ID NO: 14 (NP, B/Brisbane/60/08)

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GGTGAAACTGGGCGAATTCTATAACCAGATGATGGTCAAAGCTGGACTCAATGATGACATGGAGAGAAATCTAATCC
AAAATGCGCATGCCGTGGAAAGAATTCTATTGGCTGCCACTGATGACAAGAAAACCGAGTTCCAGAAGAAAAAGAAT
GCCAGAGATGTCAAAGAAGGGAAAGAAGAAATAGATCACAACAAAACAGGAGGCACCTTTTACAAGATGGTAAGAGA
TGATAAAACCATCTACTTCAGCCCTATAAGAATTACCTTTTTAAAAGAAGAGGTGAAAACAATGTACAAAACCACCA
TGGGGAGTGATGGCTTCAGTGGACTAAATCACATAATGATTGGGCATTCACAGATGAATGATGTCTGTTTCCAAAGA
TCAAAGGCACTAAAAAGAGTTGGACTTGATCCTTCATTAATCAGTACCTTTGCGGGAAGCACAGTCCCCAGAAGATC
AGGTGCGACTGGTGTTGCAATCAAAGGAGGTGGAACCTTAGTGGCTGAAGCCATTCGATTTATAGGAAGAGCAATGG
CAGACAGAGGGCTATTGAGAGACATCAAAGCCAAGACTGCCTATGAAAAGATTCTTCTGAATCTAAAAAACAAATGC
TCTGCGCCCCAACAAAAGGCTCTAGTTGATCAAGTGATCGGAAGCAGAAATCCGGGGATTGCAGACATTGAAGATCT
AACCCTGCTTGCTCGTAGTATGGTCGTTGTTAGGCCCTCTGTGGCAAGCAAAGTGGTGCTTCCCATAAGCATTTACG
CCAAAATACCTCAACTAGGGTTCAATGTTGAAGAGTACTCTATGGTTGGGTACGAAGCCATGGCTCTTTACAATATG
GCAACACCTGTGTCCATATTAAGAATGGGAGATGATGCAAAAGATAAATCGCAATTATTCTTCATGTCTTGCTTCGG
AGCTGCCTATGAAGACCTGAGAGTTTTGTCTGCATTAACAGGCACAGAATTCAAGCCTAGATCAGCATTAAAATGCA
AGGGTTTCCATGTTCCAGCAAAGGAACAGGTAGAAGGAATGGGAGCAGCTCTGATGTCCATCAAGCTCCAGTTTTGG
GCTCCGATGACCAGATCTGGGGGGAACGAAGTAGGTGGAGACGGAGGGTCTGGCCAAATAAGCTGCAGCCCAGTGTT
TGCAGTGGAAAGACCTATTGCTCTAAGCAAGCAAGCTGTAAGAAGAATGCTGTCAATGAATATTGAGGGACGTGATG
CAGATGTCAAAGGAAATCTACTCAAGATGATGAATGACTCAATGGCTAAGAAAACCAGTGGAAATGCTTTCATTGGG
AAGAAAATGTTTCAAATATCAGACAAAAACAAAACCAATCCCATTGAAATTCCAATTAAGCAGACCATCCCCAATTT
CTTCTTTGGGAGGGACACAGCAGAGGATTATGATGACCTCGATTATTAAGGCAACAAAATAGACACTATGACTGTGA
TTGTTTCAATACGTTTGGAATGTGGGTGTTTATTCTTATTAAAATAAATATAAAAAATGCTGTTGTTTCTACT

SEQ ID NO: 15 (M, B/Brisbane/60/08)

AGCAGAAGCACGCACTTTCTTAAAATGTCGCTGTTTGGAGACACAATTGCCTACCTGCTTTCATTGACAGAAGATGG
AGAAGGCAAAGCAGAACTAGCAGAAAAATTACACTGTTGGTTTGGTGGGAAAGAATTTGACCTAGACTCTGCCTTGG
AATGGATAAAAAACAAAAGATGCTTAACTGATATACAAAAAGCACTAATTGGTGCCTCTATATGCTTTTTAAAACCC
AAAGACCAGGAAAGAAAAAGAAGATTCATCACAGAGCCCTTATCAGGAATGGGAACAACAGCAACAAAAAAGAAAGG
CCTGATTCTGGCTGAGAGAAAAATGAGAAGATGTGTGAGCTTTCATGAAGCATTTGAAATAGCAGAAGGCCATGAAA
GCTCAGCGCTACTATACTGTCTCATGGTCATGTACCTGAATCCTGGAAATTATTCAATGCAAGTAAAACTAGGAACG
CTCTGTGCTTTATGCGAGAAACAAGCATCACATTCACACAGGGCTCATAGCAGAGCAGCGAGATCTTCAGTGCCTGG
AGTGAGACGAGAAATGCAGATGGTCTCAGCTATGAACACAGCAAAAACAATGAATGGAATGGGAAAAGGAGAAGACG
TCCAAAAGCTGGCAGAAGAGTTGCAAAGCAACATTGGAGTGCTGAGATCTCTTGGGGCAAGCCAAAAGAATGGGGAA
GGGATTGCAAAGGATGTAATGGAAGTGCTAAAGCAGAGCTCCATGGGAAATTCAGCTCTTGTGAAGAAATATCTATA
ATGCTCGAACCATTTCAGATTCTTACAATTTGTTCTTTTATCTTATCAGCTCTCCATTTCATGGCTTGGACAATAGG
GCATTTGAATCAAATAAAAAGAGGAATAAACATGAAAATACGAATAAAAGGTCCAAACAAAGAGACAATAAACAGAG
AGGTATCAATTTTGAGACACAGTTACCAAAAAGAAATCCAGGCCAAAGAAACAATGAAGGAAGTACTCTCTGACAAC
ATGGAGGTATTGAATGACCACATAATAATTGAGGGGCTTTCTGCCGAAGAGATAATAAAAATGGGTGAAACAGTTTT
GGAGATAGAAGAATTGCATTAAATTCAATTTTACTGTATTTCTTACTATGCATTTAAGCAAATTGTAATCAATGTCA
GCAAATAAACTGGAAAAAGTGCGTTGTTTCTACT

SEQ ID NO: 16 (NS, B/Brisbane/60/08)

AGCAGAAGCAGAGGATTTGTTTAGTCACTGGCAAACAGGGAAAAATGGCGAACAACAACATGACCACAACACAAATT
GAGGTGGGTCCGGGAGCAACCAATGCCACCATAAACTTTGAAGCAGGAATTCTAGAGTGCTATGAAAGGCTTTCATG
GCAAAGAGCCCTTGACTACCCTGGTCAAGACCGCCTAAACAGACTAAAGAGAAAATTAGAGTCAAGAATAAAGACTC
ACAACAAAAGTGAGCCTGAAAGTAAAAGGATGTCCCTTGAAGAGAGAAAAGCAATTGGAGTAAAAATGATGAAAGTA
CTCCTATTTATGAATCCGTCTGCTGGAATTGAAGGGTTTGAGCCATACTGTATGAAAAGTTCCTCAAATAGCAACTG
TACGAAATACAATTGGACTGATTACCCTTCAACACCAGAGAGGTGCCTTGATGACATAGAGGAAGAACCAGAGGATG
TTGATGGCCCAACTGAAATAGTATTAAGGGACATGAACAACAAAGATGCAAGGCAAAAGATAAAGGAGGAAGTAAAC
ACTCAGAAAGAAGGGAAGTTCCGTTTGACAATAAAAAGGGATATGCGTAATGTATTGTCCTTGAGAGTGTTGGTAAA
CGGAACATTCCTCAAACACCCCAATGGACACAAGTCCTTATCAACTCTGCATAGATTGAATGCATATGACCAGAGTG
GAAGGCTTGTTGCTAAACTTGTTGCCACTGATGATCTTACAGTGGAGGATGAAGAAGATGGCCATCGGATCCTCAAC
TCACTCTTCGAGCGTCTTAATGAAGGACATTCAAAGCCAATTCGAGCAGCTGAAACTGCGGTGGGAGTCTTATCCCA

ATTTGGTCAAGAGCACCGATTATCACCAGAAGAGGGAGACAATTAGACTGGTCACGGAAGAACTTTATCTTTTAAGT
AAAAGAATTGATGATAACATACTATTCCACAAAACAGTAATAGCTAACAGCTCCATAATAGCTGACATGGTTGTATC
ATTATCATTATTAGAAACATTGTATGAAATGAAGGATGTGGTTGAAGTGTACAGCAGGCAGTGCTTGTGAATTTAAA
ATAAAAATCCTCTTGTTACTACT

SEQ ID NO: 17 (HA, B/Brisbane/60/08)

AGCAGAAGCAGAGCATTTTCTAATATCCACAAAATGAAGGCAATAATTGTACTACTCATGGTAGTAACATCCAATGC
AGATCGAATCTGCACTGGGATAACATCGTCAAACTCACCACATGTCGTCAAAACTGCTACTCAAGGGGAGGTCAATG
TGACTGGTGTAATACCACTGACAACAACACCCACCAAATCTCATTTTGCAAATCTCAAAGGAACAGAAACCAGGGGG
AAACTATGCCCAAAATGCCTCAACTGCACAGATCTGGACGTAGCCTTGGGCAGACCAAAATGCACGGGGAAAATACC
CTCGGCAAGAGTTTCAATACTCCATGAAGTCAGACCTGTTACATCTGGGTGCTTTCCTATAATGCACGACAGAACAA
AAATTAGACAGCTGCCTAACCTTCTCCGAGGATACGAACATATCAGGTTATCAACCCATAACGTTATCAATGCAGAA
AATGCACCAGGAGGACCCTACAAAATTGGAACCTCAGGGTCTTGCCCTAACATTACCAATGGAAACGGATTTTTCGC
AACAATGGCTTGGGCCGTCCCAAAAAACGACAAAAACAAAACAGCAACAAATCCATTAACAATAGAAGTACCATACA
TTTGTACAGAAGGAGAAGACCAAATTACCGTTTGGGGGTTCCACTCTGACAACGAGGCCCAAATGGCAAAGCTCTAT
GGGGACTCAAAGCCCCAGAAGTTCACCTCATCTGCCAACGGAGTGACCACACATTACGTTTCACAGATTGGTGGCTT
CCCAAATCAAACAGAAGACGGAGGACTACCACAAAGTGGTAGAATTGTTGTTGATTACATGGTGCAAAAATCTGGGA
AAACAGGAACAATTACCTATCAAAGGGGTATTTTATTGCCTCAAAAGGTGTGGTGCGCAAGTGGCAGGAGCAAGGTA
ATAAAAGGATCCTTGCCTTTAATTGGAGAAGCAGATTGCCTCCACGAAAAATACGGTGGATTAAACAAAAGCAAGCC
TTACTACACAGGGGAACATGCAAAGGCCATAGGAAATTGCCCAATATGGGTGAAAACACCCTTGAAGCTGGCCAATG
GAACCAAATATAGACCTCCTGCAAAACTATTAAAGGAAAGGGGTTTCTTCGGAGCTATTGCTGGTTTCTTAGAAGGA
GGATGGGAAGGAATGATTGCAGGTTGGCACGGATACACATCCCATGGGGCACATGGAGTAGCGGTGGCAGCAGACCT
TAAGAGCACTCAAGAGGCCATAAACAAGATAACAAAAAATCTCAACTCTTTGAGTGAGCTGGAAGTAAAGAATCTTC
AAAGACTAAGCGGTGCCATGGATGAACTCCACAACGAAATACTAGAACTAGATGAGAAAGTGGATGATCTCAGAGCT
GATACAATAAGCTCACAAATAGAACTCGCAGTCCTGCTTTCCAATGAAGGAATAATAAACAGTGAAGATGAACATCT
CTTGGCGCTTGAAAGAAAGCTGAAGAAAATGCTGGGCCCCTCTGCTGTAGAGATAGGGAATGGATGCTTTGAAACCA
AACACAAGTGCAACCAGACCTGTCTCGACAGAATAGCTGCTGGTACCTTTGATGCAGGAGAATTTTCTCTCCCCACC
TTTGATTCACTGAATATTACTGCTGCATCTTTAAATGACGATGGATTGGATAATCATACTATACTGCTTTACTACTC
AACTGCTGCCTCCAGTTTGGCTGTAACACTGATGATAGCTATCTTTGTTGTTTATATGGTCTCCAGAGACAATGTTT
CTTGCTCCATCTGTCTATAAGGGAAGTTAAGCCCTGTATTTTCCTTTATTGTAGTGCTTGTTTACTTGTTGTCATTA
CAAAGAAACGTTATTGAAAAATGCTCTTGTTACTACT

SEQ ID NO: 18 (NA, B/Brisbane/60/08)

AGCAGAAGCAGAGCATCTTCTCAAAACTGAAGCAAATAGGCCAAAAATGAACAATGCTACCTTCAACTATACAAACG
TTAACCCTATTTCTCACATCAGGGGGAGTATTATTATCACTATATGTGTCAGCTTCATTATCATACTTACTATATTC
GGATATATTGCTAAAATTCTCACCAACAGAAATAACTGCACCAACAATGCCATTGGATTGTGCAAACGCATCAAATG
TTCAGGCTGTGAACCGTTCTGCAACAAAAGGGGTGACACTTCTTCTCCCAGAACCGGAGTGGACATACCCGCGTTTA
TCTTGCCCGGGCTCAACCTTTCAGAAAGCACTCCTAATTAGCCCTCATAGATTCGGAGAAACCAAAGGAAACTCAGC
TCCCTTGATAATAAGGGAACCTTTTATTGCTTGTGGACCAAATGAATGCAAACACTTTGCTCTAACCCATTATGCAG
CCCAACCAGGGGGATACTACAATGGAACAAGAGGAGACAGAAACAAGCTGAGGCATCTAATTTCAGTCAAATTGGGC
AAAATCCCAACAGTAGAAAACTCCATTTTCCACATGGCAGCATGGAGCGGGTCCGCGTGCCATGATGGTAAGGAATG
GACATATATCGGAGTTGATGGCCCTGACAATAATGCATTGCTCAAAGTAAAATATGGAGAAGCATATACTGACACAT
ACCATTCCTATGCAAACAAAATCCTAAGAACACAAGAAAGTGCCTGCAATTGCATCGGGGGAAATTGTTATCTTATG
ATAACTGATGGCTCAGCTTCAGGTGTTAGTGAATGCAGATTTCTTAAGATTCGAGAGGGCCGAATAATAAAAGAAAT
ATTTCCAACAGGAAGAGTAAAACACACTGAGGAATGCACATGCGGATTTGCCAGCAATAAAACCATAGAATGTGCCT
GTAGAGATAACAGTTACACAGCAAAAAGACCTTTTGTCAAATTAAACGTGGAGACTGATACAGCAGAAATAAGATTG
ATGTGCACAGATACTTATTTGGACACCCCCAGACCAAACGATGGAAGCATAACAGGCCCTTGTGAATCTAATGGGGA
CAAAGGGAGTGGAGGCATCAAGGGAGGATTTGTTCATCAAAGAATGGAATCCAAGATTGGAAGGTGGTACTCTCGAA
CGATGTCTAAAACTGAAAGGATGGGGATGGGACTGTATGTCAAGTATGATGGAGACCCATGGGCTGACAGTGATGCC
CTAGCTTTTAGTGGAGTAATGGTTTCAATGAAAGAACCTGGTTGGTACTCCTTTGGCTTCGAAATAAAAGATAAGAA
ATGCGATGTCCCCTGTATTGGGATAGAGATGGTACATGATGGTGGAAAAGAGACTTGGCACTCAGCAGCAACAGCCA
TTTACTGTTTAATGGGCTCAGGACAGCTGCTGTGGGACACTGTCACAGGTGTTGACATGGCTCTGTAATGGAGGAAT
GGTTGAGTCTGTTCTAAACCCTTTGTTCCTGTTTTGTTTGAACAATTGTCCTTACTAAACTTAATTGTTTCTGAAAA
ATGCTCTTGTTACTACT

SEQ ID NO: 19 (NP, B/Lee/40)

MSNMDIDSINTGTIDKKPEELTPGTSGATRPIIKPATLAPPSNKRTRNPSPERTTTSSETDIGRKIQKKQTPTEIKK
SVYNMVVKLGEFYNQMMVKAGLNDDMERNLIQNAQAVERILLAATDDKKTEYQKKRNARDVKEGKEEIDHNKTGGTF
YKMVRDDKTIYFSPIKITFLKEEVKTMYKTTMGSDGFSGLNHIMIGHSQMNDVCFQRSKALKRVGLDPSLISTFAGS
TLPRRSGTTGVAIKGGGTLVAEAIRFIGRAMADRGLLRDIKAKTAYEKILLNLKNKCSAPQQKALVDQVIGSRNPGI
ADIEDLTLLARSMIVVRPSVASKVVLPISIYAKIPQLGFNIEEYSMVGYEAMALYNMATPVSILRMGDDAKDKSQLF

FMSCFGAAYEDLRVLSALTGTEFKPRSALKCKGFHVPAKEQVEGMGAALMSIKLQFWAPMTRSGGNEVSGEGGSGQI
SCSPVFAVERPIALSKQAVRRMLSMNVEGRDADVKGNLLKMMNDSMAKKTSGNAFIGKKMFQISDKNKVNPIEIPIK
QTIPSFFFGRDTAEDYDDLDY

SEQ ID NO: 20 (PA, B/Panama/45/90)

MDTFITRNFQTTIIQKAKNTMAEFSEDPELQPAMLFNICVHLEVCYVISDMNFLDEEGKSYTALEGQGKEQNLRPQY
EVIEGMPRTIAWMVQRSLAQEHGIETPKYLADLFDYKTKRFIEVGITKGLADDYFWKKKEKLGNSMELMIFSYNQDY
SLSNESSLDEEGKGRVLSRLTELQAELSLKNLWQVLIGEEDVEKGIDFKLGQTISRLRDISVPAGFSNFEGMRSYID
NIDPKGAIERNLARMSPLVSATPKKLKWEDLRPIGPHIYNHELPEVPYNAFLLMSDELGLANMTEGKSKKPKTLAKE
CLEKYSTLRDQTDPILIMKSEKANENFLWKLWRDCVNTISNEEMSNELQKTNYAKWATGDGLTYQKIMKEVAIDDET
MCQEEPKIPNKCRVAAWVQTEMNLLSTLTSKRALDLPEIGPDVAPVEHVGSERRKYFVNEINCCKASTVMMKYVLFH
TSLLNESNASMGKYKVIPITNRVVNEKGESFDMLYGLAVKGQSHLRGDTDVVTVVTFEFSGTDPRVDSGKWPKYTVF
RIGSLFVSGREKSVYLYCRVNGTNKIQMKWGMEARRCLLQSMQQMEAIVEQESSIQGYDMTKACFKGDRVNSPKTFS
IGTQEGKLVKGSFGKALRVIFTKCLMHYVFGNAQLEGFSAESRRLLLLIQALKDRKGPWVFDLEGMYSGIEECISNN
PWVIQSAYWFNEWLGFEKEGSKVLESVDEIMNE

SEQ ID NO: 21 (PB1, B/Panama/45/90)

MNINPYFLFIDVPIQAAISTTFPYTGVPPYSHGTGTGHTIDTVIRTHEYSNKGKQYVSDITGCTMVDPTNGPLPEDN
EPSAYAQLDCVLEALDRMDEEHPGLFQAASQNAMEALMVTTVDKLTQGRQTFDWTVCRNQPAATALNTTITSFRLND
LNGADKGGLVPFCQDIIDSLDKPEMTFFSVKNIKKKLPAKNRKGFLIKRIPMKVKDRITRVEYIKRALSLNTMTKDA
ERGKLKRRAIATAGIQIRGFVLVVENLAKNICENLEQSGLPVGGNEKKAKLSNAVAKMLSNCPPGGISMTVTGDNTK
WNECLNPRIFLAMTERITRDSPIWFRDFCSIAPVLFSNKIARLGKGFMITSKTKRLKAQIPCPDLFSIPLERYNEET
RAKLKKLKPFFNEEGTASLSPGMMMGMFNMLSTVLGVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGI
NDFYRTCKLLGINMSKKKSYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMG
PATAQTAIQLFIADYRYTYKCHRGDSKVEGKRMKIIKELWENTKGRDGLLVADGGPNIYNLRNLHIPEIVLKYNLMD
PEYKGRLLHPQNPFVGHLSIEGIKEADITPAHGPVKKMDYDAVSGTHSWRTKRNRSILNTDQRNMILEEQCYAKCCN
LFEACFNSASYRKPVGQHSMLEAMAHRLRVDARLDYESGRMSKDDFEKAMAHLGEIGYI

SEQ ID NO: 22 (PB2, B/Panama/45/90)

MTLAKIELLKQLLRDNEAKTVLKQTTVDQYNIIRKFNTSRIEKNPSLRMKWAMCSNFPLALTKGDMANRIPLEYKGI
QLKTNAEDIGTKGQMCSIAAVTWWNTYGPIGDTEGFEKVYESFFLRKMRLDNATWGRITFGPVERVRKRVLLNPLTK
EMPPDEASNVIMEILFPKEAGIPRESTWIHRELIKEKREKLKGTMITPIVLAYMLERELVARRRFLPVAGATSAEFI
EMLHCLQGENWRQIYHPGGNKLTESRSQSMIVACRKIIRRSIVASNPLELAVEIANKTVIDTEPLKSCLTAIDGGDV
ACDIIRAALGLKIRQRQRFGRLELKRISGRGFKNDEEILIGNGTIQKIGIWDGEEEFHVRCGECRGILKKSKMRMEK
LLINSAKKEDMKDLIILCMVFSQDTRMFQGVRGEINFLNRAGQLLSPMYQLQRYFLNRSNDLFDQWGYEESPKASEL
HGINELMNASDYTLKGVVVTKNVIDDFSSTETEKVSITKNLSLIKRTGEVIMGANDVSELESQAQLMITYDTPKMWE
MGTTKELVQNTYQWVLKNLVTLKAQFLLGKEDMFQWDAFEAFESIIPQKMAGQYSGFARAVLKQMRDQEVMKTDQFI
KLLPFCFSPPKLRRNGEPYQFLRLVLKGGGENFIEVRKGSPLFSYNPQTEVLTICGRMMSLKGKIEDEERNRSMGNA
VLAGFLVSGKYDPDLGDFKTIEELEKLKPGEKANILLYQGKPVKVVKRKRYSALSNDISQGIKRQRMTVESMGWALS

SEQ ID NO: 23 (NP, B/Panama/45/90)

MSNMDIDGINTGTIDKTPEEITSGTSGTTRPIIRPATLAPPSNKRTRNPSPERATTSSEADVGRKTQKKQTPTEIKK
SVYNMVVKLGEFYNQMMVKAGLNDDMERNLIQNAHAVERILLAATDDKKTEFQRKKNARDVKEGKEEIDHNKTGGTF
YKMVRDDKTIYFSPIRITFLKEEVKTMYKTTMGSDGFSGLNHIMIGHSQMNDVCFQRSKALKRVGLDPSLISTFAGS
TLPRRSGATGVAIKGGGTLVAEAIRFIGRAMADRGLLRDIKAKTAYEKILLNLKNKCSAPQQKALVDQVIGSRNPGI
ADIEDLTLLARSMVVVRPSVASKVVLPISIYAKIPQLGFNVEEYSMVGYEAMALYNMATPVSILRMGDDAKDKSQLF
FMSCFGAAYEDLRVLSALTGIEFKPRSALKCKGFHVPAKEQVEGMGAALMSIKLQFWAPMTRSGGNEVGGDGGSGQI
SCSPVFAVERPIALSKQAVRRMLSMNIEGRDADVKGNLLKMMNDSMAKKTNGNAFIGKKMFQISDKNKTNPVEIPIK
QTIPNFFFGRDTAEDYDDLDY

SEQ ID NO: 24 (M_1, B/Panama/45/90)

MSLFGDTIAYLLSLTEDGEGKAELAEKLHCWFGGKEFDLDSALEWIKNKRCLTDIQKALIGASICFLKPKDQERKRR
FITEPLSGMGTTATKKKGLILAERKMRRCVSFHEAFEIAEGHESSALLYCLMVMYLNPGNYSMQVKLGTLCALCEKQ
ASHSHRAHSRAARSSVPGVRREMQMVSAMNTAKTMNGMGKGEDVQKLAEELQSNIGVLRSLGASQKNGEGIAKDVME
VLKQSSMGNSALVKKYL

SEQ ID NO: 25 (M_2, B/Panama/45/90)

MLEPFQILSICSFILSALHFMAWTIGHLNQIKRGVNMKIRIKNPNKETINREVSILRHSYQKEIQAKETMKEVLSDN
MEVLSDHIVIEGLSAEEIIKMGETVLEVEELH

SEQ ID NO: 26 (NS_1, B/Panama/45/90)

MADNMTTTQIEVGPGATNATINFEAGILECYERLSWQRALDYPGQDRLNKLKRKLESRIKTHNKSEPESKRMSLEER
KAIGVKMMKVLLFMNPSAGVEGFEPYCMKNPSNSNCPDCNWADYPPTPGKYLDGIEEEPENVGDSTEIVLRDMNNKD
ARQKIKEEVNTQKEGKFRLTIKRDIRNVLSLRVLVNGTFIKHPNGYKSLSTLHRLNAYDQSGRLVAKLVATDDLTVE
DEEDGHRILNSLFERLNEGHSKPIRAAETAVGVLSQFGQEHRLSPEERDN

SEQ ID NO: 27 (NS_2, B/Panama/45/90)

MADNMTTTQIEWRMKKMAIGSSTHSSSVLMKDIQSQFEQLKLRWESYPNLVKSTDYHQKRETIRLVTEELYLLSKRI
DDNILFHKTVIANSSIIADMIVSLSLLETLYEMKDVVEVYSRQCL

SEQ ID NO: 28 (HA, B/Panama/45/90)

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSHFANLKGTKTRGKLCPNCLNCTD
LDVALGRPMCVGTTPSAKASILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYENIRLSTQNVINAERAPGGPYRLGT
SGSCPNVTSRDGFFATMAWAVPRDNKTATNPLTVEVPYICTKGEDQITVWGFHSDDKTQMKNLYGDSNPQKFTSSAN
GVTTHYVSQIGGFPNQTEDGGLPQSGRIVVDYMVQKPGKTGTIVYQRGVLLPQKVWCASGRSKVIKGSLPLIGEADC
LHEKYGGLNKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAGWHGYT
SHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLL
SNEGIINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNITAASLND
DGLDNHTILLYYSTAASSLAVTLMIAIFIVYMVSRDNVSCSICL

SEQ ID NO: 29 (NA, B/Panama/45/90)

MLPSTIQTLTLFLTSGGVLLSLYVSASLSYLLYSDILLKFSPTEITAPTMPLDCANASNVQAVNRSATKEMTLLLPE
PEWTYPRLSCPGSTFQKALLISPHRFGETRGNSAPLTIREPFIACGPKECKHFALTHYAAQPGGYYNGTREDRNKLR
HLISVKLGKIPTVENSIFHMAAWSGSACHDGREWTYIGVDGPDSNALIKIKYGEAYTDTYHSYANNILRTQESACNC
IGGDCYLMITDGSASGISKCRFLKIREGRIIKEIFPTGRVEHTEECTCGFASNKTIECACRDNSYTAKRPFVKLNVE
TDTAEIRLMCTETYLDTPRPDDGSITGPCESNGDKGRGGIKGGFVHQRMASKIGRWYSRTMSKTERMGMELYVKYDG
DPWTDSEALAPSGVMVSMEEPGWYSFGFEIKDKKCDVPCIGIEMVHDGGKKTWHSAATAIYCLMGSGQLLWDTVTGV
DMAL

SEQ ID NO: 30 (PA, B/Panama/45/90)

AGCAGAAGCGGTGCGTTTGATTTGCCATAATGGATACTTTTATTACAAGAAACTTCCAGACTACAATAATACAAAAG
GCCAAAAACACAATGGCAGAATTTAGTGAAGATCCTGAATTACAACCAGCAATGCTATTCAACATCTGCGTCCATCT
AGAGGTTTGCTATGTAATAAGTGACATGAATTTTCTTGACGAAGAAGGAAAATCATATACAGCATTAGAAGGACAAG
GAAAAGAACAAAACTTGAGACCACAATATGAAGTAATTGAGGGAATGCCAAGAACCATAGCATGGATGGTCCAAAGA
TCCTTAGCTCAAGAGCATGGAATAGAGACTCCAAAGTATCTGGCTGATTTGTTTGATTATAAAACCAAGAGATTTAT
AGAAGTTGGAATAACAAAAGGATTGGCTGATGATTACTTTTGGAAAAAGAAAGAAAAGCTGGGAAATAGCATGGAAC
TGATGATATTCAGCTACAATCAAGACTATTCGTTAAGTAATGAATCCTCATTGGATGAGGAAGGGAAAGGGAGAGTG
CTAAGCAGACTCACAGAACTTCAGGCTGAATTAAGTCTGAAAAACCTATGGCAAGTTCTCATAGGAGAAGAAGATGT
TGAAAAGGGAATTGACTTTAAACTTGGACAAACAATATCTAGACTAAGGGATATATCTGTTCCAGCTGGTTTCTCCA
ATTTTGAAGGAATGAGGAGCTACATAGACAATATAGATCCTAAAGGAGCAATAGAAAGAAATCTAGCAAGGATGTCT
CCCTTAGTATCAGCCACACCTAAAAAGTTGAAATGGGAGGACCTAAGACCAATAGGGCCTCACATTTACAACCATGA
GTTACCAGAAGTTCCATATAATGCCTTTCTTCTAATGTCTGATGAATTGGGGCTGGCCAATATGACTGAGGGAAAGT
CCAAAAAACCGAAGACATTAGCCAAAGAATGTCTAGAAAAGTACTCAACACTACGGGATCAAACTGACCCAATATTA
ATAATGAAAAGCGAAAAAGCTAACGAAAATTTCCTATGGAAGCTGTGGAGGGACTGTGTAAATACAATAAGTAATGA
GGAAATGAGTAACGAGTTACAGAAAACCAATTATGCCAAGTGGGCCACAGGAGATGGATTAACATACCAGAAAATAA
TGAAAGAAGTAGCAATAGATGACGAAACAATGTGCCAAGAAGAGCCTAAAATCCCTAACAAATGTAGAGTGGCTGCT
TGGGTTCAAACAGAGATGAATTTATTGAGCACTCTGACAAGTAAAAGAGCTCTGGACCTACCAGAAATAGGGCCAGA
CGTAGCACCCGTGGAGCATGTAGGGAGTGAAAGAAGGAAATACTTTGTTAATGAAATCAACTGCTGTAAGGCCTCTA
CAGTTATGATGAAGTATGTGCTTTTTCACACTTCATTATTGAATGAAAGCAATGCCAGCATGGGAAAATATAAAGTA
ATACCAATAACCAATAGAGTAGTAAATGAAAAAGGAGAAAGTTTCGACATGCTTTATGGTCTGGCGGTTAAAGGACA
ATCTCATCTGAGGGGAGATACTGATGTTGTAACAGTTGTGACTTTCGAATTTAGTGGTACAGATCCCAGAGTGGACT
CAGGAAAGTGGCCAAAATATACTGTGTTTAGGATTGGCTCCCTATTTGTGAGTGGGAGGGAAAAATCTGTGTACCTA
TATTGCCGAGTGAATGGCACAAATAAGATCCAAATGAAATGGGGAATGGAAGCTAGAAGATGTCTGCTTCAATCAAT
GCAACAAATGGAAGCAATTGTTGAACAAGAATCATCGATACAAGGATATGACATGACCAAAGCTTGTTTCAAGGGAG
ACAGAGTAAATAGCCCCAAAACTTTTAGTATTGGGACTCAAGAAGGAAAACTAGTAAAAGGATCCTTTGGGAAAGCA
CTAAGAGTAATATTTACCAAATGTTTGATGCACTATGTATTTGGAAATGCCCAATTGGAGGGGTTTAGTGCCGAGTC
TAGGAGACTTCTACTGTTAATTCAAGCACTAAAGGACAGAAAGGGCCCTTGGGTGTTCGACTTAGAGGGAATGTATT
CTGGAATAGAAGAATGTATTAGTAACAACCCTTGGGTAATACAGAGTGCATACTGGTTCAATGAATGGTTGGGCTTT
GAAAAGGAGGGGAGTAAAGTATTAGAATCAGTAGATGAAATAATGAATGAATGAAAAAACATAGTACTCAATTTGGT
ACTATTTTGTTCATTATGTATCTAAACATCCAATAAAAAGAATCGAGAATCAAAAATGCACGTGTTTCTACT

SEQ ID NO: 31 (PB1, B/Panama/45/90)

AGCAGAAGCGGAGCCTTTAAGATGAATATAAATCCTTATTTTCTCTTCATAGATGTACCCATACAGGCAGCAATTTC
AACAACATTCCCATACACCGGTGTTCCCCCTTACTCCCATGGAACGGGAACAGGCCACACAATAGACACCGTGATCA
GAACACATGAGTACTCGAACAAGGGAAAACAGTATGTTTCTGACATCACAGGATGTACAATGGTAGATCCAACAAAT
GGGCCATTACCCGAAGACAATGAGCCGAGTGCCTATGCACAATTAGATTGCGTTCTGGAGGCTTTGGATAGAATGGA
TGAAGAACATCCAGGTTTGTTTCAAGCAGCCTCACAGAATGCCATGGAGGCACTAATGGTCACAACTGTAGACAAAT
TAACCCAGGGGAGACAGACTTTTGATTGGACAGTATGCAGAAACCAGCCTGCTGCAACGGCACTAAACACAACAATA
ACCTCCTTTAGGTTGAATGATTTGAATGGAGCTGACAAGGGTGGATTGGTACCCTTTTGCCAAGATATCATTGATTC
ATTGGACAAACCTGAAATGACTTTCTTCTCAGTAAAGAATATAAAGAAAAAATTGCCTGCTAAAAACAGAAAGGGTT
TCCTCATAAAGAGAATACCAATGAAAGTAAAAGACAGGATAACCAGAGTGGAATACATCAAAAGAGCATTATCATTA
AACACAATGACAAAAGATGCTGAAAGGGGCAAACTAAAAAGAAGAGCGATTGCAACCGCTGGAATACAAATCAGAGG
GTTTGTATTAGTAGTTGAAAACTTGGCTAAAAATATCTGTGAAAATCTAGAACAAAGTGGTTTGCCCGTAGGTGGAA
ATGAAAAGAAGGCCAAACTGTCAAATGCAGTGGCCAAAATGCTCAGTAACTGCCCACCAGGAGGGATCAGCATGACA
GTAACAGGAGACAATACTAAATGGAATGAATGCTTAAATCCAAGAATCTTTTTGGCTATGACTGAAAGGATAACAAG
AGACAGCCCAATTTGGTTCCGGGATTTTTGTAGTATAGCACCGGTCTTGTTCTCCAATAAAATAGCCAGATTGGGAA
AAGGATTTATGATAACAAGCAAAACAAAAAGACTGAAGGCTCAAATACCTTGTCCAGATCTGTTTAGCATACCATTA
GAAAGATATAATGAAGAAACAAGGGCAAAATTAAAAAAGCTGAAACCATTCTTCAATGAAGAAGGAACGGCATCTTT
GTCGCCTGGGATGATGATGGGAATGTTTAATATGCTATCTACCGTGTTGGGAGTAGCCGCACTAGGTATCAAAAACA
TTGGAAACAAAGAATATTTATGGGATGGACTGCAATCTTCTGATGATTTTGCTCTGTTTGTTAATGCAAAAGATGAA
GAGACATGTATGGAAGGAATAAACGACTTTTACCGAACATGTAAATTATTGGGAATAAACATGAGCAAAAAGAAAAG
TTACTGTAATGAAACTGGAATGTTTGAATTTACAAGCATGTTCTATAGAGATGGATTTGTATCTAATTTTGCAATGG
AAATTCCTTCATTTGGAGTTGCTGGAGTAAATGAATCAGCAGATATGGCAATAGGAATGACAATAATAAAGAACAAT
ATGATCAACAATGGGATGGGTCCAGCAACAGCACAAACAGCCATACAATTATTCATAGCTGATTATAGGTACACCTA
CAAATGCCACAGGGGAGATTCCAAAGTGGAAGGAAAAAGAATGAAAATTATAAAGGAGCTATGGGAAAACACTAAAG
GAAGAGATGGTCTGTTAGTGGCAGATGGTGGGCCCAACATTTACAATTTGAGAAACTTACATATCCCAGAAATAGTA
TTGAAGTACAACCTAATGGACCCTGAATACAAAGGGCGGTTACTTCATCCTCAAAATCCATTTGTAGGACATTTATC
TATTGAGGGCATCAAAGAAGCAGATATAACCCCAGCACATGGTCCCGTAAAGAAAATGGATTATGATGCAGTATCTG
GAACTCATAGTTGGAGAACCAAAAGGAACAGATCTATACTAAATACTGACCAGAGGAACATGATTCTTGAGGAACAA
TGCTACGCTAAGTGTTGCAACCTTTTTGAGGCCTGTTTTAATAGTGCATCATACAGGAAACCAGTAGGTCAGCACAG
CATGCTTGAGGCTATGGCCCACAGATTAAGAGTGGATGCACGACTAGATTATGAATCAGGAAGAATGTCAAAGGATG
ATTTTGAGAAAGCAATGGCTCACCTTGGTGAGATTGGGTACATATAAGCTCCGAAGATGTCTATGGGGTTATTGGTC
ATCATTGAATACATGTGATAAACAAATGATTAAAATGAAAAAAGGCTCGTGTTTCTACT

SEQ ID NO: 32 (PB2, B/Panama/45/90)

AGCAGAAGCGGAGCGTTTTCAAGATGACATTGGCTAAAATTGAATTGTTAAAACAACTGTTAAGGGACAATGAAGCC
AAAACAGTATTGAAACAAACAACGGTAGACCAATATAACATAATAAGAAAATTCAATACATCAAGAATTGAAAAGAA
CCCTTCATTGAGGATGAAGTGGGCAATGTGTTCTAATTTTCCCTTGGCTCTGACCAAGGGTGATATGGCAAACAGAA
TCCCCTTGGAATACAAGGGAATACAACTTAAAACAAATGCTGAAGACATAGGAACTAAAGGCCAAATGTGCTCAATA
GCAGCAGTTACCTGGTGGAATACATATGGACCAATAGGAGATACTGAAGGTTTCGAAAAGGTCTACGAAAGCTTTTT
TCTCAGAAAGATGAGACTTGACAATGCCACTTGGGGCCGAATAACTTTTGGCCCAGTTGAAAGAGTAAGAAAAAGGG
TACTGCTAAACCCTCTCACCAAGGAAATGCCTCCAGATGAAGCAAGTAATGTGATAATGGAAATATTGTTCCCTAAG
GAAGCAGGAATACCAAGAGAATCTACTTGGATACATAGGGAACTGATAAAAGAAAAAAGAGAAAAATTGAAAGGAAC
AATGATAACTCCCATTGTACTGGCATACATGCTTGAGAGAGAATTGGTTGCCAGAAGAAGGTTCCTGCCGGTGGCAG
GAGCAACATCAGCTGAGTTCATAGAAATGCTACACTGCTTACAAGGTGAAAATTGGAGACAAATATATCACCCAGGA
GGAAATAAACTAACTGAATCTAGGTCTCAATCGATGATTGTAGCTTGTAGAAAGATAATCAGAAGATCAATAGTCGC
ATCAAACCCATTAGAGCTAGCTGTAGAAATTGCAAACAAGACTGTGATAGATACTGAACCTTTAAAATCATGTCTGA
CAGCCATAGACGGAGGTGATGTAGCCTGTGACATAATAAGAGCTGCATTAGGACTAAAGATCAGACAAAGACAAAGA
TTTGGACGACTTGAACTAAAGAGAATATCAGGAAGAGGATTCAAAAATGATGAAGAAATATTAATCGGGAACGGAAC
AATACAGAAGATTGGAATATGGGACGGAGAAGAGGAGTTCCATGTAAGATGTGGTGAATGCAGGGGAATATTAAAAA
AGAGCAAAATGAGAATGGAAAAACTACTAATAAATTCAGCTAAAAAGGAAGACATGAAAGATTTAATAATCTTGTGC
ATGGTATTTTCTCAAGACACTAGGATGTTCCAAGGAGTGAGAGGAGAAATAAATTTTCTTAATAGAGCAGGCCAACT
TTTATCTCCAATGTACCAACTCCAAAGATATTTTTTGAATAGAAGCAACGATCTCTTTGATCAATGGGGGTATGAGG
AATCACCCAAAGCAAGTGAGCTACATGGAATAAATGAATTAATGAATGCATCTGACTACACTTTGAAAGGGGTTGTA
GTAACAAAAAATGTAATTGATGATTTTAGTTCTACTGAAACAGAAAAAGTATCTATAACAAAAAATCTTAGTTTAAT
AAAAAGGACTGGGGAAGTCATAATGGGGGCTAATGACGTAAGTGAATTAGAATCACAAGCTCAGCTAATGATAACAT
ATGATACACCTAAGATGTGGGAGATGGGAACAACCAAAGAACTGGTGCAAAACACCTACCAATGGGTGCTGAAAAAT
TTGGTAACACTGAAGGCTCAGTTTCTTCTAGGAAAAGAAGACATGTTCCAATGGGATGCATTTGAAGCATTTGAAAG
CATAATCCCCCAGAAGATGGCTGGCCAGTACAGTGGATTTGCAAGAGCAGTGCTCAAACAAATGAGAGACCAAGAGG
TTATGAAAACTGACCAGTTCATAAAGTTGTTGCCCTTTTGTTTCTCACCACCAAAATTAAGGAGAAATGGGGAGCCT
TATCAGTTCTTGAGGCTTGTATTGAAGGGAGGAGGAGAAAATTTCATCGAAGTAAGGAAAGGGTCCCCTCTATTCTC
TTACAATCCACAAACAGAAGTCCTAACTATATGCGGCAGAATGATGTCATTAAAAGGGAAAATTGAAGATGAAGAAA

GGAATAGATCAATGGGGAATGCAGTATTAGCGGGCTTTCTCGTTAGTGGCAAGTATGACCCAGATCTTGGAGATTTC
AAAACTATTGAAGAACTTGAAAAGCTGAAACCGGGGGGAGAAAGCAAACATCTTACTTTATCAAGGAAAGCCCGTTAA
AGTAGTTAAAAGGAAAAGATATAGTGCTTTATCCAATGACATTTCACAAGGAATTAAGAGACAAAGAATGACAGTTG
AGTCCATGGGGTGGGCCTTGAGCTAATATAAATTTATCCATTAATTCAATAAACACAATTGAGTGAAAAATGCTCGT
GTTTCTACT

SEQ ID NO: 33 (NP, B/Panama/45/90)

AGCAGAAGCACAGCATTTTCTTATTAACTTCAAGTACCAACAAAAGAACTGAAAATCAAAATGTCCAACATGGATAT
TGACGGTATCAACACTGGGACAATTGACAAAACACCGGAAGAAATAACTTCTGGAACCAGTGGGACAACCAGACCAA
TCATCAGACCAGCAACCCTTGCCCCACCAAGCAACAAACGAACCCGGAACCCATCCCCGGAAAGAGCAACCACAAGC
AGTGAAGCTGATGTCGGAAGGAAAACCCAAAAGAAACAGACCCCGACAGAGATAAAGAAGAGCGTCTACAATATGGT
AGTGAAACTGGGTGAATTCTATAACCAGATGATGGTCAAAGCTGGACTCAACGATGACATGGAGAGAAACCTAATCC
AAAATGCGCATGCTGTGGAAAGAATTCTATTGGCTGCCACTGATGACAAGAAAACTGAATTCCAGAGGAAAAAGAAT
GCCAGAGATGTCAAAGAAGGAAAAGAAGAAATAGACCACAACAAAACAGGAGGCACCTTTTACAAGATGGTAAGAGA
TGATAAAACCATCTACTTCAGCCCTATAAGAATTACCTTTTTAAAAGAAGAGGTGAAAACAATGTACAAAACCACCA
TGGGGAGTGATGGCTTCAGTGGACTAAATCACATAATGATTGGGCATTCACAGATGAATGATGTCTGTTTCCAAAGA
TCAAAGGCCCTAAAAAGAGTTGGACTTGACCCTTCATTAATCAGTACCTTTGCAGGAAGCACACTCCCCAGAAGATC
AGGTGCAACTGGTGTTGCAATCAAAGGAGGTGGAACTTTAGTGGCTGAAGCCATTCGATTTATAGGAAGAGCAATGG
CAGACAGAGGGCTATTGAGAGACATCAAAGCCAAGACTGCCTATGAAAAGATTCTTCTGAATCTAAAAAACAAATGC
TCTGCGCCCCAACAAAAGGCTCTAGTTGATCAAGTGATCGGAAGTAGAAATCCAGGGATTGCAGACATTGAAGACCT
AACCCTGCTTGCTCGTAGTATGGTCGTTGTTAGGCCCTCTGTGGCGAGCAAAGTAGTGCTTCCCATAAGCATTTATG
CTAAAATACCTCAACTAGGGTTCAATGTTGAAGAATACTCTATGGTTGGGTATGAAGCCATGGCTCTCTACAATATG
GCAACACCTGTTTCCATATTAAGAATGGGAGATGATGCAAAAGATAAATCGCAATTATTCTTCATGTCTTGCTTCGG
AGCTGCCTATGAAGACCTGAGAGTTTTGTCTGCATTAACAGGCATAGAATTCAAGCCTAGATCAGCATTAAAATGCA
AGGGTTTCCATGTTCCAGCAAAGGAACAGGTGGAAGGAATGGGGGCAGCTCTGATGTCCATCAAGCTCCAGTTTTGG
GCTCCAATGACCAGATCTGGAGGGAACGAAGTAGGTGGAGACGGAGGGTCTGGCCAAATAAGTTGCAGCCCAGTGTT
TGCAGTAGAAAGACCTATTGCTCTAAGCAAGCAAGCTGTAAGAAGAATGCTTTCAATGAATATTGAGGGACGTGATG
CAGATGTCAAAGGAAATCTACTCAAGATGATGAATGACTCAATGGCTAAGAAAACCAATGGAAATGCTTTCATTGGG
AAGAAAATGTTTCAAATATCAGACAAAAACAAAACCAATCCCGTTGAAATTCCAATTAAGCAGACCATCCCCAATTT
CTTCTTTGGGAGGGACACAGCAGAGGATTATGATGACCTCGATTATTAAAGCAACAAAATAGACACTATGACTGTGA
TTGTTTCAATACGTTTGGAATGTGGGTGTTTACTCTTATTGAAATAAATATAAAAAATGCTGTTGTTTCTACT

SEQ ID NO: 34 (M, B/Panama/45/90)

AGCAGAAGCACGCACTTTCTTAAAATGTCGCTGTTTGGAGACACAATTGCCTACCTGCTTTCATTGACAGAAGATGG
AGAAGGCAAAGCAGAACTAGCAGAAAAATTACACTGTTGGTTCGGTGGGAAAGAATTTGACCTAGACTCTGCCTTGG
AATGGATAAAAAACAAAAGATGCTTAACTGATATACAGAAAGCACTAATTGGTGCCTCTATCTGCTTTTTAAAACCA
AAAGACCAAGAAAGAAAAAGAAGATTCATCACAGAGCCCCTATCAGGAATGGGAACAACAGCAACAAAAAAGAAGGG
CCTGATTCTAGCTGAGAGAAAAATGAGAAGATGTGTGAGTTTTCATGAAGCATTTGAAATAGCAGAAGGCCATGAAA
GCTCAGCGCTACTATATTGTCTCATGGTCATGTACCTGAACCCTGGAAATTATTCAATGCAAGTAAAACTAGGAACG
CTCTGTGCTTTGTGCGAGAAACAAGCATCACATTCACACAGGGCTCATAGCAGAGCAGCAAGATCTTCAGTGCCTGG
AGTGAGGCGAGAAATGCAGATGGTCTCAGCTATGAACACAGCAAAAACAATGAATGGAATGGGAAAGGGAGAAGACG
TCCAAAAACTGGCAGAAGAGCTGCAAAGCAACATTGGAGTATTGAGATCTCTTGGGGCAAGTCAAAAGAATGGGGAA
GGAATTGCAAAGGATGTGATGGAAGTGCTAAAGCAGAGCTCTATGGGAAATTCAGCTCTTGTGAAGAAATACCTATA
ATGCTCGAACCATTTCAGATTCTTTCAATTTGTTCTTTCATCTTATCAGCTCTCCATTTCATGGCTTGGACAATAGG
GCATTTGAATCAAATAAAAAGAGGAGTAAACATGAAAATACGAATAAAAAATCCAAATAAAGAGACAATAAACAGAG
AGGTATCAATTTTGAGACACAGTTACCAAAAAGAAATCCAGGCCAAAGAAACAATGAAGGAAGTACTCTCTGACAAC
ATGGAGGTATTGAGTGACCACATAGTAATTGAGGGGCTTTCTGCTGAAGAGATAATAAAAATGGGTGAAACAGTTTT
GGAGGTAGAAGAATTGCATTAAATTCAATTTTTACTGTATTTCTTGCTATGCATTTAAGCAAATTGTAATCAATGTC
AGCAAATAAACTGGAAAAAGTGCGTTGTTTCTACT

SEQ ID NO: 35 (NS, B/Panama/45/90)

AGCAGAAGCAGAGGATTTGTTTAGTCACTGGCAAACGAAAAAATGGCGGACAACATGACCACAACACAAATTGAGGT
GGGTCCGGGAGCAACCAATGCCACCATAAACTTTGAAGCAGGAATTTTGGAGTGCTATGAAAGGCTTTCATGGCAAA
GAGCCCTTGACTACCCTGGTCAAGACCGCCTAAACAAACTAAAGAGAAAATTGGAATCAAGAATAAAGACTCACAAC
AAAAGTGAGCCAGAAAGTAAAAGGATGTCTCTTGAAGAGAGAAAAGCTATTGGGGTAAAAATGATGAAAGTGCTCCT
ATTTATGAACCCATCTGCTGGAGTTGAAGGGTTTGAGCCATATTGTATGAAAAATCCCTCCAATAGCAACTGTCCAG
ACTGCAATTGGGCTGATTACCCTCCAACACCAGGAAAGTACCTTGATGGCATAGAAGAAGAACCGGAGAATGTTGGT
GACTCAACTGAAATAGTATTAAGGGACATGAACAACAAAGATGCAAGGCAAAAGATAAAAGAGGAAGTAAACACTCA
GAAAGAAGGGAAATTCCGTTTGACAATAAAAAGGGATATACGTAATGTGTTGTCCTTGAGAGTGTTGGTAAACGGAA
CATTCATCAAGCACCCTAATGGATACAAGTCCTTATCAACTCTGCATAGATTGAATGCATATGACCAGAGTGGAAGA
CTTGTTGCTAAACTTGTTGCTACTGATGATCTTACAGTGGAGGATGAAGAAGATGGCCATCGGATCCTCAACTCACT

CTTCGAGCGTCTTAATGAAGGACATTCAAAGCCAATTCGAGCAGCTGAAACTGCGGTGGGAGTCTTATCCCAATTTG
GTCAAGAGCACCGATTATCACCAGAAGAGAGAGACAATTAGACTGGTTACGGAAGAACTTTATCTTTTAAGTAAAAG
AATTGATGATAACATATTGTTCCACAAAACAGTAATAGCCAACAGCTCCATAATAGCTGACATGATTGTATCATTAT
CATTATTGGAAACATTGTATGAAATGAAGGATGTGGTTGAAGTGTACAGCAGGCAGTGCTTGTGAATTTAAAATAAA
AATCCTCTTGTTACTACT

SEQ ID NO: 36 (NA, B/Panama/45/90)

AGCAGAAGCAGAGCATCTTCTCAAAACTGAGGCAAATAGGCCAAAAATGAACAATGCTACCTTCAACTATACAAACG
TTAACCCTATTTCTCACATCAGGGGGAGTGTTATTATCACTATATGTGTCAGCTTCACTATCATACTTACTGTATTC
GGATATATTGCTAAAATTTTCACCAACAGAAATAACTGCACCAACAATGCCATTGGATTGTGCAAACGCATCAAATG
TTCAGGCTGTGAACCGTTCTGCAACAAAAGAGATGACACTTCTTCTCCCAGAACCGGAGTGGACATACCCTCGTTTA
TCTTGCCCGGGCTCAACCTTTCAGAAAGCACTCCTAATTAGCCCTCATAGATTCGGAGAAACCAGAGGAAACTCAGC
TCCCTTGACAATAAGGGAACCTTTTATTGCTTGTGGACCAAAGGAATGCAAACACTTTGCTCTAACCCATTATGCAG
CTCAACCAGGGGGATACTACAATGGAACAAGAGAGGACAGAAACAAGCTGAGGCATCTGATTTCAGTCAAATTGGGC
AAAATACCAACAGTAGAAAACTCCATTTTCCACATGGCAGCTTGGAGCGGGTCCGCATGCCATGATGGTAGAGAATG
GACATATATCGGAGTTGATGGCCCTGACAGTAATGCATTGATCAAAATAAAATATGGAGAAGCATATACTGACACAT
ACCATTCCTATGCAAACAACATCCTAAGAACACAAGAAAGTGCCTGCAATTGCATTGGGGGAGATTGTTATCTTATG
ATAACTGATGGCTCAGCTTCAGGAATTAGTAAATGCAGATTTCTTAAGATTCGAGAGGGTCGAATAATAAAAGAAAT
ATTTCCAACAGGAAGAGTAGAACATACTGAAGAATGCACATGCGGATTTGCCAGCAACAAAACCATAGAATGTGCCT
GTAGAGATAACAGTTACACAGCAAAAAGACCCTTTGTCAAATTAAATGTGGAGACTGATACAGCTGAAATAAGATTG
ATGTGCACAGAGACTTATTTGGACACCCCCAGACCAGATGATGGAAGCATAACAGGGCCTTGCGAATCTAATGGGGA
CAAAGGGCGTGGAGGCATCAAGGGAGGATTTGTTCATCAAAGAATGGCATCCAAGATTGGAAGATGGTACTCTCGAA
CGATGTCTAAAACTGAAAGAATGGGGATGGAACTGTATGTCAAGTATGATGGAGACCCATGGACTGACAGTGAAGCC
CTTGCTCCTAGTGGAGTAATGGTTTCAATGGAAGAACCTGGTTGGTATTCTTTTGGCTTCGAAATAAAAGATAAGAA
ATGTGATGTCCCCTGTATTGGGATAGAGATGGTACACGATGGTGGAAAAAAGACTTGGCACTCAGCAGCAACAGCCA
TTTACTGTTTAATGGGCTCAGGACAATTGCTATGGGACACTGTCACAGGTGTTGATATGGCTCTGTAATGGAGGAAT
GGTTGAGTCTGTTCTAAACCCTTTGTTCCTATTTTGTTTGAATAATTGTCCTTACTGAACTTAATTGTTTCTGAAAA
ATGCTCTTGTTACTACT

SEQ ID NO: 37 (HA, B/Panama/45/90)

AGCAGAAGCAGAGCATTTTCTAATATCCACAAAATGAAGGCAATAATTGTACTACTCATGGTAGTAACATCCAACGC
AGATCGAATCTGCACTGGGATAACATCTTCAAACTCACCTCATGTGGTCAAAACAGCTACTCAAGGGGAAGTCAATG
TGACTGGTGTGATACCACTGACAACAACACCAACAAAATCTCATTTTGCAAATCTAAAAGGAACAAAGACCAGAGGG
AAACTATGCCCAAACTGTCTCAACTGCACAGATCTGGATGTGGCCTTGGGCAGACCAATGTGTGTGGGGACCACACC
TTCGGCAAAAGCTTCAATACTCCACGAAGTCAGACCTGTTACATCCGGGTGCTTTCCTATAATGCACGACAGAACAA
AAATCAGACAGCTACCCAATCTTCTCAGAGGATATGAAAATATCAGATTATCAACCCAAAACGTTATCAACGCAGAA
AGAGCACCAGGAGGACCCTACAGACTTGGAACCTCAGGATCTTGCCCTAACGTTACCAGTAGAGACGGATTCTTCGC
AACAATGGCTTGGGCTGTCCCAAGGGACAACAAAACAGCAACGAATCCACTAACAGTAGAAGTACCATACATTTGTA
CAAAAGGAGAAGACCAAATTACTGTTTGGGGGTTCCATTCTGATGACAAAACCCAAATGAAAAACCTCTATGGAGAC
TCAAATCCTCAAAAGTTCACCTCATCTGCCAATGGAGTAACCACACATTATGTTTCTCAGATTGGTGGCTTCCCAAA
TCAAACAGAAGACGGAGGGCTACCACAAAGCGGCAGAATTGTTGTTGATTACATGGTGCAAAAACCTGGGAAAACAG
GAACAATTGTCTATCAAAGAGGTGTTTTGTTGCCTCAAAAGGTGTGGTGCGCAAGTGGCAGGAGCAAGGTAATAAAA
GGGTCCTTGCCTTTAATTGGTGAAGCAGATTGCCTTCACGAAAAATACGGTGGATTAAACAAAAGCAAGCCTTACTA
CACAGGAGAACATGCAAAAGCCATAGGAAATTGCCCAATATGGGTGAAAACACCTTTGAAGCTTGCCAATGGAACCA
AATATAGACCTCCTGCAAAACTATTAAAGGAAAGGGGTTTCTTCGGAGCTATTGCTGGTTTCTTAGAAGGAGGATGG
GAAGGAATGATTGCAGGTTGGCACGGATACACATCTCATGGAGCACATGGAGTGGCAGTGGCAGCAGACCTTAAGAG
TACGCAAGAAGCCATAAACAAGATAACAAAAAATCTCAATTCTTTGAGTGAGCTAGAAGTAAAGAATCTTCAAAGAC
TAAGTGGTGCCATGGATGAACTCCACAACGAAATACTCGAGCTGGATGAGAAAGTGGATGATCTCAGAGCTGACACA
ATAAGCTCGCAAATAGAGCTTGCAGTCTTGCTTTCCAACGAAGGAATAATAAACAGTGAAGATGAGCATCTATTGGC
ACTTGAGAGAAAACTAAAGAAAATGCTGGGTCCCTCTGCTGTAGACATAGGGAATGGATGCTTCGAAACCAAACACA
AGTGCAACCAGACCTGCTTAGACAGAATAGCTGCTGGCACCTTTAATGCAGGAGAATTTTCTCTTCCCACTTTTGAT
TCACTGAATATTACTGCTGCATCTTTAAATGATGATGGATTGGATAATCATACTATACTGCTCTACTACTCAACTGC
TGCTTCTAGTTTGGCTGTAACATTGATGATAGCTATTTTTATTGTTTATATGGTCTCCAGAGACAATGTTTCTTGCT
CCATCTGTCTATAAGGAAAATTAAGCCCTGTATTTTCCTTTGTTGTAGTGCTTGTTTGCTTGTTACCATTACAAAGA
AACGTTATTGAAAAATGCTCTTGTTACTACT

SEQ ID NO: 38 (NP, B/Ann Arbor/1/66)

AGCAGAAGCACAGCATTTTCTTGTGAACTTCAAGTACCAACAAAAACTGAAAATCAAAATGTCCAACATGGATATTG
ACGGCATCAACACTGGAACAATTGACAAAACACCAGAAGAAATAACTTCCGGAACCAGTGGGGCAACCAGACCAATC
ATCAAGCCAGCAACCCTTGCCCCACCAAGCAATAAACGAACCCGAAACCCATCCCCAGAAAGGGCAACCACAAGCAG
CGAAGCGATTGTCGGAAGGAGAACCCAAAAGAAACAAACCCCGACAGAGATAAAGAAGAGCGTCTACAATATGGTAG

TGAAACTGGGTGAATTCTACAACCAGATGATGGTCAAAGCTGGACTCAACGATGACATGGAGAGAAACCTAATCCAA
AATGCACATGCTGTGGAAAGAATTCTATTGGCTGCTACTGATGACAAGAAAACTGAATACCAAAAGAAAAAGAATGC
CAGAGATGTCAAAGAAGGGAAAGAAGAAATAGACCACAACAAAACAGGAGGCACCTTTTATAAGATGGTAAGAGATG
ATAAAACCATCTACTTCAGCCCTATAAGAATTACCTTTTTAAAAGAAGAGGTGAAAACAATGTACAAGACCACCATG
GGGAGTGATGGTTTCAGTGGACTAAATCACATCATGATTGGGCATTCACAGATGAACGATGTCTGTTTCCAAAGATC
AAAGGCACTAAAAAGAGTTGGACTTGACCCTTCATTAATCAGTACTTTTGCAGGAAGCACACTCCCCAGAAGATCAG
GTGCAACTGGTGTTGCGATCAAAGGAGGTGGAACTTTAGTGGCAGAAGCCATTCGATTTATAGGAAGAGCAATGGCA
GACAGAGGGCTATTGAGAGACATCAGAGCCAAGACGGCCTATGAAAAGATTCTTCTGAATCTGAAAAACAAGTGCTC
TGCGCCCCAACAAAAGGCTCTAGTTGATCAAGTGATCGGAAGTAGAAACCCAGGGATTGCAGACATAGAAGACCTAA
CCCTGCTTGCCCGAAGCATGGTCGTTGTCAGGCCCTCTGTAGCGAGCAAAGTGGTGCTTCCCATAAGCATTAATGCT
AAAATACCTCAACTAGGGTTCAATGTTGAAGAATACTCTATGGTTGGGTATGAAGCCATGGCTCTTTATAATATGGC
AACACCTGTTTCCATATTAAGAATGGGAGACGATGCAAAAGATAAATCACAATTATTCTTCATGTCTTGCTTTGGAG
CTGCCTATGAAGACCAAAGAGTTTTGTCTGCACTAACCGGCACAGAATTCAAGCCTAGGTCAGCATTAAAGTGCAAG
GGTTTCCACGTTCCAGCAAAGGAGCAAGTGGAAGGAATGGGGGCAGCTCTGATGTCCATCAAGCTCCAGTTTTGGGC
CCCAATGACCAGATCTGGGGGGAACGAAGTAGGTGGAGACGGAGGGTCTGGTCAAATAAGTTGCAGCCCCGTGTTTG
CAGTAGAGAGACCTATTGCTCTAAGCAAGCAAGCTGTAAGAAGAATGCTGTCAATGAATATTGAGGGACGTGATGCA
GATGTCAAAGGAAATCTACTCAAGATGATGAATGATTCAATGGCTAAGAAAACCAATGGAAATGCTTTCATTGGGAA
GAAAATGTTTCAAATATCAGACAAAAACAAAATCAATCCCGTTGATATTCCAATTAAGCAGACCATCCCCAATTTCT
TCTTTGGGAGGGACACAGCAGAGGATTATGATGACCTCGATTATTAAAGCAACAAAATAGACACTATGGCTGTGACT
GTTTCAGTACGTTTGGAATGTGGGTGTTTACTCTTATTGAAATAAATGTAAAAAATGCTGTTGTTTCTACT

SEQ ID NO: 39 (NP, B/Ann Arbor/1/66 – alternative sequence)

AGCAGAAGCACAGCATTTTCTTGTGAACTTCAAGTACCAACAAAAACTGAAAATCAAAATGTCCAACATGGATATTG
ACGGCATCAACACTGGAACAATTGACAAAACACCAGAAGAAATAACTTCCGGAACCAGTGGGGCAACCAGACCAATC
ATCAAGCCAGCAACCCTTGCCCCACCAAGCAATAAACGAACCCGAAACCCATCCCCAGAAAGGGCAACCACAAGCAG
CGAAGCGATTGTCGGAAGGAGAACCCAAAAGAAACAAACCCCGACAGAGATAAAGAAGAGCGTCTACAATATGGTAG
TGAAACTGGGTGAATTCTACAACCAGATGATGGTCAAAGCTGGACTCAACGATGACATGGAGAGAAACCTAATCCAA
AATGCACATGCTGTGGAAAGAATTCTATTGGCTGCTACTGATGACAAGAAAACTGAATACCAAAAGAAAAAGAATGC
CAGAGATGTCAAAGAAGGGAAAGAAGAAATAGACCACAACAAAACAGGAGGCACCTTTTATAAGATGGTAAGAGATG
ATAAAACCATCTACTTCAGCCCTATAAGAATTACCTTTTTAAAAGAAGAGGTGAAAACAATGTACAAGACCACCATG
GGGAGTGATGGTTTCAGTGGACTAAATCACATCATGATTGGGCATTCACAGATGAACGATGTCTGTTTCCAAAGATC
AAAGGCACTAAAAAGAGTTGGACTTGACCCTTCATTAATCAGTACTTTTGCAGGAAGCACACTCCCCAGAAGATCAG
GTGCAACTGGTGTTGCGATCAAAGGAGGTGGAACTTTAGTGGCAGAAGCCATTCGATTTATAGGAAGAGCAATGGCA
GACAGAGGGCTATTGAGAGACATCAGAGCCAAGACGGCCTATGAAAAGATTCTTCTGAATCTGAAAAACAAGTGCTC
TGCGCCCCAACAAAAGGCTCTAGTTGATCAAGTGATCGGAAGTAGAAACCCAGGGATTGCAGACATAGAAGACCTAA
CCCTGCTTGCCCGAAGCATGGTCGTTGTCAGGCCCTCTGTAGCGAGCAAAGTGGTGCTTCCCATAAGCATTAATGCT
AAAATACCTCAACTAGGGTTCAATGTTGAAGAATACTCTATGGTTGGGTATGAAGCCATGGCTCTTTATAATATGGC
AACACCTGTTTCCATATTAAGAATGGGAGACGATGCAAAAGATAAATCACAATTATTCTTCATGTCTTGCTTTGGAG
CTGCCTATGAAGACCAAAGAGTTTTGTCTGCACTAACCGGCACAGAATTCAAGCCTAGGTCAGCATTAAAGTGCAAG
GGTTTCCACGTTCCAGCAAAGGAGCAAGTGGAAGGAATGGGGGCAGCTCTGATGTCCATCAAGCTCCAGTTTTGGGC
CCCAATGACCAGATCTGGGGGGAACGAAGTAGGTGGAGACGGAGGGTCTGGTCAAATAAGTTGCAGCCCCGTGTTTG
CAGTAGAGAGACCTATTGCTCTAAGCAAGCAAGCTGTAAGAAGAATGCTGTCAATGAATATTGAGGGACGTGATGCA
GATGTCAAAGGAAATCTACTCAAGATGATGAATGATTCAATGGCTAAGAAAACCAATGGAAATGCTTTCATTGGGAA
GAAAATGTTTCAAATATCAGACAAAAACAAAATCAATCCCGTTGATATTCCAATTAAGCAGACCATCCCCAATTTCT
TCTTTGGGAGGGACACAGCAGAGGATTATGATGACCTCGATTATTAAAGCAACAAAATAGACACTATGGCTGTGACT
GTTTCAGTACGTTTGGAATGTGGGTGTTTACTCTTATTGAAATAAATGTAAAAAATGCTGTTGTTTCTACT

SEQ ID NO: 40 (PB2, A/New Caledonia/20/1999)

ATGGAAAGAATAAAAGAGCTAAGGAATCTGATGTCACAATCTCGCACTCGCGAGATACTTACAAAAACTACTGTAGA
CCACATGGCCATAATCAAGAAATACACATCAGGAAGACAGGAGAAAAACCCATCACTTAGAATGAAATGGATGATGG
CAATGAAATACCCAATTACAGCAGATAAAAGGATAACGGAAATGATTCCTGAAAGAAATGAGCAAGGACAGACATTA
TGGAGTAAAGTGAATGATGCCGGATCAGACCGAGTGATGATATCACCCCTGGCTGTGACATGGTGGAACAGAAATGG
ACCAGTGGCAAGTACTATTCACTATCCAAAAATCTACAAAACTTACTTTGAAAAGGTTGAAAGGTTAAAACATGGAA
CCTTTGGCCCTGTACACTTTAGAAACCAAGTCAAAATACGCCGAAGAGTCGACATAAATCCTGGTCATGCAGACCTC
AGCGCCAAGGAGGCACAGGATGTAATTATGGAAGTTGTTTTCCCTAATGAAGTGGGAGCCAGAATACTAACATCAGA
ATCGCAATTAACGATAACCAAGGAGAAAAAAGAAGAACTCCAGAATTGCAAAATTTCCCCTTTGATGGTTGCATACA
TGTTAGAGAGGGAACTTGTCCGCAAAACGAGATTTCTCCCGGTTGCTGGTGGAACAAGCAGTGTGTACATTGAAGTT
TTGCATTTAACACAGGGGACATGCTGGGAGCAGATGTACACTCCAGGTGGGGAGGTGAGGAATGATGATGTTGATCA
AAGCCTAATTATTGCTGCTAGGAACATAGTGAGAAGAGCTGCAGTATCAGCAGATCCACTAGCATCTTTATTAGAAA
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AATGGCCCTGAGTCAGTGTTGATCAATACCTATCAGTGGATCATCAGAAACTGGGAGACTGTTAAAATTCAGTGGTC
TCAGAACCCTACAATGCTATACAATAAAATGGAATTCGAGCCATTTCAGTCTCTAGTCCCTAAGGCCATTAGAGGCC
AATACAGTGGGTTTGTTAGAACTCTATTTCAACAAATGAGGGATGTGCTTGGGACCTTTGACACAACTCAGATAATA
AAACTTCTTCCCTTTGCAGCCGCTCCACCAAAGCAAAGTAGAATGCAATTCTCATCATTGACTGTGAATGTGAGGGG
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SEQ ID NO: 41 (encodes the same amino acid sequence as SEQ ID NO: 40)

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SEQ ID NO: 42 (HA, BX-35)

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SEQ ID NO: 43 (NP, B/Lee/40)

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SEQ ID NO: 44 (NP, B/Ann Arbor/1/66)

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SEQ ID NO: 45 (NP, B/Ann Arbor/1/66)

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QTIPNFFFGRDTAEDYDDLDY

CLAIMS

1. A method of preparing a reassortant influenza B virus comprising steps of:

   (i) introducing into a culture host one or more expression construct(s) which encode(s) the viral segments required to produce an influenza B virus wherein the expression construct(s) encode the HA segment from a first influenza B virus and the NP and/or PB2 segment from a second influenza B virus which is a B/Victoria/2/87-like strain; and

   (ii) culturing the culture host in order to produce a reassortant influenza B virus.

2. A method of preparing a reassortant influenza B virus comprising steps of:

   (i) introducing into a culture host one or more expression construct(s) which encode(s) the viral segments required to produce an influenza B virus wherein the expression construct(s) encode the HA segment from a first influenza B virus and the NP segment from a second influenza B virus which is not B/Lee/40 or B/Ann Arbor/1/66 or B/Panama/45/90; and

   (ii) culturing the culture host in order to produce a reassortant influenza B virus.

3. The method of claim 2, wherein the NP and PB2 segments are from the second influenza B virus.

4. The method of any one of claims 2 or 3, wherein the second influenza B virus is a B/Victoria/2/87-like strain.

5. The method of any one of claims 1 to 4 wherein the PA, PB1, PB2, NP, NS and M segments are from the second influenza B virus.

6. The method of any one of claims 1 to 5, wherein the reassortant influenza B virus comprises backbone segments from two or more influenza B strains.

7. The method of claim 6, wherein at least one backbone segment is from a B/Yamagata/16/88-like strain.

8. A method of preparing a reassortant influenza B virus comprising steps of

   (i) introducing into a culture host one or more expression construct(s) which encode(s) the viral segments required to produce an influenza B virus comprising the HA segment from a B/Yamagata/16/88-like strain and at least one backbone segment from a B/Victoria/2/87-like strain; and

   (ii) culturing the culture host in order to produce a reassortant influenza B virus.

9. The method of claim 8, wherein two, three, four, five or six backbone segments are from the B/Victoria/2/87-like strain.

10. The method of claim 8, wherein the ratio of segments from the B/Victoria/2/87-like strain and the B/Yamagata/16/88-like strain is 7:1, 6:2, 4:4, 3:5 or 1:7.

11. A method of preparing a reassortant influenza B virus comprising steps of

(i) introducing into a culture host one or more expression construct(s) which encode(s) the viral segments required to produce an influenza B virus comprising viral segments from a B/Victoria/2/87-like strain and a B/Yamagata/16/88-like strain, wherein the ratio of segments from the B/Victoria/2/87-like strain and the B/Yamagata/16/88-like strain is 1:7, 2:6, 3:5, 4:4, 5:3, 6:2 or 7:1; and

(ii) culturing the culture host in order to produce a reassortant influenza B virus.

12. The method of any one of claims 10 or 11, wherein the ratio is 7:1, 6:2, 4:4, 3:5 or 1:7.

13. The method of any one of claims 1 or 4-12, wherein the B/Victoria/2/87-like strain is B/Brisbane/60/08.

14. The method of any one of claims 7-13, wherein the B/Yamagata/16/88-like strain is B/Panama/45/90.

15. The method of any preceding claim, wherein the NP segment encodes a protein which has at least 97%, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 4.

16. The method of any preceding claim, wherein the PB2 segment encodes a protein which has at least 97%, at least 98%, at least 99% or 100% identity to the sequence of SEQ ID NO: 3.

17. The method of any preceding claim, wherein the NS segment encodes a protein which has at least 97%, at least 98%, at least 99% or 100% identity with the sequence of SEQ ID NO: 35 and/or wherein the M1 segment encodes a protein which has at least 97%, at least 98%, at least 99% or 100% identity with the sequence of SEQ ID NO: 34.

18. The method of any preceding claim wherein the reassortant influenza B virus comprises:

a) a PA protein which has at least 97% identity, at least 98% identity, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 1; and/or

b) a PB1 protein which has at least 97% identity, at least 98% identity , at least 99% identity or 100% identity to the sequence of SEQ ID NO: 2; and/or

c) a M1 protein which has at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 5; and/or

d) a M2 protein which has at least 97% identity, at least 98% identity , at least 99% identity or 100% identity to the sequence of SEQ ID NO: 6; and/or

e) a NS1 protein which has at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 7; and/or

f) a NS2 protein which has at least 97% identity, at least 98%, at least 99% identity or 100% identity to the sequence of SEQ ID NO: 8.

19. A method of preparing a reassortant influenza B virus comprising steps of

(i) introducing into a culture host one or more expression construct(s) which encode(s) the viral segments required to produce an influenza B virus comprising:

a) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 16 and the M segment of SEQ ID NO: 15; or

b) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34; or

c) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 32, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 16 and the M segment of SEQ ID NO: 15; or

d) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 16 and the M segment of SEQ ID NO: 15, or

e) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34;

f) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 33, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

g) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 32, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

h) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 33, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

i) the PA segment of SEQ ID NO: 11, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 32, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

j) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 12, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34, or

k) the PA segment of SEQ ID NO: 30, the PB1 segment of SEQ ID NO: 31, the PB2 segment of SEQ ID NO: 13, the NP segment of SEQ ID NO: 14, the NS segment of SEQ ID NO: 35 and the M segment of SEQ ID NO: 34; and

(ii) culturing the culture host in order to produce a reassortant influenza B virus.

20. The method of any one of claims 1 to 19, further comprising the step (iii) of purifying the reassortant virus obtained in step (ii).

21. A reassortant influenza B virus obtainable by the method of any one of claims 1 to 20.

22. The method of any one of claims 1 to 20, wherein the culture host is an embryonated hen egg.

23. The method of any one of claims 1 to 20, wherein the culture host is a cell.

24. The method of claim 23, wherein the cell is an MDCK, Vero or PerC6 cell.

25. The method of any one of claims 23 or 24, wherein the cell grows adherently.

26. The method of any one of claims 23 or 24, wherein the cell grows in suspension.

27. The method of claim 26, wherein the MDCK cell is cell line MDCK 33016 (DSM ACC2219).

28. A method of preparing a vaccine, comprising the steps of (a) preparing a virus by the method of any one of claims 1 to 27 and (b) preparing a vaccine from the virus.

29. A method of preparing a vaccine, comprising the step of preparing a vaccine from the virus of claim 21.

30. The method of any one of claims 28 or 29, wherein step (b) involves inactivating the virus.

31. The method of any one of claims 28 to 30, wherein the vaccine is a whole virion vaccine.

32. The method of one of claims 28 to 30, wherein the vaccine is a split virion vaccine.

33. The method of any one of claims 28 to 30, wherein the vaccine is a surface antigen vaccine.

34. The method of any one of claims 28 to 30, wherein the vaccine is a virosomal vaccine.

35. The method of any one of claims 28 to 34, wherein the vaccine contains less than 10ng of residual host cell DNA per dose.

36. The method of any one of claims 28 to 35 wherein the vaccine comprises an antigen from an influenza A strain.

37. The method of claim 36, wherein the influenza A strain is of the H1, H2, H5, H7 or H9 subtype.

38. A vaccine obtainable by the method of any one of claims 28 to 37.

39. An expression system comprising one or more expression construct(s) comprising the vRNA encoding segments of an influenza B virus of claim 21.

40. A host cell comprising the expression system of claim 39.

41. The host cell of claim 40, wherein the host cell is a mammalian cell.

42. The host cell of claim 41, wherein the host cell is an MDCK, Vero or PerC6 cell.

FIG. 1

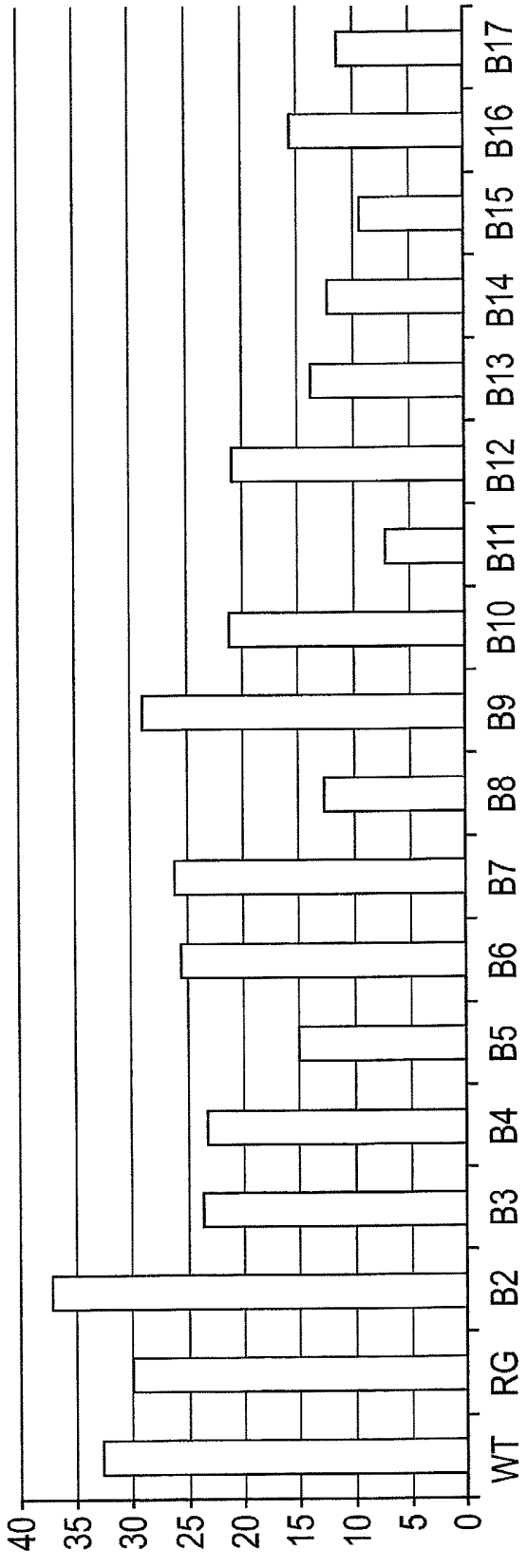
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FIG. 2

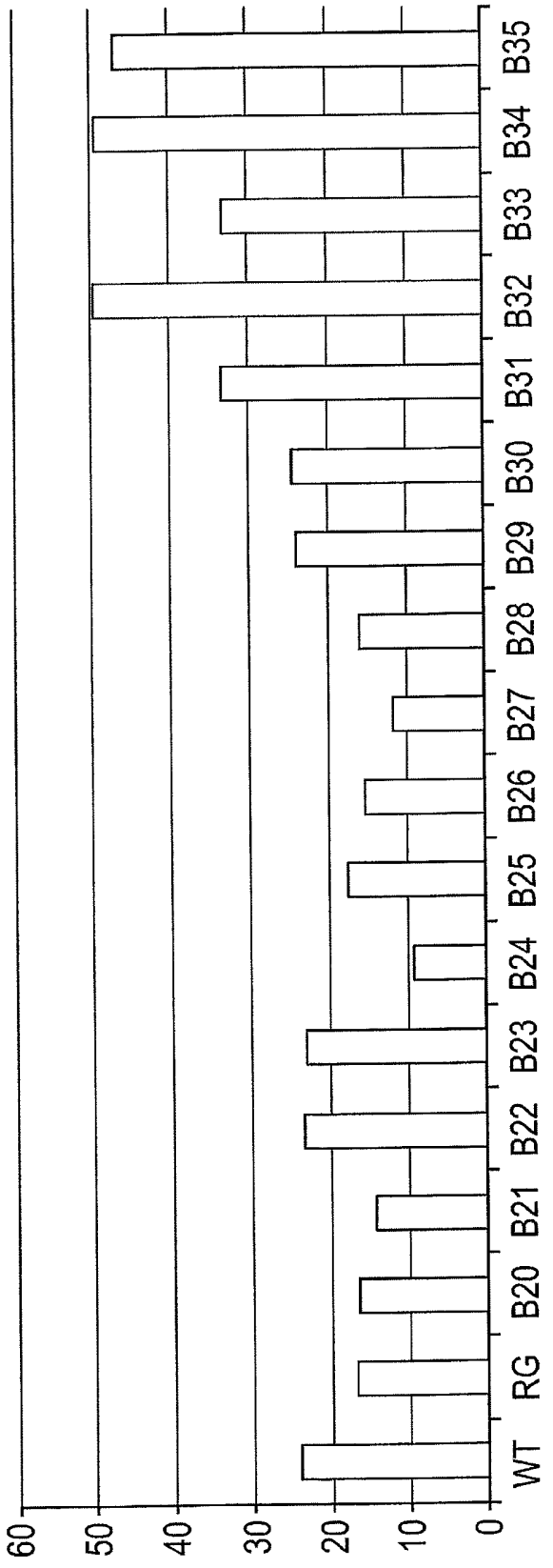
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FIG. 3A

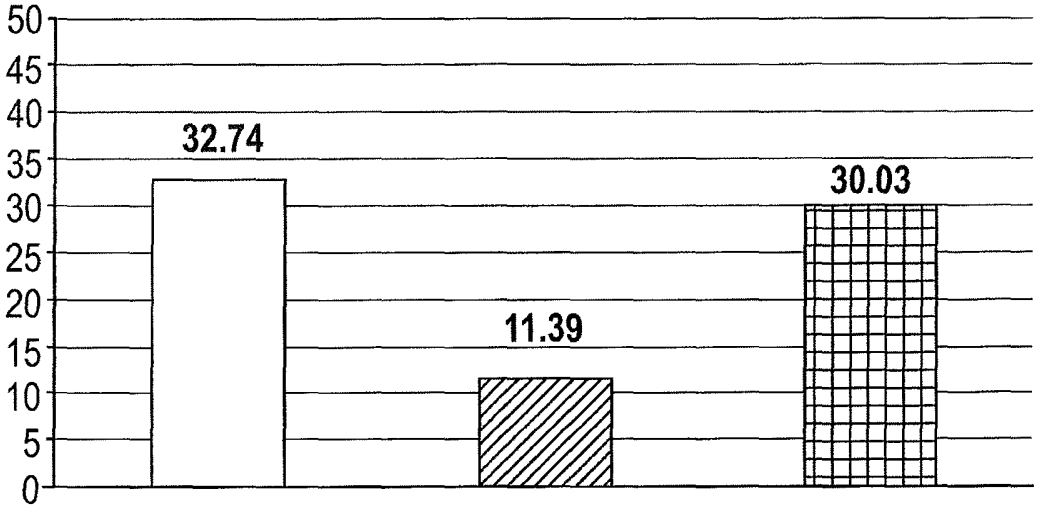
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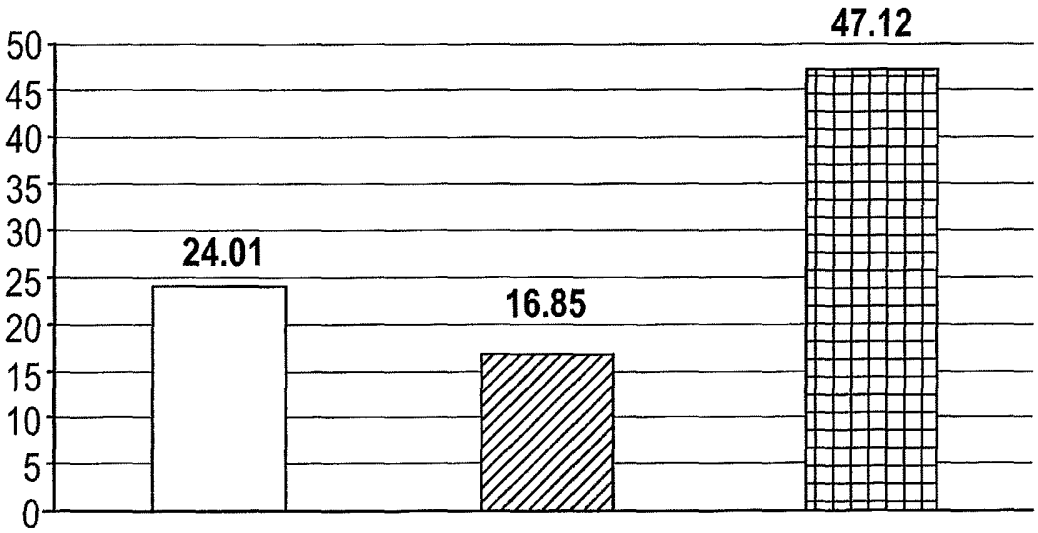
FIG. 3B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47.12

FIG. 3C

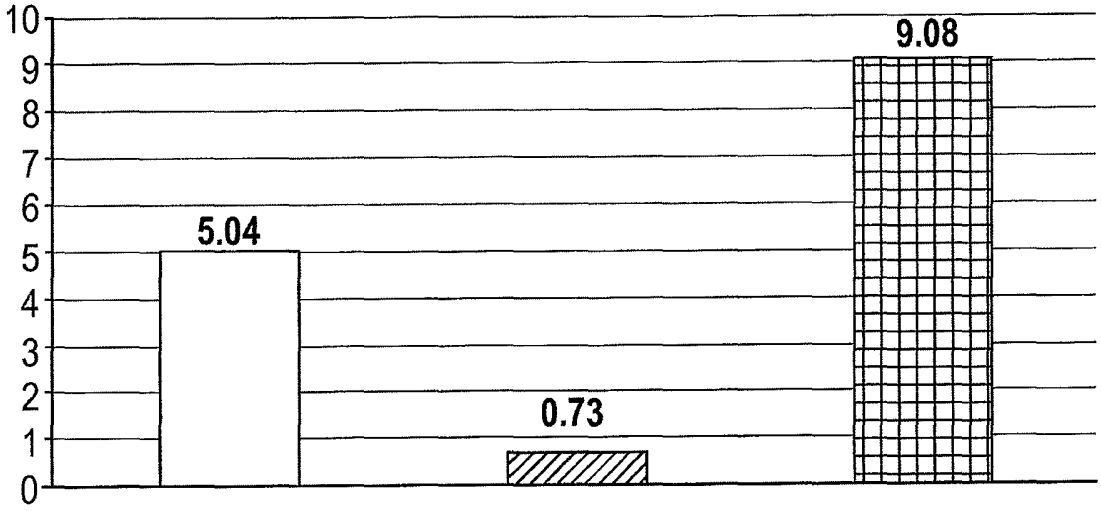


FIG. 3D

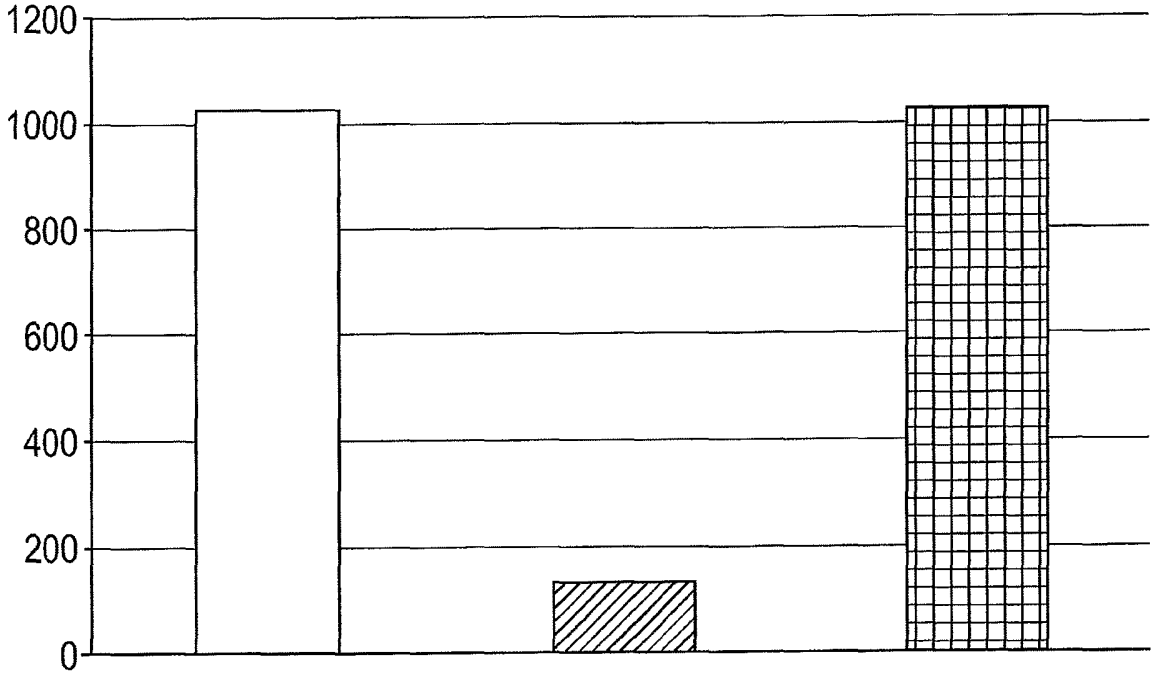
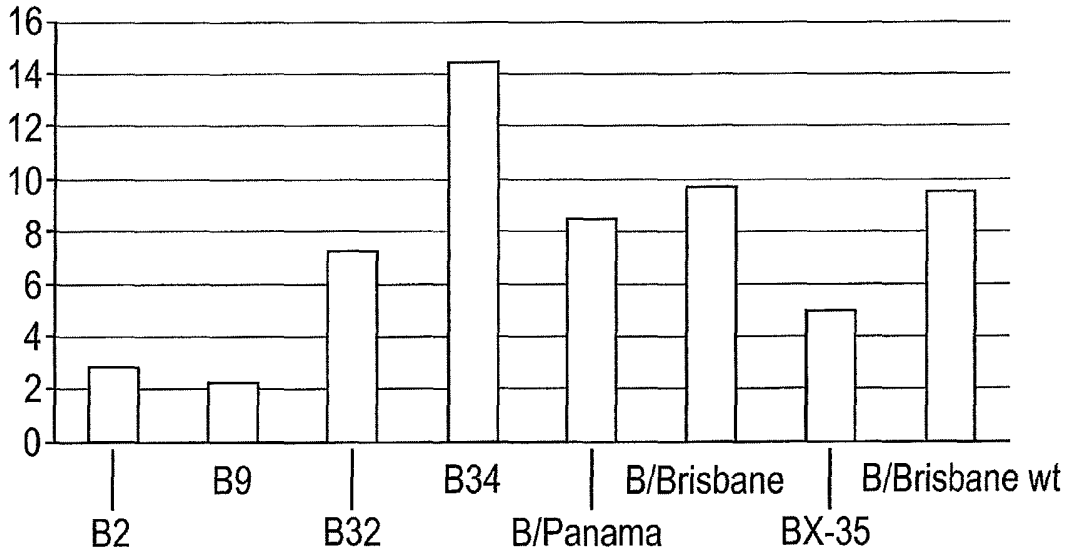


FIG. 4A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10
8
6
4
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B34
B/Panama
B/Brisbane
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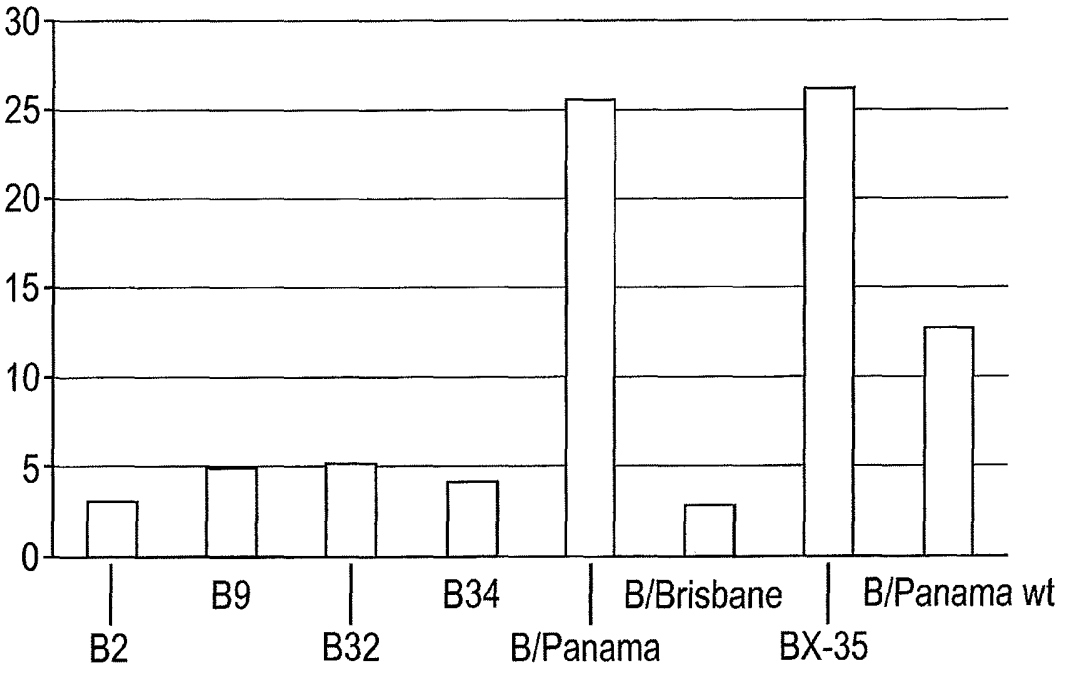
B/Brisbane wt

FIG. 4B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10
5
0
B2
B9
B32
B34
B/Panama
B/Brisbane
BX-35
B/Panama wt

FIG. 4C

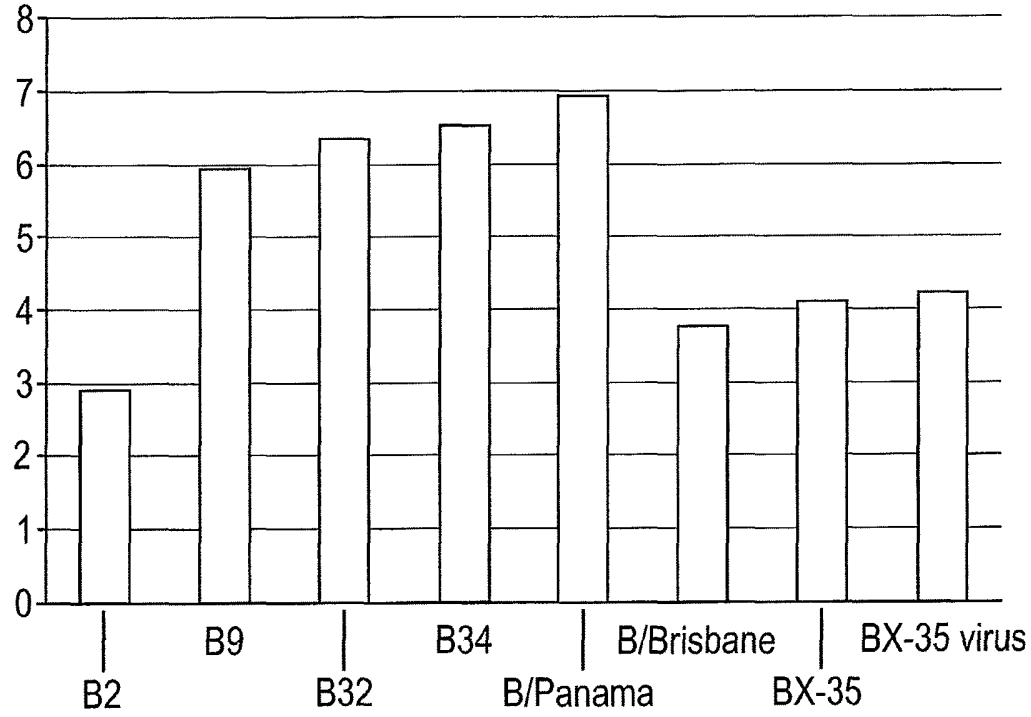

8
7
6
5
4
3
2
1
0
B2
B9
B32
B34
B/Panama
B/Brisbane
BX-35
BX-35 virus

FIG. 4D

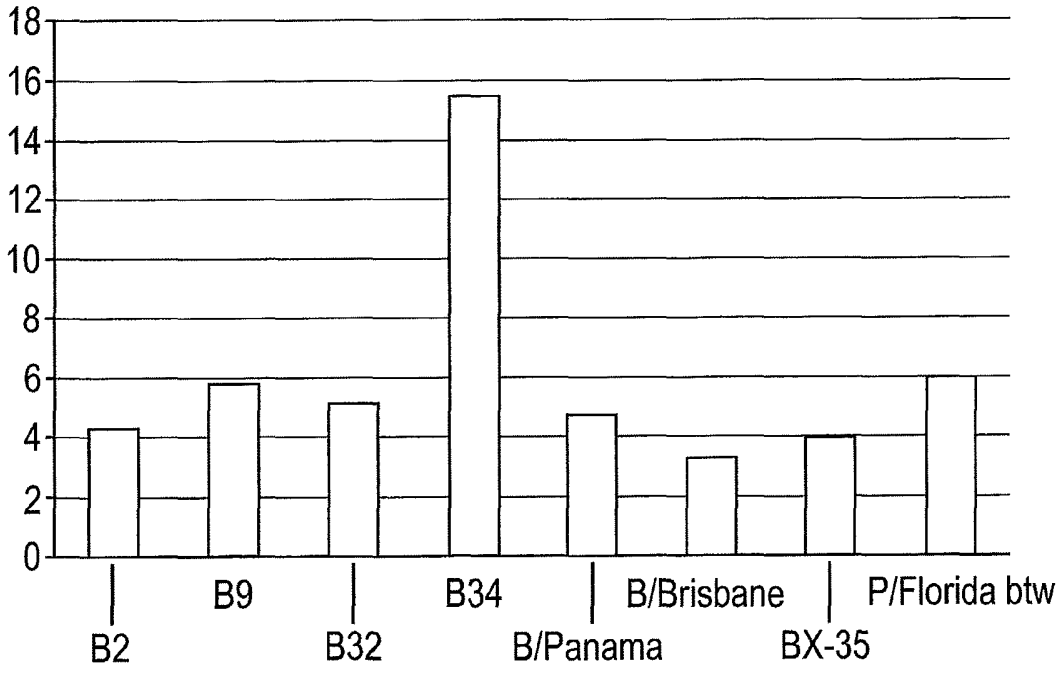

18
16
14
12
10
8
6
4
2
0
B2
B9
B32
B34
B/Panama
B/Brisbane
BX-35
P/Florida btw

FIG. 5A

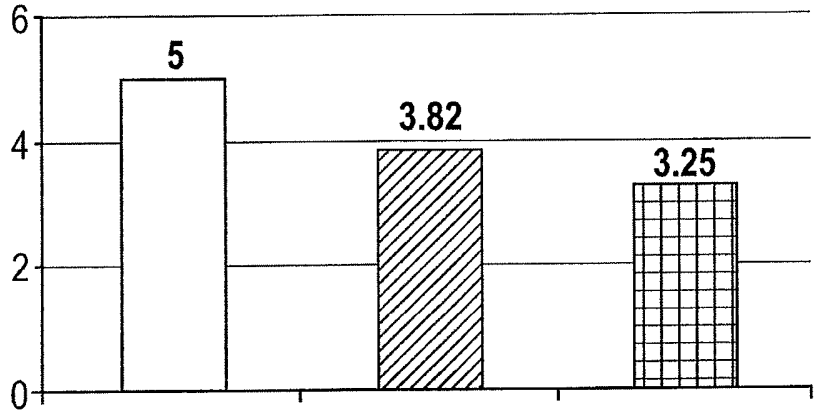


FIG. 5B

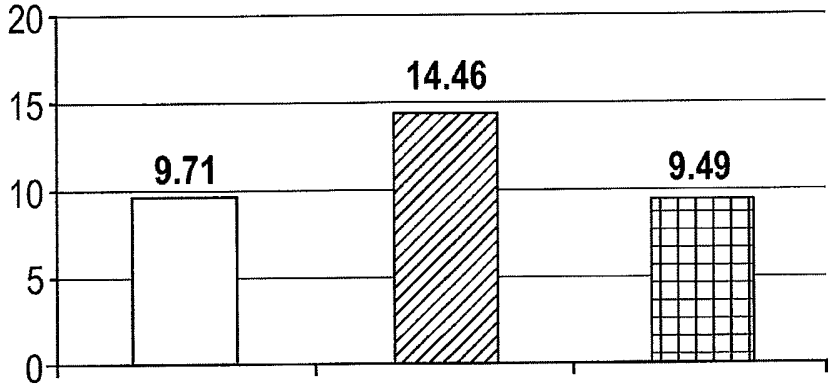


FIG. 5C

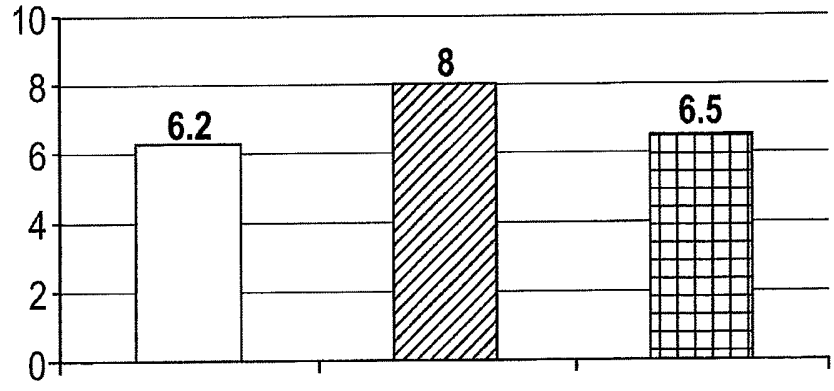


FIG. 5D

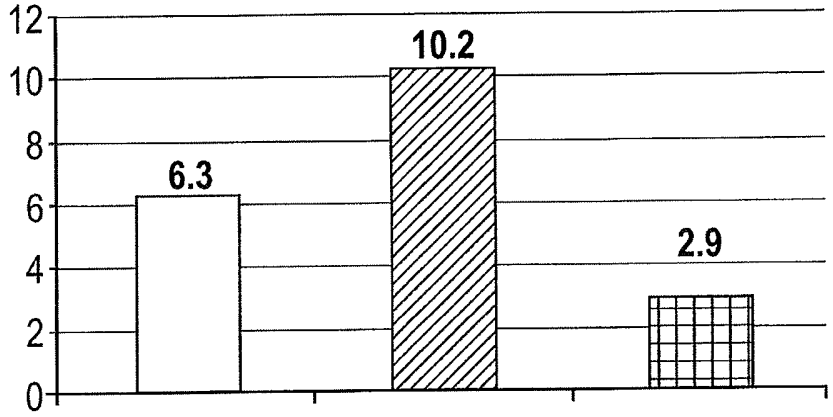


FIG. 5E

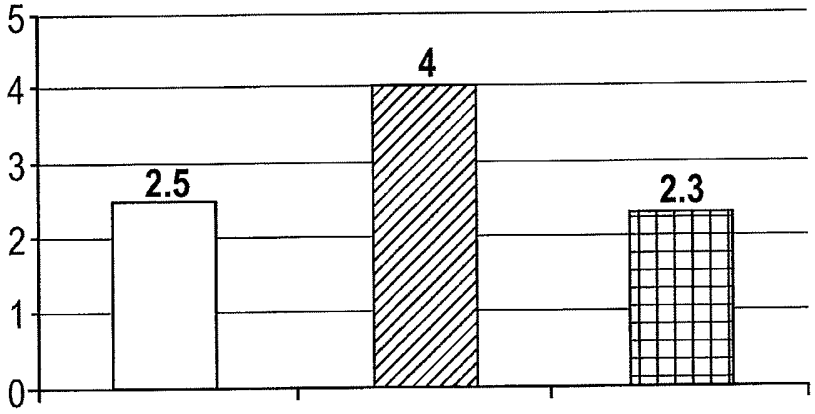


FIG. 5F

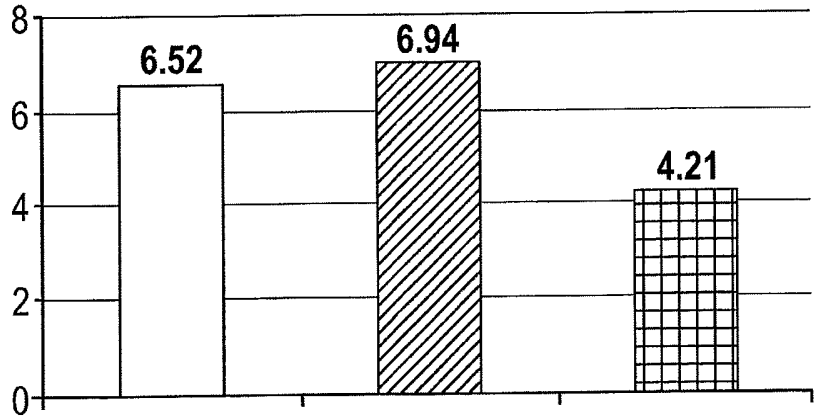


FIG. 5G

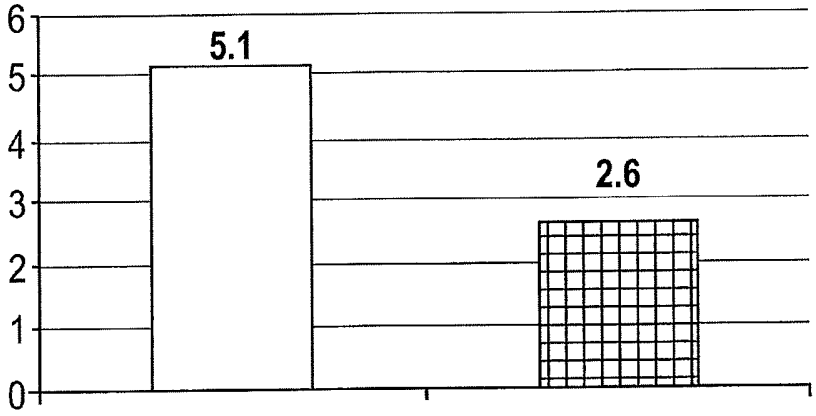


FIG. 5H

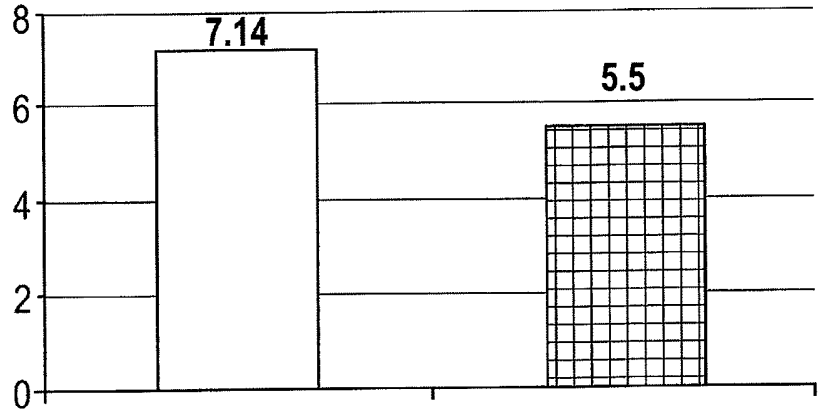


FIG. 6A

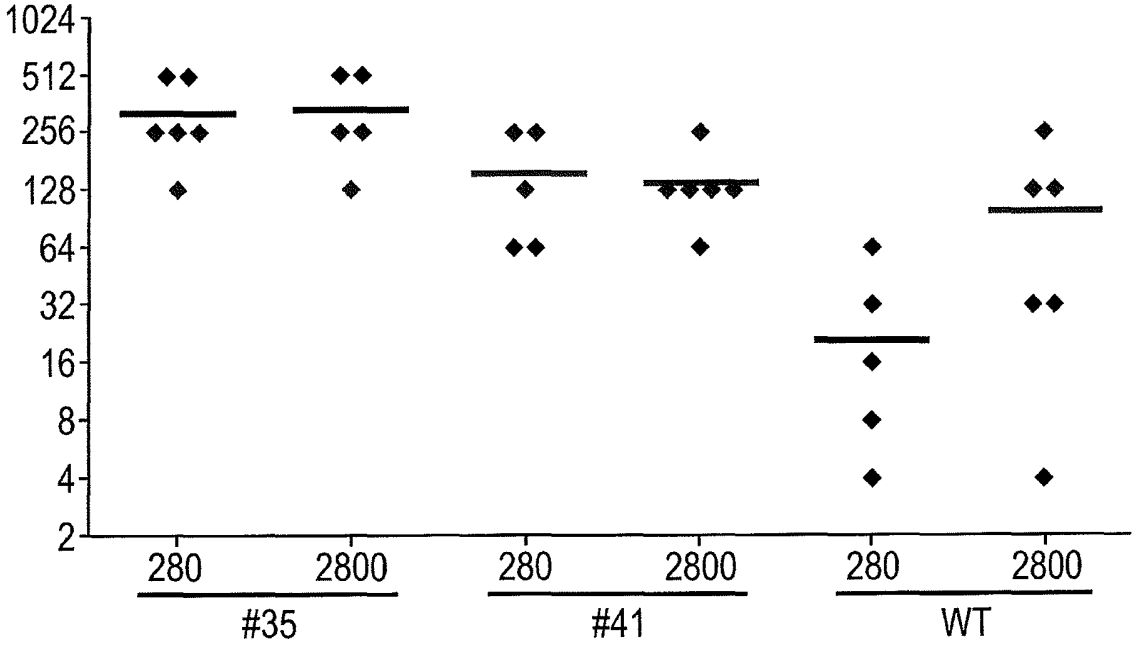
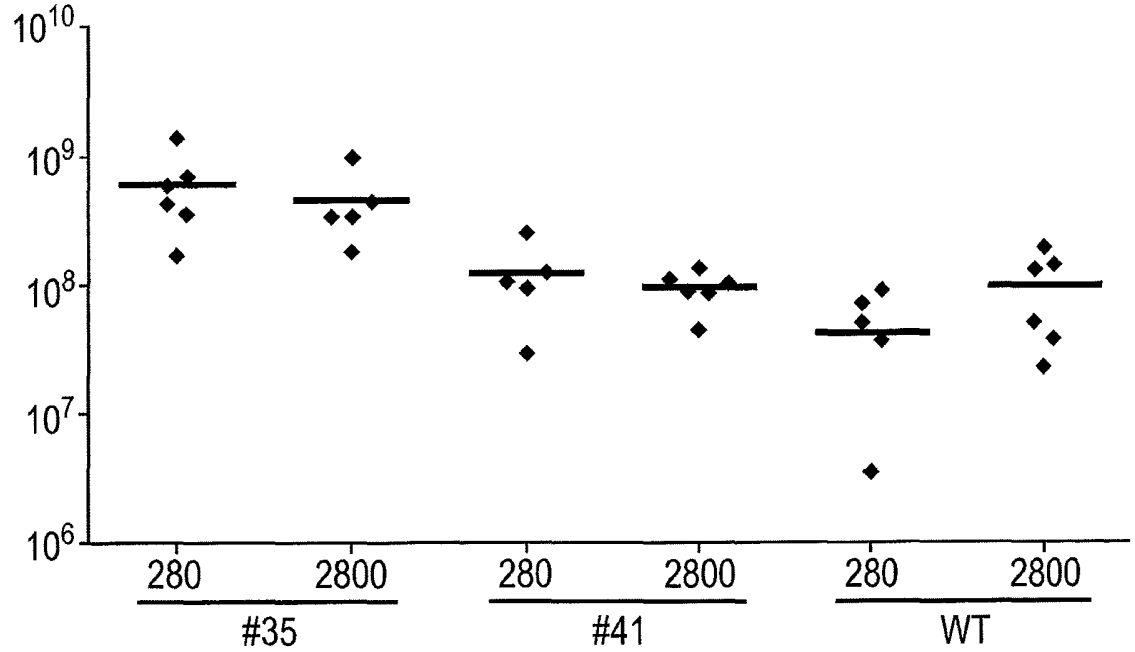

1024
512
256
128
64
32
16
8
4
2
280
2800
280
2800
280
2800
#35
#41
WT

FIG. 6B


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
280
2800
280
2800
280
2800
#35
#41
WT

摘要

提供了用於生產重配乙型流感病毒的新的流感供體毒株。