

## REPLICATION-DEFECTIVE FLAVIVIRUS VACCINES AND VACCINE VECTORS

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### Field of the Invention

This invention relates to replication-defective flavivirus vaccines and vaccine vectors, and corresponding compositions and methods.

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### Background of the Invention

Flaviviruses are distributed worldwide and represent a global public health problem. Flaviviruses also have a significant impact as veterinary pathogens. Flavivirus pathogens include yellow fever (YF), dengue types 1-4 (DEN1-4), Japanese encephalitis (JE), West Nile (WN), tick-borne encephalitis (TBE), and other viruses from the TBE serocomplex, such as Kyasanur Forest disease (KFD) and Omsk hemorrhagic fever (OHF) viruses. Vaccines against YF [live attenuated vaccine (LAV) strain 17D], JE [inactivated vaccines (INV) and LAV], and TBE (INV) are available. No licensed human vaccines are currently available against DEN and WN. Veterinary vaccines have been in use including, for example, vaccines against WN in horses (INV, recombinant and live chimeric vaccines), JE (INV and LAV) to prevent encephalitis in horses and stillbirth in pigs in Asia, louping ill flavivirus (INV) to prevent neurologic disease in sheep in the UK, and TBE (INV) used in farm animals in Czech Republic (INV) (Monath and Heinz, Flaviviruses, in Fields et al. Eds., Fields Virology, 3rd Edition, Philadelphia, New York, Lippincott-Raven Publishers, 1996, pp. 961-1034).

Tick-borne encephalitis (TBE) is the most important tick-borne viral disease of humans. It is endemic in parts of Europe and Northern Asia, causing more than 10,000 hospitalizations annually, with a case-fatality rate 0.5–1.5% in Europe and 6–40% in Siberia and the Far East. A significant proportion of patients suffer from long-lasting neuropsychiatric sequelae. Inactivated vaccines produced in chick embryo cell cultures have proven effective in preventing the disease. For example, an 86% vaccination coverage of the Austrian population (the highest among European countries) has resulted

in an approximately 90% reduction of hospitalized cases (Heinz and Kunz, Arch. Virol. Suppl. 18:201-205, 2004). The inactivated vaccines are expensive and require three inoculations for primary immunization. Periodic boosters (every 2-5 years) are required to maintain immunity. Therefore, a less costly TBE vaccine, which is effective after one-  
5 two doses and provides durable, such as life-long immunity (similar to that achieved by YF 17D immunization) is needed, and indeed has been identified by the WHO as a major priority. Development of TBE LAV candidates in the past several decades by means of empirical or rational attenuation of TBE virus parent *per se* or chimerization of TBE or Langat (LGT, a naturally attenuated flavivirus that is closely related (serologically) to  
10 TBE) viruses with dengue 4 virus has faced difficulties due to problems with residual virulence of candidates and/or low immunogenicity/overattenuation (Wright et al., Vaccine 26:882-890, 2008; Maximova et al., J. Virol. 82:5255-5268, 2008; Rummyantsev et al., Vaccine 24:133-143, 2006; Kofler et al., Arch. Virol. Suppl. 18:191-200, 2004; and references therein).

15 Flaviviruses are small, enveloped, plus-strand RNA viruses transmitted primarily by arthropod vectors (mosquitoes or ticks) to natural hosts, which are primarily vertebrate animals, such as various mammals, including humans, and birds. The flavivirus genomic RNA molecule is about 11,000 nucleotides (nt) in length and encompasses a long open reading frame (ORF) flanked by 5' and 3' untranslated terminal regions (UTRs) of about  
20 120 and 500 nucleotides in length, respectively. The ORF encodes a polyprotein precursor that is cleaved co- and post-translationally to generate individual viral proteins. The proteins are encoded in the order: C-prM/M-E-NS1-NS2A/2B-NS3-NS4A/4B-NS5, where C (core/capsid), prM/M (pre-membrane/membrane), and E (envelope) are the structural proteins, i.e., the components of viral particles, and the NS proteins are non-  
25 structural proteins, which are involved in intracellular virus replication. Flavivirus replication occurs in the cytoplasm. Upon infection of cells and translation of genomic RNA, processing of the polyprotein starts with translocation of the prM portion of the polyprotein into the lumen of endoplasmic reticulum (ER) of infected cells, followed by translocation of E and NS1 portions, as directed by the hydrophobic signals for the prM,  
30 E, and NS1 proteins. Amino-termini of prM, E, and NS1 proteins are generated by

cleavage with cellular signalase, which is located on the luminal side of the ER membrane, and the resulting individual proteins remain carboxy-terminally anchored in the membrane. Most of the remaining cleavages, in the nonstructural region, are carried out by the viral NS2B/NS3 serine protease. The viral protease is also responsible for  
5 generating the C-terminus of the mature C protein found in progeny virions. Newly synthesized genomic RNA molecules and the C protein form a dense spherical nucleocapsid, which becomes surrounded by cellular membrane in which the E and prM proteins are embedded. The mature M protein is produced by cleavage of prM shortly prior to virus release by cellular furin or a similar protease. E, the major protein of the  
10 envelope, is the principal target for neutralizing antibodies, the main correlate of immunity against flavivirus infection. Virus-specific cytotoxic T-lymphocyte (CTL) response is the other key attribute of immunity. Multiple CD8+ and CD4+ CTL epitopes have been characterized in various flavivirus structural and non-structural proteins. In addition, innate immune responses contribute to both virus clearance and regulating the  
15 development of adaptive immune responses and immunologic memory.

In addition to the inactivated (INV) and live-attenuated (LAV) vaccines against flaviviruses discussed above, other vaccine platforms have been developed. One example is based on chimeric flaviviruses that include yellow fever virus capsid and non-structural sequences and prM-E proteins from other flaviviruses, to which immunity is sought. This  
20 technology has been used to develop vaccine candidates against dengue (DEN), Japanese encephalitis (JE), West Nile (WN), and St. Louis encephalitis (SLE) viruses (see, e.g., U.S. Patent Nos. 6,962,708 and 6,696,281). Yellow fever virus-based chimeric flaviviruses have yielded highly promising results in clinical trials.

Another flavivirus vaccine platform is based on the use of pseudoinfectious  
25 virus (PIV) technology (Mason et al., Virology 351:432-443, 2006; Shustov et al., J. Virol. 21:11737-11748, 2007; Widman et al., Adv. Virus. Res. 72:77-126, 2008; Suzuki et al., J. Virol. 82:6942-6951, 2008; Suzuki et al., J. Virol. 83:1870-1880, 2009; Ishikawa et al., Vaccine 26:2772-2781, 2008; Widman et al., Vaccine 26:2762-2771, 2008). PIVs are replication-defective viruses attenuated by a deletion(s). Unlike live flavivirus  
30 vaccines, they undergo a single round replication *in vivo* (or optionally limited rounds, for

two-component constructs; see below), which may provide benefits with respect to safety. PIVs also do not induce viremia and systemic infection. Further, unlike inactivated vaccines, PIVs mimic whole virus infection, which can result in increased efficacy due to the induction of robust B- and T-cell responses, higher durability of immunity, and decreased dose requirements. Similar to whole viruses, PIV vaccines target antigen-presenting cells, such as dendritic cells, stimulate toll-like receptors (TLRs), and induce balanced Th1/Th2 immunity. In addition, PIV constructs have been shown to grow to high titers in substrate cells, with little or no cytopathic effect (CPE), allowing for high-yield manufacture, optionally employing multiple harvests and/or expansion of infected substrate cells.

The principles of the PIV technology are illustrated in Figs. 1 and 2. There are two variations of the technology. In the first variation, a single-component pseudoinfectious virus (s-PIV) is constructed with a large deletion in the capsid protein (C), rendering mutant virus unable to form infectious viral particles in normal cells (Fig. 1). The deletion does not remove the first ~20 codons of the C protein, which contain an RNA cyclization sequence, and a similar number of codons at the end of C, which encode a viral protease cleavage site and the signal peptide for prM. The s-PIV can be propagated, e.g., during manufacture, in substrate (helper) cell cultures in which the C protein is supplied in trans, e.g., in stably transfected cells producing the C protein (or a larger helper cassette including C protein), or in cells containing an alphavirus replicon [e.g., a Venezuelan equine encephalitis virus (VEE) replicon] expressing the C protein or another intracellular expression vector expressing the C protein. Following inoculation in vivo, e.g., after immunization, the PIV undergoes a single round of replication in infected cells in the absence of trans-complementation of the deletion, without spread to surrounding cells. The infected cells produce empty virus-like particles (VLPs), which are the product of the prM-E genes in the PIV, resulting in the induction of neutralizing antibody response. A T-cell response should also be induced via MHC I presentation of viral epitopes. This approach has been applied to YF 17D virus and WN viruses and WN/JE and WN/DEN2 chimeric viruses (Mason et al., *Virology* 351:432-443, 2006;

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Suzuki et al., J. Virol. 83:1870-1880, 2009; Ishikawa et al., Vaccine 26:2772-2781, 2008; Widman et al., Vaccine 26:2762-2771, 2008; WO 2007/098267; WO 2008/137163).

In the second variation, a two-component PIV (d-PIV) is constructed (Fig. 2). Substrate cells are transfected with two defective viral RNAs, one with a deletion in the C gene and another lacking the prM-E envelope protein genes. The two defective genomes complement each other, resulting in accumulation of two types of PIVs in the cell culture medium (Shustov et al., J. Virol. 21:11737-11748, 2007; Suzuki et al., J. Virol. 82:6942-6951, 2008). Optionally, the two PIVs can be manufactured separately in appropriate helper cell lines and then mixed in a two-component formulation. The latter may offer an advantage of adjusting relative concentrations of the two components, increasing immunogenicity and efficacy. This type of PIV vaccine should be able to undergo a limited spread in vivo due to coinfection of some cells at the site of inoculation with both components. The spread is expected to be self-limiting as there are more cells in tissues than viral particles produced by initially coinfecting cells. In addition, a relatively high MOI is necessary for efficient co-infection, and cells outside of the inoculation site are not expected to be efficiently coinfecting (e.g., in draining lymph nodes). Cells infected with the  $\Delta$ C PIV alone produce the highly immunogenic VLPs. Coinfecting cells produce the two types of packaged defective viral particles, which also stimulate neutralizing antibodies. The limited infection is expected to result in a stronger neutralizing antibody response and T-cell response compared to s-PIVs. To decrease chances of recombination during manufacture or in vivo, including with circulating flaviviruses, viral sequences can be modified in both s-PIVs and d-PIVs using, e.g., synonymous codon replacements, to reduce nucleotide sequence homologies, and mutating the complementary cyclization 5' and 3' elements.

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### Summary of the Invention

In one aspect, there is provided a replication-deficient pseudoinfectious flavivirus comprising a yellow fever or West Nile virus genome comprising (i) a deletion in the nucleotide sequence encoding amino acids 26-93 or 31-100 or 31-93 or 26-100 of capsid (C) protein, and one or more deletions within nucleotide sequences encoding a pre-membrane (prM) and/or envelope (E) protein, and (ii) a sequence encoding one or more heterologous pathogen immunogens inserted in the place of or in combination with the one or more deletions of the pre-membrane (prM) and/or envelope (E) protein, wherein said pathogen is selected from the group consisting of a rabies virus, *Borrelia burgdorferi*, an influenza virus, a human immunodeficiency virus, a simian immunodeficiency virus, a human papilloma virus, a respiratory syncytial virus, a malaria parasite, and *Mycobacterium tuberculosis*.

The disclosure provides replication-deficient or defective pseudoinfectious flaviviruses including a flavivirus genome that includes (i) one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1

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(NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5), and (ii) sequences encoding one or more heterologous pathogen, cancer, or allergy-related immunogens. For example, the deletion/mutation can be within capsid (C) sequences; pre-membrane (prM) and/or envelope (E) sequences; capsid (C), pre-membrane (prM), and envelope (E) sequences; or non-structural protein 1 (NS1) sequences.

The heterologous immunogen can be, for example, from a pathogen selected from the group consisting of a rabies virus (e.g., a rabies virus G protein epitope), *Borrelia burgdorferi* (e.g., OspA immunogen or an immunogenic fragment thereof), a tick (e.g., a tick saliva protein selected from the group consisting of 64TRP, Isac, and Salp20, or an immunogenic fragment thereof), an influenza virus (e.g., an influenza virus M2, hemagglutinin (HA), or neuraminidase (NA) epitope, or an immunogenic fragment thereof), a human immunodeficiency virus (e.g., a codon-optimized HIV gag, tat/nef, or gp120 protein, or an immunogenic fragment thereof), a simian immunodeficiency virus, a human papilloma virus (e.g., an HPV16 or HPV18 capsid protein L1 or L2, or an immunogenic fragment thereof), a respiratory syncytial virus (e.g., a respiratory syncytial virus F or G glycoprotein), malaria parasite, and *Mycobacterium tuberculosis* (also see below).

The replication-deficient pseudoinfectious flaviviruses can include sequences encoding a pre-membrane (prM) and/or envelope (E) protein. Further, the replication-deficient pseudoinfectious flavivirus genomes can be selected from those of yellow fever virus, West Nile virus, tick-borne encephalitis virus, Langat virus, Japanese encephalitis virus, dengue virus, and St. Louis encephalitis virus, attenuated strains thereof, and chimeras thereof (also see below). In various examples, the chimeras include pre-membrane (prM) and envelope (E) sequences of a first flavivirus (e.g., a tick-borne encephalitis virus or a Langat virus), and capsid (C) and non-structural sequences of a second, different flavivirus (e.g., a yellow fever, a West Nile, or Langat virus).

The replication-deficient pseudoinfectious flavivirus genomes can be packaged in particles including pre-membrane (prM) and envelope (E) sequences from a flavivirus that is the same or different from that of the genomes. Further, the sequences encoding

the heterologous immunogens can be inserted in the place of, or in combination with, the deletion(s) or mutation(s) of the one or more proteins.

The sequences encoding the heterologous immunogens can be inserted in the flavivirus genomes within sequences encoding the envelope (E) protein, within sequences  
5 encoding the non-structural 1 (NS1) protein, within sequences encoding the pre-membrane (prM) protein, intergenically between sequences encoding the envelope (E) protein and non-structural protein 1 (NS1), intergenically between non-structural protein 2B (NS2B) and non-structural protein 3 (NS3), and/or as a bicistronic insertion in the 3' untranslated region of the flavivirus genome.

10 In another aspect there is provided a pharmaceutical composition comprising the replication-deficient pseudoinfectious flavivirus of the present invention and a pharmaceutically acceptable carrier or diluent.

The disclosure also includes compositions including a first replication-deficient pseudoinfectious flavivirus, as described above, and a second (or further), different  
15 replication-deficient pseudoinfectious flavivirus including a genome that includes one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5). In these compositions, the one or more proteins encoded by the sequences in  
20 which the deletion(s) or mutation(s) occur in the second, different replication-deficient pseudoinfectious flavivirus are different from the one or more proteins encoded by the sequences in which the deletion(s) occur in the first replication-deficient pseudoinfectious flavivirus.

In a further aspect, there is provided the pharmaceutical composition of the  
25 present invention for use in inducing an immune response against the heterologous pathogen immunogen in a subject.

In another aspect, there is provided the pharmaceutical composition of the present invention for use in inducing an immune response against the heterologous pathogen immunogen in a subject wherein the subject is at risk of but does not have an infection by said pathogen.

5           In yet another aspect, there is provided the pharmaceutical composition of the present invention for use in inducing an immune response against the heterologous pathogen immunogen in a subject wherein the subject has an infection by said pathogen.

          The disclosure further includes methods of inducing immune responses to an immunogen in a subject, which involves administering to the subject one or more  
10   replication-deficient pseudoinfectious flavivirus and/or composition as described herein to the subject. In various examples, the subject is at risk of but does not have an infection by the pathogen or a disease or condition associated with the cancer or allergy-related immunogen. In other examples, the subject has an infection by the pathogen or a disease or condition associated with the cancer or allergy-related immunogen. The disclosure  
15   thus includes prophylactic and therapeutic methods. In these methods, the immunogen can be from, for example, a pathogen selected from the group consisting of a rabies virus, *Borrelia burgdorferi*, a tick, an influenza virus, a human immunodeficiency virus, a

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simian immunodeficiency virus, a human papilloma virus, a respiratory syncytial virus, malaria parasite, and *Mycobacterium tuberculosis* (also see below). Further, the methods can be for inducing an immune response against a protein encoded by the flavivirus genome, in addition to the source of the immunogen. In various examples, the subject is  
5 at risk of but does not have an infection by the flavivirus corresponding to the genome of the pseudoinfectious flavivirus, which includes sequences encoding a flavivirus pre-membrane and/or envelope protein. In other examples, the subject has an infection by the flavivirus corresponding to the genome of the pseudoinfectious flavivirus, which includes sequences encoding a flavivirus pre-membrane and/or envelope protein.

10 The disclosure also includes live, attenuated chimeric flaviviruses including a yellow fever virus in which sequences encoding pre-membrane and envelope proteins are replaced with sequences encoding pre-membrane and envelope proteins of a tick-borne encephalitis virus or a Langat virus, and the signal sequence between the capsid and pre-membrane proteins of the chimeric flavivirus includes a hybrid of yellow fever virus and  
15 tick-borne encephalitis or Langat virus capsid/pre-membrane signal sequences, or a variant thereof. In various examples, the capsid/pre-membrane signal sequence of the chimeric flavivirus includes yellow fever virus sequences in the amino terminal region and tick-borne encephalitis or Langat virus sequences in the carboxy terminal region (see below).

20 Further, the disclosure includes live, attenuated chimeric flaviviruses including a West Nile virus in which sequences encoding pre-membrane and envelope proteins are replaced with sequences encoding pre-membrane and envelope proteins of a tick-borne encephalitis or a Langat virus, and the signal sequence between the capsid and pre-membrane proteins of the chimeric flavivirus includes a tick-borne encephalitis or a  
25 Langat virus capsid/pre-membrane signal sequence, or a variant thereof.

The disclosure also includes pharmaceutical compositions including one or more pseudoinfectious flavivirus, composition, or live, attenuated flavivirus as described herein, and a pharmaceutically acceptable carrier or diluent. Further, the compositions can include an adjuvant.

Also included in the disclosure are replication-deficient pseudoinfectious flaviviruses including a flavivirus genome including one or more deletion(s) or mutation(s) in nucleotide sequences encoding non-structural protein 1 (NS1), non-structural protein 3 (NS3), or non-structural protein 5 (NS5).

5           In one aspect, there is provided a nucleic acid molecule corresponding to the genome of a pseudoinfectious flavivirus of the present invention.

Further, the disclosure includes nucleic acid molecules corresponding to the genome of a pseudoinfectious flavivirus, or the genome the live, attenuated flavivirus, as described herein, and complements thereof.

10           In one aspect, there is provided a method of making a replication-deficient pseudoinfectious flavivirus of the present invention, the method comprising introducing a nucleic acid molecule of the present invention into a cell that expresses the protein corresponding to any sequences deleted from the flavivirus genome of the replication-deficient pseudoinfectious flavivirus.

15           The disclosure also provides methods of making replication-deficient pseudoinfectious flaviviruses as described herein, involving introducing one or more nucleic acid molecules, as described above, into a cell that expresses the protein(s) corresponding to any sequences deleted from the flavivirus genome of the replication-deficient pseudoinfectious flaviviruses. In these methods, the protein can be expressed in  
20           the cell from the genome of a second (or further), different, replication-deficient pseudoinfectious flavivirus. In other examples, the protein is expressed from a replicon (e.g., an alphavirus replicon, such as a Venezuelan Equine Encephalitis virus replicon; see below).

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The disclosure also includes compositions containing two or more replication-deficient pseudoinfectious flaviviruses, in which two of the replication-deficient pseudoinfectious flaviviruses are selected from the groups consisting of: (a) a replication-deficient pseudoinfectious flavivirus including a genome containing Japanese encephalitis virus sequences, and a replication-deficient pseudoinfectious flavivirus including a genome containing dengue virus sequences; (b) a replication-deficient pseudoinfectious flavivirus including a genome containing yellow fever virus sequences, and a replication-deficient pseudoinfectious flavivirus including a genome containing dengue virus sequences; and (c) a replication-deficient pseudoinfectious flavivirus including a genome containing tick-borne encephalitis or Langat virus sequences and an inserted sequence encoding a *Borrelia burgdorferi* immunogen, and a replication-deficient pseudoinfectious flavivirus including a genome containing tick-borne encephalitis or Langat virus sequences and an inserted sequence encoding a tick saliva protein immunogen, or a replication-deficient pseudoinfectious flavivirus including a genome containing

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tick-borne encephalitis or Langat virus sequences and inserted sequences encoding a *Borrelia burgdorferi* immunogen and a tick saliva protein immunogen.

Pharmaceutical compositions including the live, attenuated chimeric flaviviruses described herein are also included in the disclosure. Further, the disclosure includes methods of inducing an immune response to tick-borne encephalitis virus or Langat virus in a subject, involving administering to the subject such a pharmaceutical composition. In various examples, the subject does not have but is at risk of developing infection by tick-borne encephalitis virus or Langat virus. In other examples, the subject is infected with tick-borne encephalitis virus or Langat virus.

The disclosure further includes replication-deficient pseudoinfectious flaviviruses including a flavivirus genome including one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5), wherein the flavivirus genome includes yellow fever virus sequences in which sequences encoding pre-membrane and envelope proteins are replaced with sequences encoding pre-membrane and envelope proteins of a tick-borne encephalitis virus or a Langat virus, and sequences encoding the signal sequence between the capsid and pre-membrane proteins of the flavivirus genome include a hybrid of sequences encoding yellow fever virus and tick-borne encephalitis or Langat virus capsid/pre-membrane signal sequences, or a variant thereof. In various examples, the sequences encoding the capsid/pre-membrane signal sequence of the flavivirus genome include yellow fever virus sequences in the 5' region and tick-borne encephalitis or Langat virus sequences in the 3' region.

Further, the disclosure includes replication-deficient pseudoinfectious flaviviruses including a flavivirus genome including one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5), wherein the flavivirus genome includes West Nile virus sequences in which sequences encoding pre-membrane and envelope proteins are replaced with sequences encoding pre-membrane and envelope

proteins of a tick-borne encephalitis or a Langat virus, and the sequences encoding the signal sequence between the capsid and pre-membrane proteins of the flavivirus genome include sequences encoding a tick-borne encephalitis or a Langat virus capsid/pre-membrane signal sequence, or a variant thereof.

5           In addition, the disclosure includes replication-deficient pseudoinfectious flaviviruses including a flavivirus genome including one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5), wherein any capsid (C)  
10   and non-structural (NS) proteins in the flavivirus genome are from Langat virus and any pre-membrane (prM) and envelope (E) proteins are from a tick-borne encephalitis virus.

          By “replication-deficient pseudoinfectious flavivirus” or “PIV” is meant a flavivirus that is replication-deficient due to a deletion or mutation in the flavivirus genome. The deletion or mutation can be, for example, a deletion of a large sequence,  
15   such as most of the capsid protein, as described herein (with the cyclization sequence remaining; see below). In other examples, sequences encoding different proteins (e.g., prM, E, NS1, NS3, and/or NS5; see below) or combinations of proteins (e.g., prM-E or C-prM-E) are deleted. This type of deletion may be advantageous if the PIV is to be used a vector to deliver a heterologous immunogen, as the deletion can permit insertion of  
20   sequences that may be, for example; at least up to the size of the deleted sequence. In other examples, the mutation can be, for example, a point mutation, provided that it results in replication deficiency, as discussed above. Because of the deletion or mutation, the genome does not encode all proteins necessary to produce a full flavivirus particle. The missing sequences can be provided in trans by a complementing cell line that is  
25   engineered to express the missing sequence (e.g., by use of a replicon; s-PIV; see below), or by co-expression of two replication-deficient genomes in the same cell, where the two replication-deficient genomes, when considered together, encode all proteins necessary for production (d-PIV system; see below).

          Upon introduction into cells that do not express complementing proteins, the  
30   genomes replicate and, in some instances, generate “virus-like particles,” which are

released from the cells and are able to leave the cells and be immunogenic, but cannot infect other cells and lead to the generation of further particles. For example, in the case of a PIV including a deletion in capsid protein encoding sequences, after infection of cells that do not express capsid, VLPs including prM-E proteins are released from the cells.

5 Because of the lack of capsid protein, the VLPs lack capsid and a nucleic acid genome. In the case of the d-PIV approach, production of further PIVs is possible in cells that are infected with two PIVs that complement each other with respect to the production of all required proteins (see below).

The invention provides several advantages. For example, the PIV vectors and  
10 PIVs of the invention are highly attenuated and highly efficacious after one-to-two doses, providing durable immunity. Further, unlike inactivated vaccines, PIVs mimic whole virus infection, which can result in increased efficacy due to the induction of robust B- and T-cell responses, higher durability of immunity, and decreased dose requirements. In addition, similar to whole viruses, PIV vaccines target antigen-presenting cells, such as  
15 dendritic cells, stimulate toll-like receptors (TLRs), and induce balanced Th1/Th2 immunity. PIV constructs have also been shown to grow to high titers in substrate cells, with little or no CPE, allowing for high-yield manufacture, optionally employing multiple harvests and/or expansion of infected substrate cells. Further, the PIV vectors of the invention provide an option for developing vaccines against non-flavivirus pathogens for  
20 which no vaccines are currently available.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

#### Brief Description of the Drawings

25 Fig. 1 is a schematic illustration of single component PIV (s-PIV) technology.

Fig. 2 is a schematic illustration of two-component PIV (d-PIV) technology.

Fig. 3 is a schematic illustration of a general experimental design for testing immunogenicity and efficacy of PIVs in mice.

Fig. 4 is a graph comparing the humoral immune response induced by PIV-WN  
30 (RV-WN) with that of YF/WN LAV (CV-WN) in mice.

Fig. 5 is a series of graphs showing the results of challenging hamsters immunized with PIV-YF (RV-YF), YF17D, PIV-WN (RV-WN), and YF/WN LAV (CVWN) with hamster-adapted Asibi (PIV-YF and YF17D vaccinees) and wild type WN-NY99 (PIV-WN and YF/WN LAV vaccinees).

5 Fig. 6 is a table showing YF/TBE and YF/LGT virus titers and plaque morphology obtained with the indicated chimeric flaviviruses.

Fig. 7 is a table showing WN/TBE PIV titers and examples of immunofluorescence of cells containing the indicated PIVs.

Fig. 8 is a set of graphs showing the replication kinetics of YF/TBE LAV and PIV-WN/TBE in Vero and BHK cell lines (CV-Hypr = YF/Hypr LAV; CV-LGT = YF/LGT LAV; RV-WN/TBEV = PIV-WN/TBEV).

Fig. 9 is a series of graphs showing survival of mice inoculated IC with PIV-TBE and YF/TBE LAV constructs in a neurovirulence test (3.5 week old ICR mice; RV-WN/Hypr = PIV-WN/TBE(Hypr); CV-Hypr = YF/TBE(Hypr) LAV; CV-LGT = YF/LGT LAV).

Fig. 10 is a graph showing survival of mice inoculated IP with PIV-WN/TBE(Hypr) (RV-WN/Hypr), YF/TBE(Hypr) LAV (CV-Hypr), and YF/LGT LAV (CV-LGT) constructs and YF17D in a neuroinvasiveness test (3.5 week old ICR mice).

Fig. 11 is a series of graphs illustrating morbidity in mice measured by dynamics of body weight loss after TBE virus challenge, for groups immunized with s-PIV-TBE candidates (upper left panel), YF/TBE and YF/LGT chimeric viruses (upper right panel), and controls (YF 17D, human killed TBE vaccine, and mock; bottom panel).

Fig. 12 is a schematic representation of PIV constructs expressing rabies virus G protein, as well as illustration of packaging of the constructs to make pseudoinfectious virus and immunization.

Fig. 13 is a schematic representation of insertion designs resulting in viable/expressing constructs (exemplified by rabies G).

Fig. 14 is series of images showing immunofluorescence analysis and graphs showing growth curves of cells transfected with the indicated PIV-WN constructs ( $\Delta$ C-Rabies G,  $\Delta$ PrM-E-Rabies G, and  $\Delta$ C-PrM-E-Rabies G).

Fig. 15 is a series of images showing immunofluorescence analysis of RabG expressed on the plasma membranes of Vero cells transfected with the indicated PIV constructs ( $\Delta$ C-Rabies G,  $\Delta$ PrM-E-Rabies G, and  $\Delta$ C-PrM-E-Rabies G).

Fig. 16 is a schematic illustration of a PIV-WN-rabies G construct and a series of images showing that this construct spreads in helper cells, but not in naïve cells.

Fig. 17 is a series of graphs showing stability of the rabies G protein gene in PIV-WN vectors.

Fig. 18 is a set of images showing a comparison of spread of single-component vs. two-component PIV-WN-rabies G variants in Vero cells.

Fig. 19 is a set of immunofluorescence images showing expression of full-length RSV F protein (strain A2) by the  $\Delta$ prM-E component of d-PIV-WN in helper cells after transfection.

#### Detailed Description of the Invention

The invention provides replication-defective or deficient pseudoinfectious virus (PIV) vectors including flavivirus sequences, which can be used in methods for inducing immunity against heterologous pathogen, cancer, and allergy-related immunogens inserted into the vectors as well as, optionally, the vectors themselves. The invention also includes compositions including combinations of PIVs and/or PIV vectors, as described herein, and methods of using such compositions to induce immune responses against inserted immunogen sequences and/or sequences of the PIVs themselves. Further, the invention includes particular PIVs and live, attenuated chimeric flaviviruses including tick-borne encephalitis virus sequences, and related vectors, compositions, and methods of use. The PIV vectors, PIVs, live attenuated chimeric flaviviruses, compositions, and methods of the invention are described further below.

#### PIV Vectors and PIVs

The PIV vectors and PIVs of the invention can be based on the single- or two-component PIVs described above (also see WO 2007/098267 and WO 2008/137163). Thus, for example, in the case of single component PIVs, the PIV vectors and PIVs can

include a genome including a large deletion in capsid protein encoding sequences and be produced in a complementing cell line that produces capsid protein in trans (single component; Fig. 1 and Fig. 12). According to this approach, most of the capsid-encoding region is deleted, which prevents the PIV genome from producing infectious progeny in normal cell lines (i.e., cell lines not expressing capsid sequences) and vaccinated subjects. The capsid deletion typically does not disrupt RNA sequences required for genome cyclization (i.e., the sequence encoding amino acids in the region of positions 1-26), and/or the prM sequence required for maturation of prM to M. In specific examples, the deleted sequences correspond to those encoding amino acids 26-100, 26-93, 31-100, or 31-93 of the C protein.

Single component PIV vectors and PIVs can be propagated in cell lines that express either C or a C-prM-E cassette, where they replicate to high levels. Exemplary cell lines that can be used for expression of single component PIV vectors and PIVs include BHK-21 (e.g., ATCC CCL-10), Vero (e.g., ATCC CCL-81), C7/10, and other cells of vertebrate or mosquito origin. The C or C-prM-E cassette can be expressed in such cells by use of a viral vector-derived replicon, such as an alphavirus replicon (e.g., a replicon based on Venezuelan Equine Encephalitis virus (VEEV), Sindbis virus, Semliki Forest virus (SFV), Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV), or Ross River virus). To decrease the possibility of productive recombination between the PIV vectors/PIVs and complementing sequences, the sequences in the replicons (encoding C, prM, and/or E) can include nucleotide mutations. For example, sequences encoding a complementing C protein can include an unnatural cyclization sequence. The mutations can result from codon optimization, which can provide an additional benefit with respect to PIV yield. Further, in the case of complementing cells expressing C protein sequences (and not a C-prM-E cassette), it may be beneficial to include an anchoring sequence at the carboxy terminus of the C protein including, for example, about 20 amino acids of prM (see, e.g., WO 2007/098267).

The PIV vectors and PIVs of the invention can also be based on the two-component genome technology described above. This technology employs two partial genome constructs, each of which is deficient in expression of at least one protein

required for productive replication (capsid or prM/E) but, when present in the same cell, result in the production of all components necessary to make a PIV. Thus, in one example of the two-component genome technology, the first component includes a large deletion of C, as described above in reference to single component PIVs, and the second component includes a deletion of prM and E (Fig. 2 and Fig. 12). In another example, the first component includes a deletion of C, prM, and E, and the second component includes a deletion of NS1 (Fig. 12). Both components can include cis-acting promoter elements required for RNA replication and a complete set of non-structural proteins, which form the replicative enzyme complex. Thus, both defective genomes can include a 5'-untranslated region and at least about 60 nucleotides (Element 1) of the following, natural protein-coding sequence, which comprises an amino-terminal fragment of the capsid protein. This sequence can be followed by a protease cleavage sequence such as, for example, a ubiquitin or foot-and-mouth disease virus (FAMDV)-specific 2A protease sequence, which can be fused with either capsid or envelope (prM-E) coding sequences. Further, artificial, codon optimized sequences can be used to exclude the possibility of recombination between the two defective viral genomes, which could lead to formation of replication-competent viruses (see, e.g., WO 2008/137163). Use of the two-component genome approach does not require the development of cell lines expressing complementing genomes, such as the cells transformed with replicons, as discussed above in reference to the single component PIV approach. Exemplary cell lines that can be used in the two-component genome approach include Vero (e.g., ATCC CCL-81), BHK-21 (e.g., ATCC CCL-10), C7/10, and other cells of vertebrate or mosquito origin.

Additional examples of d-PIV approaches that can be used in the invention are based on use of complementing genomes including deletions in NS3 or NS5 sequences. A deletion in, e.g., NS1, NS3, or NS5 proteins can be used as long as several hundred amino acids in the ORF, removing the entire chosen protein sequence, or as short as 1 amino acid inactivating protein enzymatic activity (e.g., NS5 RNA polymerase activity, NS3 helicase activity, etc.). Alternatively, point amino acid changes (as few as 1 amino acid mutation, or optionally more mutations) can be introduced into any NS protein, inactivating enzymatic activity. In addition, several  $\Delta$ NS deletions can be combined in

one helper molecule. The same heterologous gene, i.e., expressed by the first d-PIV component, can be expressed in place or in combination with the NS deletion(s) in the second component, increasing the amount of expressed immunogen. Notably, the insertion capacity of the helper will increase proportionally to the size of NS deletion(s).  
5 Alternatively, a different foreign immunogen(s) can be inserted in place of deletion(s) of the helper to produce multivalent vaccines.

Further, additional approaches that can be used in making PIV vectors and PIVs for use in the present invention are described, for example, in WO 99/28487, WO 03/046189, WO 2004/108936, US 2004/0265338, US 2007/0249032, and U.S. Patent  
10 No. 7,332,322.

The PIV vectors and PIVs of the invention can be comprised of sequences from a single flavivirus type (e.g., tick-borne encephalitis (TBE, e.g., strain Hypr), Langat (LGT), yellow fever (e.g., YF17D), West Nile, Japanese encephalitis, dengue (serotype 1-4), St. Louis encephalitis, Kunjin, Rocio encephalitis, Ilheus, Central European  
15 encephalitis, Siberian encephalitis, Russian Spring-Summer encephalitis, Kyasanur Forest Disease, Omsk Hemorrhagic fever, Louping ill, Powassan, Negishi, Absettarov, Hansalova, and Apoi viruses), or can comprise sequences from two or more different flaviviruses. Sequences of some strains of these viruses are readily available from generally accessible sequence databases; sequences of other strains can be easily  
20 determined by methods well known in the art. In the case of PIV vectors and PIVs including sequences of more than one flavivirus, the sequences can be those of a chimeric flavivirus, as described above (also see, e.g., U.S. Patent No. 6,962,708; U.S. Patent No. 6,696,281; and U.S. Patent No. 6,184,024). In certain examples, the chimeras include pre-membrane and envelope sequences from one flavivirus (such as a flavivirus to which  
25 immunity may be desired), and capsid and non-structural sequences from a second, different flavivirus. In one specific example, the second flavivirus is a yellow fever virus, such as the vaccine strain YF17D. Other examples include the YF/TBE, YF/LGT, WN/TBE, and WN/LGT chimeras described below. Another example is an LGT/TBE chimera based on LGT virus backbone containing TBE virus prM-E proteins. A PIV  
30 vaccine based on this genetic background would have an advantage, because LGT

replicates very efficiently in vitro and is highly attenuated and immunogenic for humans. Thus, a chimeric LGT/TBE PIV vaccine is expected to provide a robust specific immune response in humans against TBE, particularly due to inclusion of TBE prM-E genes.

Vectors of the invention can be based on PIV constructs or live, attenuated  
5 chimeric flaviviruses as described herein (in particular, YF/TBE, YF/LGT, WN/TBE, and WN/LGT; see below). Use of PIV constructs as vectors provides particular advantages in certain circumstances, because these constructs by necessity include large deletions, which render the constructs more amenable to accommodation of insertions that are at least up to the size of the deleted sequences, without there being a loss in replication  
10 efficiency. Thus, PIV vectors in general can comprise very small insertions (e.g., in the range 6-10, 11-20, 21-100, 101-500, or more amino acid residues combined with the  $\Delta$ C deletion or other deletions), as well as relatively large insertions or insertions of intermediate size (e.g., in the range 501-1000, 1001-1700, 1701-3000, or 3001-4000 or more residues). In contrast, in certain examples, it may be advantageous to express  
15 relatively short sequences in live attenuated viruses, particularly if the insertions are made in the absence of a corresponding deletion. Additional information concerning insertion sites that can be used in the invention is provided below. In addition, as discussed further below, expression of non-flavivirus immunogens in PIVs and chimeric flaviviruses of the invention can result in dual vaccines that elicit protective immunity against both a  
20 flavivirus vector virus pathogen and a target heterologous immunogen (e.g., a pathogen (such as a bacterial, viral, parasite, or fungal pathogen), cancer, or allergy-related immunogen).

As discussed above, the PIV vectors and PIVs of the invention can comprise sequences of chimeric flaviviruses, for example, chimeric flaviviruses including pre-  
25 membrane and envelope sequences of a first flavivirus (e.g., a flavivirus to which immunity is sought), and capsid and non-structural sequences of a second, different flavivirus, such as a yellow fever virus (e.g., YF17D; see above and also U.S. Patent No. 6,962,708; U.S. Patent No. 6,696,281; and U.S. Patent No. 6,184,024). Further, chimeric flaviviruses of the invention, used as a source for constructing PIVs, or as  
30 vaccines/vaccine vectors per se, can optionally include one or more specific attenuating

mutations (e.g., E protein mutations, prM protein mutations, deletions in the C protein, and/or deletions in the 3'UTR), such as any of those described in WO 2006/116182. For example, the C protein or 3'UTR deletions can be directly applied to YF/TBE or YF/LGT chimeras. Similar deletions can be designed and introduced in other chimeric LAV  
5 candidates such as based on LGT/TBE, WN/TBE, and WN/LGT genomes. With respect to E protein mutations, attenuating mutations similar to those described for YF/WN chimera in WO 2006/116182 can be designed, e.g., based on the knowledge of crystal structure of the E protein (Rey et al., Nature 375(6529):291-298, 1995), and employed. Further, additional examples of attenuating E protein mutations described for TBE virus  
10 and other flaviviruses are provided in Table 9. These can be similarly introduced into chimeric vaccine candidates.

The invention also provides new, particular chimeric flaviviruses, which can be used as a basis for the design of PIV vectors and PIVs, as live attenuated chimeric flavivirus vectors, and as vaccines against the source(s) of the pre-membrane and  
15 envelope components of the chimeras. These chimeras include tick-borne encephalitis (TBE) virus or related prM-E sequences. Thus, the chimeras can include prM-E sequences from, for example, the Hypr strain of TBE or Langat (LGT) virus. Capsid and non-structural proteins of the chimeras can include those from yellow fever virus (e.g., YF17D) or West Nile virus (e.g., NY99).

20 A central feature of these exemplary YF/TBE, YF/LGT, WN/TBE, and WN/LGT chimeras is the signal sequence between the capsid and prM proteins. As is shown in the Examples, below, we have found that, in the case of YF-based PIV chimeras, it is advantageous to use a signal sequence comprising yellow fever and TBE sequences (see below). In one example, the signal sequence includes yellow fever sequences in the  
25 amino terminal region (e.g., SHDVLTQFLIL) and TBE sequences in the carboxy terminal region (e.g., GMLGMTIA), resulting in the sequence SHDVLTQFLILGMLGMTIA. We have also found that, in the case of WN-based PIV chimeras, it is advantageous to use a signal sequence comprising TBE sequences (e.g., GGTDWMSWLLVIGMLGMTIA). The invention thus includes YF/TBE, YF/LGT,  
30 WN/TBE, and WN/LGT chimeras, both PIVs and LAVs, which include the above-noted

signal sequences, or variants thereof having, e.g., 1-8, 2-7, 3-6, or 4-5 amino acid substitutions, deletions, or insertions, which do not substantially interfere with processing at the signal sequence. In various examples, the substitutions are "conservative substitutions," which are characterized by replacement of one amino acid residue with another, biologically similar residue. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine, or methionine for another, or the substitution of one polar residue for another, such as between arginine and lysine, between glutamic and aspartic acids, or between glutamine and asparagine and the like. Additional information concerning these chimeras is provided below, in the Examples.

#### Insertion Sites

Sequences encoding immunogens can be inserted at one or more different sites within the vectors of the invention. Relatively short peptides can be delivered on the surface of PIV or LAV glycoproteins (e.g., prM, E, and/or NS1 proteins) and/or in the context of other proteins (to induce predominantly B-cell and T-cell responses, respectively). Other inserts, including larger portions of foreign proteins, as well as complete proteins, can be expressed intergenically, at the N- and C-termini of the polyprotein, or bicistronically (e.g., within the ORF under an IRES or in the 3'UTR under an IRES; see, e.g., WO 02/102828, WO 2008/036146, WO 2008/094674, WO 2008/100464, WO 2008/115314, and below for further details). In PIV constructs, there is an additional option of inserting a foreign amino acid sequence directly in place of introduced deletion(s). Insertions can be made in, for example,  $\Delta$ C,  $\Delta$ prM-E,  $\Delta$ C-prM-E,  $\Delta$ NS1,  $\Delta$ NS3, and  $\Delta$ NS5. Thus, in one example, in the case of s-PIVs and the  $\Delta$ C component of d-PIVs, immunogen-encoding sequences can be inserted in place of deleted capsid sequences. Immunogen-encoding sequences can also, optionally, be inserted in place of deleted prM-E sequences in the  $\Delta$ prM-E component of d-PIVs. In another example, the sequences are inserted in place of or combined with deleted sequences in  $\Delta$ C-prM-E constructs. Examples of such insertions are provided in the Examples section, below.

In the case of making insertions into PIV deletions, the insertions can be made with a few (e.g., 1, 2, 3, 4, or 5) additional vector-specific residues at the N- and/or C-termini of the foreign immunogen, if the sequence is simply fused in-frame (e.g., ~20 first a.a. and a few last residues of the C protein if the sequence replaces the  $\Delta$ C deletion),  
5 or without, if the foreign immunogen is flanked by appropriate elements well known in the field (e.g., viral protease cleavage sites; cellular protease cleavage sites, such as signalase, furin, etc.; autoprotease; termination codon; and/or IRES elements).

If a protein is expressed outside of the continuous viral open reading frame (ORF), e.g., if vector and non-vector sequences are separated by an internal ribosome  
10 entry site (IRES), cytoplasmic expression of the product can be achieved or the product can be directed towards the secretory pathway by using appropriate signal/anchor segments, as desired. If the protein is expressed within the vector ORF, important considerations include cleavage of the foreign protein from the nascent polyprotein sequence, and maintaining correct topology of the foreign protein and all viral proteins (to  
15 ensure vector viability) relative to the ER membrane, e.g., translocation of secreted proteins into the ER lumen, or keeping cytoplasmic proteins or membrane-associated proteins in the cytoplasm/in association with the ER membrane.

In more detail, the above-described approaches to making insertions can employ the use of, for instance, appropriate vector-derived, insert-derived, or unrelated signal and  
20 anchor sequences included at the N and C termini of glycoprotein inserts. Standard autoproteases, such as FMDV 2A autoprotease (~20 amino acids) or ubiquitin (gene ~500 nt), or flanking viral NS2B/NS3 protease cleavage sites can be used to direct cleavage of an expressed product from a growing polypeptide chain, to release a foreign protein from a vector polyprotein, and to ensure viability of the construct. Optionally,  
25 growth of the polyprotein chain can be terminated by using a termination codon, e.g., following a foreign gene insert, and synthesis of the remaining proteins in the constructs can be re-initiated by incorporation of an IRES element, e.g., the encephalomyocarditis virus (EMCV) IRES commonly used in the field of RNA virus vectors. Viable recombinants can be recovered from helper cells (or regular cells for d-PIV versions).  
30 Optionally, backbone PIV sequences can be rearranged, e.g., if the latter results in more

efficient expression of a foreign gene. For example, a gene rearrangement has been applied to TBE virus, in which the prM-E genes were moved to the 3' end of the genome under the control of an IRES (Orlinger et al., J. Virol. 80:12197-12208, 2006).

Translocation of prM-E or any other genes can be applied to PIV flavivirus vaccine candidates and expression vectors, according to the invention.

Additional details concerning different insertion sites that can be used in the invention are as follows (also see WO 02/102828, WO 2008/036146, WO 2008/094674, WO 2008/100464, WO 2008/115314, as noted above). Peptide sequences can be inserted within the envelope protein, which is the principle target for neutralizing antibodies. The sequences can be inserted into the envelope in, for example, positions corresponding to amino acid positions 59, 207, 231, 277, 287, 340, and/or 436 of the Japanese encephalitis virus envelope protein (see, e.g., WO 2008/115314 and WO 02/102828). To identify the corresponding loci in different flaviviruses, the flavivirus sequences are aligned with that of Japanese encephalitis virus. As there may not be an exact match, it should be understood that, in non-JE viruses, the site of insertion may vary by, for example, 1, 2, 3, 4, or 5 amino acids, in either direction. Further, given the identification of such sites as being permissive in JE, they can also vary in JE by, for example, 1, 2, 3, 4, or 5 amino acids, in either direction. Additional permissive sites can be identified using methods such as transposon mutagenesis (see, e.g., WO 02/102828 and WO 2008/036146). The insertions can be made at the indicated amino acids by insertion just C-terminal to the indicated amino acids (i.e., between amino acids 51-52, 207-208, 231-232, 277-278, 287-288, 340-341, and 436-437), or in place of short deletions (e.g., deletions of 1, 2, 3, 4, 5, 6, 7, or 8 amino acids) beginning at the indicated amino acids (or within 1-5 positions thereof, in either direction).

In addition to the envelope protein, insertions can be made into other virus proteins including, for example, the membrane/pre-membrane protein and NS1 (see, e.g., WO 2008/036146). For example, insertions can be made into a sequence preceding the capsid/pre-membrane cleavage site (at, e.g., -4, -2, or -1) or within the first 50 amino acids of the pre-membrane protein (e.g., at position 26), and/or between amino acids 236 and 237 of NS1 (or in regions surrounding the indicated sequences, as described above).

In other examples, insertions can be made intergenically. For example, an insertion can be made between E and NS1 proteins and/or between NS2B and NS3 proteins (see, e.g., WO 2008/100464). In one example of an intergenic insertion, the inserted sequence can be fused with the C-terminus of the E protein of the vector, after the C-terminal  
5 signal/anchor sequence of the E protein, and the insertion can include a C-terminal anchor/signal sequence, which is fused with vector NS1 sequences. In another example of an intergenic insertion, the inserted sequences, with flanking protease cleavage sites (e.g., YF 17D cleavage sites), can be inserted into a unique restriction site introduced at the NS2B/NS3 junction (WO 2008/100464).

10 In other examples, a sequence can be inserted in the context of an internal ribosome entry site (IRES, e.g., an IRES derived from encephalomyocarditis virus; EMCV), as noted above, such as inserted in the 3'-untranslated region (WO 2008/094674). In one example of such a vector, employing, for example, yellow fever virus sequences, an IRES-immunogen cassette can be inserted into a multiple cloning site  
15 engineered into the 3'-untranslated region of the vector, e.g., in a deletion (e.g., a 136 nucleotide deletion in the case of a yellow fever virus-based example) after the polyprotein stop codon (WO 2008/094674).

Details concerning the insertion of rabies virus G protein and full-length respiratory syncytial virus (RSV) F protein into s-PIV and d-PIV vectors of the invention  
20 are provided below in Example 3. The information provided in Example 3 can be applied in the context of other vectors and immunogens described herein.

### Immunogens

PIVs (s-PIVs and d-PIVs) based on flavivirus sequences and live, attenuated  
25 chimeric flaviviruses (e.g., YF/TBE, YF/LGT, WN/TBE, and WN/LGT), as described above, can be used in the invention to deliver foreign (e.g., non-flavivirus) pathogen (e.g., viral, bacterial, fungal, and parasitic pathogens), cancer, and allergy-related immunogens. As discussed further below, in certain examples, it may be advantageous to target several pathogens occupying the same ecological niche, in a particular geographical region.

30 Specific, non-limiting examples of such immunogens are provided as follows.

In addition to TBE virus, ticks are known to transmit another major disease, Lyme disease. Thus, in a first example, PIVs of the invention, such as PIVs including TBE/LGT sequences, as well as chimeric flaviviruses including TBE sequences (e.g., YF/TBE, YF/LGT, WN/TBE, LGT/TBE, and WN/LGT; in all instances where "TBE" is indicated, this includes the option of using the Hypr strain), can be used as vectors to deliver protective immunogens of the causative agent of Lyme disease (tick-borne spirochete *Borrelia burgdorferi*). This combination, targeting both infectious agents (TBE and *B. burgdorferi*) is advantageous, because TBE and Lyme disease are both tick-borne diseases. The PIV approaches can be applied to chimeras (e.g., YF/TBE, YF/LGT, WN/TBE, or WN/LGT), according to the invention, as well as to non-chimeric TBE and LGT viruses. An exemplary immunogen from *B. burgdorferi* that can be used in the invention is OspA (Gipson et al., Vaccine 21:3875-3884, 2003). Optionally, to increase safety and/or immunogenicity, OspA can be mutated to reduce chances of autoimmune responses and/or to eliminate sites for unwanted post-translational modification in vertebrate animal cells, such as N-linked glycosylation, which may affect immunogenicity of the expression product. Mutations that decrease autoimmunity can include, e.g., those described by Willett et al., Proc. Natl. Acad. Sci. U.S.A. 101:1303-1308, 2004. In one example, FTK-OspA, a putative cross-reactive T cell epitope, *Bb* OspA<sub>165-173</sub> (YVLEGTLTA) is altered to resemble the corresponding peptide sequence of *Borrelia afzelli* (FTLEGKLVAN). In FTK-OspA, the corresponding sequence is FTLEGKLTAA.

The sequence of OspA is as follows:

```

1  mkkyllgigl ilaliackqn vssldeknsv svdlpgemkv lvskekknkg
kydliatvdk
61  lelkgtsdkn ngsgvlegvk adsksvklti sddlqgttle vfkedgkttlv
25  skkvtskdks
121  steekfnekf evsekiitra dgtrleytgi ksdgsgkake vlkgyvlegt
ltaekttlvv
181  kegtvtlskn isksgevsve lndtdssaat kktawnsqt stltitvnsk
ktdkdlvftke
30  241  ntitvqqyds ngtklegsav eitkldeikn alk

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The full-length sequence and/or immunogenic fragments of the full-length sequence can be used in the present invention. Exemplary fragments can include one or more of domains 1 (amino acids 34-41), 2 (amino acids 65-75), 3 (amino acids 190-220), and 4 (amino acids 250-270) (Jiang et al., Clin. Diag. Lab. Immun. 1(4):406-412, 1994).

Thus, for example, a peptide comprising any one (or more) of the following sequences (which include sequence variations that can be included in the sequence listed above, in any combination) can be delivered: LPGE/GM/IK/T/GVL; GTSDKN/S/DNGSGV/T; N/H/EIS/P/L/A/SK/NSGEV/IS/TV/AE/ALN/DDT/SD/NS/TS/TA/Q/RATKKTA/GA/K/

5 TWN/DS/AG/N/KT; SN/AGTK/NLEGS/N/K/TA VEIT/KK/TLD/KEI/LKN.

In addition to *B. burgdorferi* immunogens, tick saliva proteins, such as 64TRP, Isac, and Salp20, can be expressed, e.g., to generate a vaccine candidate of trivalent-specificity (TBE+Lyme disease+ticks). Alternatively, tick saliva proteins can be expressed instead of *B. burgdorferi* immunogens in TBE sequence-containing vectors. In

10 addition, there are many other candidate tick saliva proteins that can be used for tick vector vaccine development according to the invention (Francischetti et al., Insect Biochem. Mol. Biol. 35:1142-1161, 2005). One or more of these immunogens can be expressed in s-PIV-TBE. However, d-PIV-TBE may also be selected, because of its large insertion capacity. In addition to PIV-TBE, other PIV vaccines can be used as vectors,

15 e.g., to protect from Lyme disease and another flavivirus disease, such as West Nile virus. Expression of these immunogens can be evaluated in cell culture, and immunogenicity/protection examined in available animal models (e.g., as described in Gipson et al., Vaccine 21:3875-3884, 2003; Labuda et al., Pathog. 2(e27):0251-0259, 2006). Immunogens of other pathogens can be similarly expressed, in addition to Lyme

20 disease and tick immunogens, with the purpose of making multivalent vaccine candidates. Exemplary tick saliva immunogens that can be used in the invention include the following:

#### 64TRP (AF469170)

25 MKAFFVLSLL STAALTNAAR AGRLGSDLDLT FGRVHGNYLYA GIERAGPRGY PGLTASIGGE  
VGARLGGRAG VGVSSYGYGY PSWGYPYGGY GGYGGYGGYG GYDQGFSGAY GGYPGYYGY  
YPSGYGGYG GSYGGSYGGS YTYPNVRASA GAAA

#### Isac (AF270496)

30 MRTAFTCALL AISFLGSPCS SSLEDGLEQDT IVETTTONLY ERHYRNHSG L CGAQYRNSSH  
AEAVYNCTLN HLPVNVNATW EGIRHRINKT IPQFVKLICN FTVAMPQEFY LVYMGSDGNS  
DFEEDKESTG TDEDSNTGSS AAKVTEALI IEAEENCTAH ITGWTETPT TLEPTTESQF EAIP

Salp20 (EU008559)

MRTALTCALL AISFLGSPCS SSEGGLEKDS RVETTTONLY ERYRKHPLG CGAQYRNSSH  
AEAVYNCTLS LLPLSVNTTW EGIRHRINKT IPEFVNLCN FTVAMPDQFY LVYMGSGNGS  
YSEEDDGKT GSSAAVQVTE QLIQAEENC TAHITGWTTE APTTLEPTTE TQFEALS

5

Additional details concerning the TBE-related PIVs and LAVs are provided in Example 2, below.

The invention further provides PIV and LAV-vectored vaccines against other non-flavivirus pathogens, including vaccines having dual action, eliciting protective immunity  
10 against both flavivirus (as specified by the vector envelope proteins) and non-flavivirus pathogens (as specified by expressed immunologic determinant(s)). These are similar to the example of PIV-TBE-Lyme disease-tick vector vaccines described above. As mentioned above, such dual-action vaccines can be developed against a broad range of pathogens by expression of immunogens from, for example, viral, bacterial, fungal, and  
15 parasitic pathogens, and immunogens associated with cancer and allergy. As specific non-limiting examples, we describe herein the design and biological properties of PIV vectored-rabies and -respiratory syncytial virus (RSV) vaccine candidates constructed by expression of rabies virus G protein or full-length RSV F protein in place of or in combination with various deletions in one- and two-component PIV vectors (see Example  
20 3, below).

As is demonstrated in the Examples, below, s-PIV constructs may be advantageously used to stably deliver relatively short foreign immunogens (similar to Lyme disease agent OspA protein and tick saliva proteins), because insertions are combined with a relatively short  $\Delta C$  deletion. Two-component PIV vectors may be  
25 advantageously used to stably express relatively large immunogens, such as rabies G protein and RSV F, as the insertions in such vectors are combined with, for example, large  $\Delta prM-E$ ,  $\Delta C-prM-E$ , and/or  $\Delta NS1$  deletions. Some of the d-PIV components can be manufactured and used as vaccines individually, for instance, the PIV-RSV F construct described below containing a  $\Delta C-prM-E$  deletion. In this case, the vaccine induces an  
30 immune response (e.g., neutralizing antibodies) predominantly against the expressed protein, but not against the flavivirus vector virus pathogen. In other examples of the invention, dual immunity is obtained by having immunity induced both to vector and

insert components. Additionally, because of the large insertion capacity of PIV vectors, and the option of using two-component genomes, PIV vectors offer the opportunity to target several non-flavivirus pathogens simultaneously, e.g., by expressing foreign immunogens from two different non-flavivirus pathogens in the two components of a d-PIV.

In addition to the RSV F protein, rabies G protein, Lyme disease protective immunogens, and tick saliva proteins, as examples of foreign immunogens described above, other foreign immunogens can be expressed to target respective diseases including, for example, influenza virus type A and B immunogens. In these examples, a few short epitopes and/or whole genes of viral particle proteins can be used, such as the M2, HA, and NA genes of influenza A, and/or the NB or BM2 genes of influenza B. Shorter fragments of M2, NB, and BM2, corresponding for instance to M2e, the extracellular fragment of M2, can also be used. In addition, fragments of the HA gene, including epitopes identified as HA0 (23 amino acids in length, corresponding to the cleavage site in HA) can be used. Specific examples of influenza-related sequences that can be used in the invention include PAKLLKERGFFGAIAGFLE (HA0), PAKLLKERGFFGAIAGFLEGSGC (HA0), NNATFNVTNVNPISHIRGS (NBe), MSLLTEVETPIRNEWGCRCNDSSD (M2e), MSLLTEVETPTRNEWECRCSDSSD (M2e), MSLLTEVETLTRNGWGCRCSDDSSD (M2e), EVETPTRN (M2e), SLLTEVETPIRNEWGCRCNDSSD (M2e), and SLLTEVETPIRNEWGCR (M2e). Additional M2e sequences that can be used in the invention include sequences from the extracellular domain of BM2 protein of influenza B (consensus MLEPFQ, e.g., LEPFQILSISGC), and the M2e peptide from the H5N1 avian flu (MSLLTEVETLTRNGWGCRCSDDSSD).

Other examples of pathogen immunogens that can be delivered in the vectors of the invention include codon-optimized SIV or HIV gag (55 kDa), gp120, gp160, SIV mac239-rev/tat/nef genes or analogs from HIV, and other HIV immunogens; immunogens from HPV viruses, such as HPV16, HPV18, etc., e.g., the capsid protein L1 which self-assembles into HPV-like particles, the capsid protein L2 or its immunodominant portions (e.g., amino acids 1-200, 1-88, or 17-36), the E6 and E7 proteins which are involved in

transforming and immortalizing mammalian cells fused together and appropriately mutated (fusion of the two genes creates a fusion protein, referred to as E6E7Rb<sup>-</sup>, that is about 10-fold less capable of transforming fibroblasts, and mutations of the E7 component at 2 residues renders the resulting fusion protein mutant incapable of inducing transformation (Boursnell et al., Vaccine 14:1485-1494, 1996). Other immunogens include protective immunogens from HCV, CMV, HSV2, viruses, malaria parasite, *Mycobacterium tuberculosis* causing tuberculosis, *C. difficile*, and other nosocomial infections, that are known in the art, as well as fungal pathogens, cancer immunogens, and proteins associated with allergy that can be used as vaccine targets.

Foreign immunogen inserts of the invention can be modified in various ways. For instance, codon optimization is used to increase the level of expression and eliminate long repeats in nucleotide sequences to increase insert stability in the RNA genome of PIV vectors. Immunogenicity can be increased by chimerization of proteins with immunostimulatory moieties well known in the art, such as TLR agonists, stimulatory cytokines, components of complement, heat-shock proteins, etc. (e.g., reviewed in "Immunopotentiators in Modern Vaccines," Schijns and O'Hagan Eds., 2006, Elsevier Academic Press: Amsterdam, Boston).

With respect to construction of dual vaccines against rabies and other flavivirus diseases, other combinations, such as TBE + rabies, YF + rabies, etc., can be of interest both for human and veterinary use in corresponding geographical regions, and thus can be similarly generated. Possible designs of expression constructs are not limited to those described herein. For example deletions and insertions can be modified, genetic elements can be rearranged, or other genetic elements (e.g. non-flavivirus, non-rabies signals for secretion, intracellular transport determinants, inclusion of or fusion with immunostimulatory moieties such as cytokines, TLR agonists such as flagellin, multimerization components such as leucine zipper, and peptides that increase the period of protein circulation in the blood) can be used to facilitate antigen presentation and increase immunogenicity. Further, such designs can be applied to s-PIV and d-PIV vaccine candidates based on vector genomes of other flaviviruses, and expressing

immunogens of other pathogens, e.g., including but not limited to pathogens described in elsewhere herein.

Other examples of PIV and LAV vectors of the invention including combination vaccines such as DEN+Chikungunya virus (CHIKV) and YF+CHIKV. CHIKV, an  
5 alphavirus, is endemic in Africa, South East Asia, Indian subcontinent and the Islands, and the Pacific Islands and shares ecological/geographical niches with YF and DEN1-4. It causes serious disease primarily associated with severe pain (arthritis, other symptoms similar to DEN) and long-lasting sequelae in the majority of patients (Simon et al., Med. Clin. North Am. 92:1323-1343, 2008; Seneviratne et al., J. Travel Med. 14:320-325,  
10 2007). Other examples of PIV and LAV vectors of the invention include YF+Ebola or DEN+Ebola, which co-circulate in Africa.

Immunogens for the above-noted non-flavivirus pathogens, sequences of which are well known in the art, may include glycoprotein B or a pp65/IE1 fusion protein of CMV (Reap et al., Vaccine 25(42):7441-7449, 2007; and references therein), several TB  
15 proteins (reviewed in Skeiky et al., Nat. Rev. Microbiol. 4(6):469-476, 2006), malaria parasite antigens such as RTS,S (a pre-erythrocytic circumsporozoite protein, CSP) and others (e.g., reviewed in Li et al., Vaccine 25(14):2567-2574, 2007), CHIKV envelope proteins E1 and E2 (or the C-E2-E1, E2-E1 cassettes), HCV structural proteins C-E1-E2 forming VLPs (Ezelle et al., J. Virol. 76(23):12325-12334, 2002) or other proteins to  
20 induce T-cell responses, Ebola virus glycoprotein GP (Yang et al., Virology 377(2):255-264, 2008).

In addition to the immunogens described above, the vectors described herein may include one or more immunogen(s) derived from or that direct an immune response against one or more viruses (e.g., viral target antigen(s)) including, for example, a dsDNA  
25 virus (e.g., adenovirus, herpesvirus, epstein-barr virus, herpes simplex type 1, herpes simplex type 2, human herpes virus simplex type 8, human cytomegalovirus, varicella-zoster virus, poxvirus); ssDNA virus (e.g., parvovirus, papillomavirus (e.g., E1, E2, E3, E4, E5, E6, E7, E8, BPV1, BPV2, BPV3, BPV4, BPV5, and BPV6 (*In Papillomavirus and Human Cancer*, edited by H. Pfister (CRC Press, Inc. 1990)); Lancaster et al., Cancer  
30 Metast. Rev. pp. 6653-6664, 1987; Pfister et al., Adv. Cancer Res. 48:113-147, 1987));

dsRNA viruses (e.g., reovirus); (+)ssRNA viruses (e.g., picornavirus, coxsackie virus, hepatitis A virus, poliovirus, togavirus, rubella virus, flavivirus, hepatitis C virus, yellow fever virus, dengue virus, west Nile virus); (-)ssRNA viruses (e.g., orthomyxovirus, influenza virus, rhabdovirus, paramyxovirus, measles virus, mumps virus, parainfluenza virus, rhabdovirus, rabies virus); ssRNA-RT viruses (e.g., retrovirus, human immunodeficiency virus (HIV)); and dsDNA-RT viruses (e.g. hepadnavirus, hepatitis B). Immunogens may also be derived from other viruses not listed above but available to those of skill in the art.

With respect to HIV, immunogens may be selected from any HIV isolate. As is well-known in the art, HIV isolates are now classified into discrete genetic subtypes. HIV-1 is known to comprise at least ten subtypes (A, B, C, D, E, F, G, H, J, and K). HIV-2 is known to include at least five subtypes (A, B, C, D, and E). Subtype B has been associated with the HIV epidemic in homosexual men and intravenous drug users worldwide. Most HIV-1 immunogens, laboratory adapted isolates, reagents and mapped epitopes belong to subtype B. In sub-Saharan Africa, India, and China, areas where the incidence of new HIV infections is high, HIV-1 subtype B accounts for only a small minority of infections, and subtype HIV-1 C appears to be the most common infecting subtype. Thus, in certain embodiments, it may be desirable to select immunogens from HIV-1 subtypes B and/or C. It may be desirable to include immunogens from multiple HIV subtypes (e.g., HIV-1 subtypes B and C, HIV-2 subtypes A and B, or a combination of HIV-1 and HIV-2 subtypes) in a single immunological composition. Suitable HIV immunogens include ENV, GAG, POL, NEF, as well as variants, derivatives, and fusion proteins thereof, for example.

Immunogens may also be derived from or direct an immune response against one or more bacterial species (spp.) (e.g., bacterial target antigen(s)) including, for example, *Bacillus* spp. (e.g., *Bacillus anthracis*), *Bordetella* spp. (e.g., *Bordetella pertussis*), *Borrelia* spp. (e.g., *Borrelia burgdorferi*), *Brucella* spp. (e.g., *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*), *Campylobacter* spp. (e.g., *Campylobacter jejuni*), *Chlamydia* spp. (e.g., *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*), *Clostridium* spp. (e.g., *Clostridium botulinum*, *Clostridium difficile*,

Clostridium perfringens, Clostridium tetani), Corynebacterium spp. (e.g., Corynebacterium diphtheriae), Enterococcus spp. (e.g., Enterococcus faecalis, enterococcus faecum), Escherichia spp. (e.g., Escherichia coli), Francisella spp. (e.g., Francisella tularensis), Haemophilus spp. (e.g., Haemophilus influenza), Helicobacter spp. (e.g., Helicobacter pylori), Legionella spp. (e.g., Legionella pneumophila),  
 5 Leptospira spp. (e.g., Leptospira interrogans), Listeria spp. (e.g., Listeria monocytogenes), Mycobacterium spp. (e.g., Mycobacterium leprae, Mycobacterium tuberculosis), Mycoplasma spp. (e.g., Mycoplasma pneumoniae), Neisseria spp. (e.g., Neisseria gonorrhea, Neisseria meningitidis), Pseudomonas spp. (e.g., Pseudomonas aeruginosa),  
 10 Rickettsia spp. (e.g., Rickettsia rickettsii), Salmonella spp. (e.g., Salmonella typhi, Salmonella typhimurium), Shigella spp. (e.g., Shigella sonnei), Staphylococcus spp. (e.g., Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, coagulase negative staphylococcus (e.g., U.S. Patent No. 7,473,762)), Streptococcus spp. (e.g., Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes),  
 15 Treponema spp. (e.g., Treponema pallidum), Vibrio spp. (e.g., Vibrio cholerae), and Yersinia spp. (Yersinia pestis). Immunogens may also be derived from or direct the immune response against other bacterial species not listed above but available to those of skill in the art.

Immunogens may also be derived from or direct an immune response against one  
 20 or more parasitic organisms (spp.) (e.g., parasite target antigen(s)) including, for example, Ancylostoma spp. (e.g., A. duodenale), Anisakis spp., Ascaris lumbricoides, Balantidium coli, Cestoda spp., Cimicidae spp., Clonorchis sinensis, Dicrocoelium dendriticum, Dicrocoelium hospes, Diphyllbothrium latum, Dracunculus spp., Echinococcus spp. (e.g., E. granulosus, E. multilocularis), Entamoeba histolytica, Enterobius vermicularis,  
 25 Fasciola spp. (e.g., F. hepatica, F. magna, F. gigantica, F. jacksoni), Fasciolopsis buski, Giardia spp. (Giardia lamblia), Gnathostoma spp., Hymenolepis spp. (e.g., H. nana, H. diminuta), Leishmania spp., Loa loa, Metorchis spp. (M. conjunctus, M. albidus), Necator americanus, Oestroidea spp. (e.g., botfly), Onchocercidae spp., Opisthorchis spp. (e.g., O. viverrini, O. felinus, O. guayaquilensis, and O. noverca), Plasmodium spp. (e.g., P. falciparum), Protofasciola robusta, Parafasciolopsis fasciomorphae, Paragonimus

westermani, *Schistosoma* spp. (e.g., *S. mansoni*, *S. japonicum*, *S. mekongi*, *S. haematobium*), *Spirometra erinaceieuropaei*, *Strongyloides stercoralis*, *Taenia* spp. (e.g., *T. saginata*, *T. solium*), *Toxocara* spp. (e.g., *T. canis*, *T. cati*), *Toxoplasma* spp. (e.g., *T. gondii*), *Trichobilharzia regenti*, *Trichinella spiralis*, *Trichuris trichiura*, *Trombiculidae* spp., *Trypanosoma* spp., *Tunga penetrans*, and/or *Wuchereria bancrofti*. Immunogens may also be derived from or direct the immune response against other parasitic organisms not listed above but available to those of skill in the art.

Immunogens may be derived from or direct the immune response against tumor target antigens (e.g., tumor target antigens). The term tumor target antigen (TA) may include both tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs), where a cancerous cell is the source of the antigen. A TA may be an antigen that is expressed on the surface of a tumor cell in higher amounts than is observed on normal cells or an antigen that is expressed on normal cells during fetal development. A TSA is typically an antigen that is unique to tumor cells and is not expressed on normal cells. TAs are typically classified into five categories according to their expression pattern, function, or genetic origin: cancer-testis (CT) antigens (i.e., MAGE, NY-ESO-1); melanocyte differentiation antigens (e.g., Melan A/MART-1, tyrosinase, gp100); mutational antigens (e.g., MUM-1, p53, CDK-4); overexpressed 'self' antigens (e.g., HER-2/neu, p53); and viral antigens (e.g., HPV, EBV). Suitable TAs include, for example, gp100 (Cox et al., *Science* 264:716-719, 1994), MART-1/Melan A (Kwakami et al., *J. Exp. Med.*, 180:347-352, 1994), gp75 (TRP-1) (Wang et al., *J. Exp. Med.*, 186:1131-1140, 1996), tyrosinase (Wolfel et al., *Eur. J. Immunol.*, 24:759-764, 1994), NY-ESO-1 (WO 98/14464; WO 99/18206), melanoma proteoglycan (Hellstrom et al., *J. Immunol.*, 130:1467-1472, 1983), MAGE family antigens (e.g., MAGE-1, 2, 3, 4, 6, and 12; Van der Bruggen et al., *Science* 254:1643-1647, 1991; U.S. Patent No. 6,235,525), BAGE family antigens (Boel et al., *Immunity* 2:167-175, 1995), GAGE family antigens (e.g., GAGE-1,2; Van den Eynde et al., *J. Exp. Med.* 182:689-698, 1995; U.S. Patent No. 6,013,765), RAGE family antigens (e.g., RAGE-1; Gaugler et al., *Immunogenetics* 44:323-330, 1996; U.S. Patent No. 5,939,526), N-acetylglucosaminyltransferase-V (Guilloux et al., *J. Exp. Med.* 183:1173-1183, 1996), p15 (Robbins et al., *J. Immunol.*

154:5944-5950, 1995),  $\beta$ -catenin (Robbins et al., J. Exp. Med., 183:1185-1192, 1996), MUM-1 (Coulie et al., Proc. Natl. Acad. Sci. U.S.A. 92:7976-7980, 1995), cyclin dependent kinase-4 (CDK4) (Wolfel et al., Science 269:1281-1284, 1995), p21-*ras* (Fossum et al., Int. J. Cancer 56:40-45, 1994), BCR-*abl* (Bocchia et al., Blood 85:2680-2684, 1995), p53 (Theobald et al., Proc. Natl. Acad. Sci. U.S.A. 92:11993-11997, 1995), p185 HER2/neu (erb-B1; Fisk et al., J. Exp. Med., 181:2109-2117, 1995), epidermal growth factor receptor (EGFR) (Harris et al., Breast Cancer Res. Treat, 29:1-2, 1994), carcinoembryonic antigens (CEA) (Kwong et al., J. Natl. Cancer Inst., 85:982-990, 1995) U.S. Patent Nos. 5,756,103; 5,274,087; 5,571,710; 6,071,716; 5,698,530; 6,045,802; EP 263933; EP 346710; and EP 784483; carcinoma-associated mutated mucins (e.g., MUC-1 gene products; Jerome et al., J. Immunol., 151:1654-1662, 1993); EBNA gene products of EBV (e.g., EBNA-1; Rickinson et al., Cancer Surveys 13:53-80, 1992); E7, E6 proteins of human papillomavirus (Ressing et al., J. Immunol. 154:5934-5943, 1995); prostate specific antigen (PSA; Xue et al., The Prostate 30:73-78, 1997); prostate specific membrane antigen (PSMA; Israeli et al., Cancer Res. 54:1807-1811, 1994); idiotypic epitopes or antigens, for example, immunoglobulin idiotypes or T cell receptor idiotypes (Chen et al., J. Immunol. 153:4775-4787, 1994); KSA (U.S. Patent No. 5,348,887), kinesin 2 (Dietz, et al., Biochem. Biophys. Res. Commun. 275(3):731-738, 2000), HIP-55, TGF $\beta$ -1 anti-apoptotic factor (Toomey et al., Br. J. Biomed. Sci. 58(3):177-183, 2001), tumor protein D52 (Bryne et al., Genomics 35:523-532, 1996), H1FT, NY-BR-1 (WO 01/47959), NY-BR-62, NY-BR-75, NY-BR-85, NY-BR-87, and NY-BR-96 (Scanlan, M. Serologic and Bioinformatic Approaches to the Identification of Human Tumor Antigens, in *Cancer Vaccines 2000*, Cancer Research Institute, New York, NY), and/or pancreatic cancer antigens (e.g., SEQ ID NOs: 1-288 of U.S. Patent No. 7,473,531). Immunogens may also be derived from or direct the immune response against include TAs not listed above but available to one of skill in the art.

In addition to the specific immunogen sequences listed above, the invention also includes the use of analogs of the sequences. Such analogs include sequences that are, for example, at least 80%, 90%, 95%, or 99% identical to the reference sequences, or fragments thereof. The analogs also include fragments of the reference sequences that

include, for example, one or more immunogenic epitopes of the sequences. Further, the analogs include truncations or expansions of the sequences (e.g., insertion of additional/repeat immunodominant/helper epitopes) by, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11-20, etc., amino acids on either or both ends. Truncation may remove immunologically unimportant or interfering sequences, e.g., within known structural/immunologic domains, or between domains; or whole undesired domains can be deleted; such modifications can be in the ranges 21-30, 31-50, 51-100, 101-400, etc. amino acids. The ranges also include, e.g., 20-400, 30-100, and 50-100 amino acids.

#### 10 Cocktails

The invention also includes compositions including mixtures of two or more PIVs and/or PIV vectors, as described herein. As discussed above, use of such mixtures or cocktails may be particularly advantageous when induction of immunity to more than one immunogen and/or pathogen is desired. This may be useful, for example, in vaccination against different flaviviruses that may be endemic to the region in which the vaccine recipient resides. This may also be useful in the context of administration of multiple immunogens against the same target.

Non-limiting examples of PIV cocktails included in the invention are those including PIV-JE + PIV-DEN, and PIV-YF + PIV-DEN. In both of these examples, the PIVs for either or both components can be single or dual component PIVs, as described above. In addition, in the case of the PIV-DEN, the PIV can include sequences of just one dengue serotype selected from the group consisting of dengue serotypes 1-4, or the cocktail can include PIVs expressing sequences from two, three, or all four of the serotypes. Further, the TBE/*Borrelia burgdorferi*/tick saliva protein (e.g., 64TRP, Isac, Salp20) vaccines described herein can be based on including the different immunogens within a single PIV or live attenuated flavivirus, or can be based on mixtures of PIVs (or LAVs), which each include one or more of the immunogens. The cocktails of the invention can be formulated as such or can be mixed just prior to administration.

### Use, Formulation, and Administration

The invention includes the PIV vectors, PIVs, LAV vectors, and LAVs, as well as corresponding nucleic acid molecules, pharmaceutical or vaccine compositions, and methods of their use and preparation. The PIV vectors, PIVs, LAV vectors, and LAVs of the invention can be used, for example, in vaccination methods to induce an immune response to TBE or other flavivirus, and/or another expressed immunogen, as described herein. These methods can be prophylactic, in which case they are carried out on subjects (e.g., human subjects or other mammalian subjects) not having, but at risk of developing infection or disease caused by TBE or another flavivirus and/or a pathogen from which the other expressed immunogen is derived. The methods can also be therapeutic, in which they are carried out on subjects already having an infection by one or more of the relevant pathogens. Further, the viruses and vectors can be used individually or in combination with one another or other vaccines. The subjects treated according to the methods of the invention include humans, as well as non-human mammals (e.g., livestock, such as, cattle, pigs, horses, sheep, and goats, and domestic animals, including dogs and cats).

Formulation of the PIV vectors, PIVs, LAV vectors, and LAVs of the invention can be carried out using methods that are standard in the art. Numerous pharmaceutically acceptable solutions for use in vaccine preparation are well known and can readily be adapted for use in the present invention by those of skill in this art (see, e.g., *Remington's Pharmaceutical Sciences* (18<sup>th</sup> edition), ed. A. Gennaro, 1990, Mack Publishing Co., Easton, PA). In two specific examples, the PIV vectors, PIVs, LAV vectors, and LAVs are formulated in Minimum Essential Medium Earle's Salt (MEME) containing 7.5% lactose and 2.5% human serum albumin or MEME containing 10% sorbitol. However, the PIV vectors, PIVs, LAV vectors, and LAVs can simply be diluted in a physiologically acceptable solution, such as sterile saline or sterile buffered saline.

The PIV vectors, PIVs, LAV vectors, and LAVs of the invention can be administered using methods that are well known in the art, and appropriate amounts of the viruses and vectors to be administered can readily be determined by those of skill in the art. What is determined to be an appropriate amount of virus to administer can be

determined by consideration of factors such as, e.g., the size and general health of the subject to whom the virus is to be administered. For example, in the case of live, attenuated viruses of the invention, the viruses can be formulated as sterile aqueous solutions containing between  $10^2$  and  $10^8$ , e.g.,  $10^3$  to  $10^7$ , infectious units (e.g., plaque-forming units or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml. PIVs  
5 can be administered at similar doses and in similar volumes; PIV titers however are usually measured in, e.g., focus-forming units determined by immunostaining of foci, as these defective constructs tend not to form virus-like plaques. Doses can range between  $10^2$  and  $10^8$  FFU and administered in volumes of 0.1 to 1.0 ml.

10 All viruses and vectors of the invention can be administered by, for example, intradermal, subcutaneous, intramuscular, intraperitoneal, or oral routes. In specific examples, dendritic cells are targeted by intradermal or transcutaneous administration, by use of, for example, microneedles or microabrasion devices. Further, the vaccines of the invention can be administered in a single dose or, optionally, administration can involve  
15 the use of a priming dose followed by a booster dose that is administered, e.g., 2-6 months later, as determined to be appropriate by those of skill in the art. Optionally, PIV vaccines can be administered via DNA or RNA immunization using methods known to those skilled in the art (Chang et al., Nat. Biotechnol. 26:571-577, 2008; Kofler et al., Proc. Natl. Acad. Sci. U.S.A. 101:1951-1956, 2004).

20 Optionally, adjuvants that are known to those skilled in the art can be used in the administration of the viruses and vectors of the invention. Adjuvants that can be used to enhance the immunogenicity of the viruses include, for example, liposomal formulations, synthetic adjuvants, such as (e.g., QS21), muramyl dipeptide, monophosphoryl lipid A, polyphosphazine, CpG oligonucleotides, or other molecules that appear to work by  
25 activating Toll-like Receptor (TLR) molecules on the surface of cells or on nuclear membranes within cells. Although these adjuvants are typically used to enhance immune responses to inactivated vaccines, they can also be used with live or replication-defective vaccines. Both agonists of TLRs or antagonists may be useful in the case of live or replication-defective vaccines. The vaccine candidates can be designed to express TLR  
30 agonists. In the case of a virus delivered via a mucosal route, for example, orally,

mucosal adjuvants such as the heat-labile toxin of *E. coli* (LT) or mutant derivations of LT can be used as adjuvants. In addition, genes encoding cytokines that have adjuvant activities can be inserted into the vaccine candidates. Thus, genes encoding desired cytokines, such as GM-CSF, IL-2, IL-12, IL-13, IL-5, etc., can be inserted together with  
5 foreign immunogen genes to produce a vaccine that results in enhanced immune responses, or to modulate immunity directed more specifically towards cellular, humoral, or mucosal responses (e.g., reviewed in “Immunopotentiators in Modern Vaccines”, Schijns and O’Hagan Eds., 2006, Elsevier Academic Press: Amsterdam, Boston, etc.). Optionally, a patch containing a layer of an appropriate toxin-derived adjuvant, can be  
10 applied over the injection site. Toxin promotes local inflammation attracting lymphocytes, which leads to a more robust immune response.

### Examples

Additional details concerning the invention are provided in the Examples, below.  
15 In the Examples, experiments are described in which PIVs based on WN, JE, and YF viruses (see, e.g., WO 2007/098267 and WO 2008/137163) were tested. Firstly, we demonstrated that the constructs are significantly more attenuated in a sensitive suckling mouse neurovirulence model (zero mortality at all tested doses) as compared to available LAV controls (YF 17D, YF/JE LAV, and YF/WN LAV). We demonstrated for the first  
20 time that d-PIV constructs were avirulent in this model and thus that two-component PIVs do not undergo uncontrolled (unlimited) spread in vivo and cannot cause clinical signs. Secondly, we performed comparisons of the immunogenicity and efficacy of the PIVs and the LAVs, and demonstrated that PIV vaccines can induce immune response comparable to LAVs and be equally efficacious (e.g., as observed for PIV-WN and  
25 YF/WN LAV pair of vaccines). In one pair examined, YF 17D LAV was significantly more immunogenic than PIV-YF. Thus, production of VLPs can vary between different, similarly designed PIV constructs. Specifically, we propose that PIV-YF does not generate a large amount of YF VLPs compared to PIV-WN (WN VLPs), and that increased production of VLPs can be achieved by genetic modifications at the C/prM  
30 junction in suboptimal PIV constructs. Specifically, the C/prM junction is an important

location in the flavivirus polyprotein orchestrating the formation of viral envelope and synthesis of viral proteins (Yamshchikov and Compans, *Virology* 192:38-51, 1993; Amberg and Rice, *J. Virol.* 73:8083-8094, 1999; Stocks and Lobigs, *J. Virol.* 72:2141-2149, 1998). We propose that secretion of VLPs in PIV infected cells (in contrast to  
5 production of viral particles in whole viruses) can be increased by uncoupling of the viral protease and signalase cleavages at the junction, or use of a strong heterologous signal peptide (tPA, etc.) in place of the signal for prM, or by mutagenesis of the signal for prM. The efficiency of signalase cleavage at the C/prM junction of flaviviruses is low (Stocks and Lobigs, *J. Virol.* 72:2141-2149, 1998), e.g., as predicted by SignalP 3.0 on-line  
10 program. It is expected that more efficient cleavage efficiency can be achieved by analysis of specific amino acid substitutions near the cleavage site with SignalP 3.0 (e.g., as described in application WO 2008/100464), followed by incorporation of chosen mutation(s) into PIV genomes, recovery of PIV progeny and measuring VLP secretion. Non-flavivirus signals are inserted by methods standard in the art. Uncoupling between  
15 the viral protease and signalase cleavages can be achieved by ablating the viral cleavage site by any non-conservative mutation (e.g., RRS in YF17D C to RRA or GRS or RSS, etc.), or deletion of the entire site or some of its 3 residues. If necessary, formation of free N-terminus of the signal of foreign protein can be achieved by using such elements as autoprotease, or termination codon followed by an IRES. Alternatively, the native AUG  
20 initiation codon of C can be ablated (in constructs where C protein sequence is unnecessary, e.g.,  $\Delta$ C PIV) and AUG placed in front of foreign gene. Optimization of vector signal can be performed by random mutagenesis, e.g., by insertion of synthetic randomized sequence followed by identification of viable PIV variants with increased VLP secretion.

25 We also discovered that PIV constructs were substantially more immunogenic in hamsters when administered by the IP route, as compared to the subcutaneous route. We concluded that this was most likely due to better targeting of antigen presenting cells in lymphoid tissues, which are abundant in the abdomen, but not abundant in tissues underlying the skin. Based on these observations, we concluded that efficient targeting of  
30 PIVs to dendritic cells, abundant in the skin, can be achieved by cutaneous inoculation,

e.g., via skin microabrasion or intradermal injection using microneedles (Dean et al., Hum Vaccin. 1:106-111, 2005).

Further, we have carried out experiments to show the feasibility of administering mixtures, or cocktails, of different PIVs, such as those described herein (e.g., JE+DEN  
5 and YF+DEN). In order to administer cocktails, it is important to verify that there is no interference between co-administered components, and that a balanced immune response is induced. Several PIV mixtures were used to immunize rodents and immune responses were compared to PIV constructs administered individually. No interference was observed in mixtures, and thus cocktail PIV vaccines are feasible. Such formulations  
10 may be of particular significance in geographical regions where different flaviviruses co-circulate. This could be also used to simultaneously administer several PIV-based vaccines against non-flavivirus pathogens.

Further, we have demonstrated that no neutralizing antibody response is induced against packaging envelope after at least two doses of PIV (and thus antibodies are  
15 elicited against VLPs secreted from infected cells). This was demonstrated using the helper ( $\Delta$ prM-E) component of a d-PIV (see in Fig. 2) packaged individually, or by measuring neutralizing antibodies to heterologous packaging envelopes (e.g., to the WN envelope used to package PIV-JE in helper cells providing WN-specific C-prM-E proteins in trans). The latter observations support sequential use of different PIV  
20 vaccines manufactured in a universal helper packaging cells line, and sequential use of different recombinant PIV-vectored vaccines in the same individual, as discussed above. In addition, we confirmed previous observations that PIV constructs can be stably propagated to high yields in vitro, and that no recombination restoring whole virus occurs after prolonged passaging in substrate cells (Mason et al., Virology 351:432-443, 2006;  
25 Shustov et al., J. Virol. 21:11737-11748, 2007).

These and other aspects of the invention are further described in the Examples, below.

### Example 1. Pseudoinfectious virus platform development studies

#### *Attenuation in suckling mouse neurovirulence (NV) model*

Materials used in the studies described below are described in Table 1 and the  
5 references cited therein. These include s-PIV-WN (based on wt WN virus strain NY99  
sequences), s-PIV-JE, s-PIV-WN/JE (based on wt WN virus backbone and prM-E genes  
from wt JE virus Nakayama strain), s-PIV-YF/WN (YF 17D backbone and prM-E genes  
from WN virus), and s-PIV-YF (based on YF 17D sequences). Additional materials  
include d-PIV-YF (YF d-PIV, grown in regular BHK cells (Shustov et al., J. Virol.  
10 21:11737-11748, 2007), and two-component d-PIV-WN (grown in regular Vero cells;  
Suzuki et al., J. Virol. 82:6942-6951, 2008).

Attenuation of these PIV prototypes was compared to LAVs YF 17D, a chimeric  
YF/JE virus, and a chimeric YF/WN virus in suckling mouse NV test (IC inoculation)  
using highly susceptible 5-day old ICR mice (the chimeric viruses include yellow fever  
15 capsid and non-structural sequences, and JE or WN prM-E sequences). None of the  
animals that received PIV constructs showed clinical signs or died, while mortality was  
observed in animals inoculated with LAVs (Table 2). The YF 17D virus is neurovirulent  
for mice of all ages, while the chimeric vaccines are not neurovirulent for adult mice, but  
can cause dose-dependent mortality in more sensitive suckling mice (Guirakhoo et al.,  
20 Virology 257:363-372, 1999; Arroyo et al., J. Virol. 78:12497-12507, 2004).  
Accordingly, 90%-100% of suckling mice that received doses as low as 1 PFU of YF 17D  
died. YF/JE and YF/WN LAVs caused partial mortality at much higher doses ( $> 2 \log_{10}$   
PFU and  $3 \log_{10}$  PFU, respectively), with longer average survival time (AST) of animals  
that died, as expected. Thus, PIV constructs are completely avirulent in this sensitive  
25 model (at least 20,000 - 200,000 times less neurovirulent than the licensed YF 17D  
vaccine).

The YF d-PIV and WN d-PIV caused no mortality or clinical signs. Thus, the  
two-component PIV variants that theoretically could spread within brain tissue from cells  
co-infected by both of their components did not cause disease. Moreover, we tried to  
30 detect the d-PIVs in the brains of additional animals in this experiment, sacrificed on day  
6 post-inoculation by titration, and detected none (brain tissues from 10 and 11 mice that

received 4 log<sub>10</sub> FFU of YF d-PIV and WN d-PIV, respectively, were homogenized and used for titration). Thus, the d-PIVs did not cause spreading infection characteristic of whole virus. YF/JE LAV has been shown to replicate in the brain of adult ICR mice inoculated by the IC route with a peak titer of ~ 6 log<sub>10</sub> PFU/g on day 6, albeit without clinical signs (Guirakhoo et al., Virology 257:363-372, 1999). Co-infection of cells with components of a d-PIV is clearly a less efficient process than infection with whole virus. The data show that d-PIV replication in vivo is quickly brought under control by innate immune responses (and adaptive responses in older animals).

#### 10 *Immunogenicity/efficacy in mice and hamsters*

Immunogenicity/efficacy of the PIV prototypes described above was compared to that of chimeric LAV counterparts and YF 17D in mice and Syrian hamsters. The general experiment design is illustrated in Fig. 3 (mice, IP immunization). Experiments in hamsters were performed similarly (plus-minus a few days, SC or IP inoculation with doses indicated below). 3.5-week old ICR mice (for s-PIV-WN and -YF, YF/WN LAV, and YF 17D groups) or C57/BL6 mice (for s-PIV-JE and YF/JE LAV groups) were immunized IP with graded doses of PIV constructs (4-6 log<sub>10</sub> FFU/dose) or chimeric LAV and YF 17D LAV controls (4 log<sub>10</sub> PFU). Select PIV-WN, -JE and -YF groups were boosted on day 21 with 5 log<sub>10</sub> FFU of corresponding constructs (Table 3).

Neutralizing antibody responses were determined in animal sera by standard PRNT<sub>50</sub> against YF/WN or /JE LAVs, or YF 17D viruses. PIV-WN induced very high WN-specific neutralizing antibody responses in all groups, with or without boost, as evidenced by PRNT<sub>50</sub> titers determined in pools of sera from immunized animals on days 20 and 34, which was comparable to that in the YF/WN LAV control group. Accordingly, animals immunized with both PIV-WN and YF/WN LAV were protected from lethal challenge on day 35 with wt WN virus (IP, 270 LD<sub>50</sub>), but not mock-immunized animals (Table 3).

When WN neutralizing antibodies were measured in sera from individual mice, high uniformity of immune responses was observed (Fig. 4). Thus, single-round PIV vaccines can be as immunogenic and efficacious as corresponding LAVs. PIV-JE was also highly immunogenic (black mice), while immunogenicity of PIV-YF was significantly lower

compared to the YF 17D control (ICR mice). Yet, dose-dependent protection of PIV-YF immunized animals (but not mock-immunized animals) was observed following a severe lethal IC challenge with wt YF strain Asibi virus (500 LD<sub>50</sub>) (Table 3), which is in agreement with the knowledge that neutralizing antibody titers as low 1:10 are protective against flavivirus infections.

The YF 17D control virus was highly immunogenic (e.g., PRNT<sub>50</sub> titer 1:1,280 on day 34), and thus it is able to infect cells and replicate efficiently *in vivo*, and its envelope is a strong immunogen. Therefore, it is unlikely that low immunogenicity of PIV-YF was due to its inability to infect cells or replicate efficiently in infected cells *in vivo*. We believe that the low immunogenicity of PIV-YF (e.g., compared to PIV-WN) was most likely due to a low-level production of YF-specific VLPs in PIV-YF infected cells (while VLP secretion is high in PIV-WN infected cells). As discussed above, we propose that immunogenicity of PIV-YF can be significantly increased, e.g., by appropriate modifications at the C/prM junction, e.g., by uncoupling the two protease cleavages that occur at this junction (viral protease and signalase cleavages), and/or by using a strong heterologous signal [e.g., rabies virus G protein signal, or eukaryotic tissue plasminogen activator (tPA) signal (Malin et al., *Microbes and Infection*, 2:1677-1685, 2000), etc.] in place of the YF signal for prM.

A similar experiment was performed in ~4.5-week old Syrian hamsters, to compare immunogenicity of PIV constructs to LAV controls in this model. Animals were immunized SC with graded doses of the test articles (Table 4). PIV-WN was highly immunogenic, e.g., WN-specific PRNT<sub>50</sub> titers on day 38 (pre-challenge) were 1:320, 1:640, and 1:1280 in groups that received 5, 6, and 6 (prime)+5 (boost) log<sub>10</sub> FFU doses, respectively. This was somewhat lower compared to YF/WN LAV 4 log<sub>10</sub> PFU control (≥ 1:2560). PIV-JE and -YF induced detectable specific neutralizing antibody responses, albeit with lower titers compared to YF/JE LAV and YF 17D controls. All animals immunized with PIV-WN and YF/WN were solidly protected from lethal challenge with wt WN virus as evidenced by the absence of mortality and morbidity (e.g., loss of body weight after challenge), as well as absence or a significant reduction of postchallenge WN virus viremia. Mock-immunized animals were not protected (Table 4). PIV-JE and -WN

protected animals from respective challenge in dose-dependent fashion. Protective efficacy in this experiment is additionally illustrated in Fig. 5. For example, high post-challenge YF virus (hamster adapted Asibi strain) viremia was observed in mock immunized animals, peaking on day 3 at a titer of  $> 8 \log_{10}$  PFU/ml (upper left panel); all of the animals lost weight, and 1 out of 4 died (upper right panel). In contrast, viremia was significantly reduced or absent in hamsters immunized with PIV-YF (two doses; despite relatively low neutralizing titers) or YF 17D; none of these animals lost weight. Similarly, animals immunized with PIV-WN or YF/WN LAV were significantly or completely protected in terms of post-challenge WN virus viremia and body weight loss/mortality, in contrast to mock controls (compare in bottom panels). Thus, high immunogenicity/efficacy of PIV was demonstrated in a second animal model.

In another hamster experiment, animals were immunized with PIV constructs by the IP route, with two doses. Table 5 compares neutralizing immune responses (specific for each vaccine) determined in pooled sera of hamsters in the above-described experiment (SC inoculation) to those after IP immunization, for PIV-WN, - YF/WN, - WN/JE, and -YF after the first dose (days 20-21) and second dose (days 34-38). A clear effect of the immunization route was observed both after the 1<sup>st</sup> and 2<sup>nd</sup> doses. For instance, for PIV-WN after 1<sup>st</sup> dose, SC immunization resulted in WN-specific PRNT50 titer of 1:40, while IP inoculation resulted in much higher titer 1:320 (and after the 2<sup>nd</sup> dose, titers were similar). A more pronounced effect was observed for other constructs after both the 1<sup>st</sup> and 2<sup>nd</sup> doses. Interestingly, PIV-YF/WN was very highly immunogenic by IP route (titer 1:320 after 1<sup>st</sup> IP dose vs. 1:20 by SC, and 1:1,280 after 2<sup>nd</sup> dose vs. 1:160 by SC). Similarly, immunogenicity of PIV-JE was significantly increased (e.g., JE-specific titer of 1:640 after two IP doses). Thus, better targeting of lymphoid cells, specifically antigen-presenting cells (which are more abundant in the abdomen as opposed to tissues under the skin), is an important consideration for use of PIV vaccines. In humans, efficient targeting of dendritic cells of the skin, increasing the magnitude of immune response, can be achieved by intradermal delivery, which we thus propose for a route for PIV immunization of humans.

In the above-described experiments, we also determined whether a neutralizing

antibody response was induced against packaging envelopes (as opposed to response to VLPs encoded by PIV constructs and secreted by infected cells). No WN-specific neutralizing antibodies were detected by PRNT<sub>50</sub> in animals immunized with 5 log<sub>10</sub> FFU of the second component of WN d-PIV, containing the ΔC-prM-E deletion and thus not encoding VLPs, but packaged into the WN envelope in BHK-CprME(WN) helper cells, and no YF-specific neutralizing activity was found in sera from animals immunized with 4 log<sub>10</sub> FFU of the second component of YF d-PIV packaged in YF envelope. No YF-specific neutralizing response was induced by two doses of PIV-YF/WN packaged into YF envelope, and similarly, no WN-specific response was induced by two doses of PIV-JE packaged into WN envelope. The absence of neutralizing response against packaging envelopes permits manufacturing different PIV vaccines in one (universal) manufacturing helper cell line, or immunization of one individual with different recombinant vaccines based on the same vector, according to the present invention.

#### 15 *PIV cocktails*

Because PIVs undergo a single (optionally several, but limited) round(s) of replication in vivo, we considered that mixtures of different PIV vaccines can be administered without interference between individual constructs in the mixture (cocktail). To elucidate whether PIV vaccines can be used in cocktail formulations, immune responses in mice and hamsters to several PIV constructs given as mixtures were compared to the same constructs given individually. Similar results were obtained in both animal models. Results of mouse experiments are shown in Table 6. Similar anti-JE neutralizing antibody titers were observed in pools of sera from animals that were given one or two doses of either PIV-JE + PIV-WN mixture or PIV-JE alone (1:20 vs. 1:80 and 1:640 vs. 1:160, for one and two doses, respectively). Similarly, WN-specific titers against PIV-JE + PIV-WN mixture and PIV-WN alone were similar (1:320 vs. 1:640 and 1:5,120 vs. 1:5,120 for one and 2 doses, respectively). No or little cross-specific response was induced by either PIV-JE or -WN. The result was also confirmed by measuring PRNT<sub>50</sub> titers in sera from individual animals. Thus, it is clear that PIV vaccines can be efficiently administered as cocktails, inducing immunity against two or

more flavivirus pathogens. In addition, as discussed above, various cocktails can be made between non-flavivirus PIV vaccines, or between any of flavivirus and non-flavivirus PIV vaccines.

#### 5 *In vitro studies*

Different PIV prototypes were serially passaged up to 10 times in helper BHK cells, for s-PIVs, or in regular Vero cells, for d-PIVs. Samples harvested after each passage were titrated in Vero cells by immunostaining. Constructs grew to high titers, and no recombination restoring whole virus was observed. For instance, PIV-WN  
10 consistently grew to titers 7-8 log<sub>10</sub> FFU/ml in BHK-CprME(WN) helper cells (containing a VEE replicon expressing the WN virus C-prM-E proteins), and WN d-PIV grew to titers exceeding 8 log<sub>10</sub> FFU/ml in Vero cells, without recombination.

#### **Example 2. PIV-TBE**

15 PIV-TBE vaccine candidates can be assembled based entirely on sequences from wt TBE virus or the closely serologically related Langat (LGT) virus (naturally attenuated virus, e.g., wt strain TP-21 or its empirically attenuated variant, strain E5), or based on chimeric sequences containing the backbone (capsid and non-structural sequences) from YF 17D or other flaviviruses, such as WN virus, and the prM-E envelope protein genes  
20 from TBE, LGT, or other serologically related flaviviruses from the TBE serocomplex. YF/TBE LAV candidates are constructed based on the backbone from YF 17D and the prM-E genes from TBE or related viruses (e.g., the E5 strain of LGT), similar to other chimeric LAV vaccines.

Construction of PIV-TBE and YF/TBE LAV vaccine prototypes was performed  
25 by cloning of appropriate genetic elements into plasmids for PIV-WN (Mason et al., Virology 351:432-443, 2006; Suzuki et al., J. Virol. 82:6942-6951, 2008), or plasmids for chimeric LAVs (e.g., pBSA-AR1, a single-plasmid version of infectious clone of YF/JE LAV; WO 2008/036146), respectively, using standard methods in the art of reverse genetics. The prM-E sequences of TBE virus strain Hypr (GenBank accession number  
30 U39292) and LGT strain E5 (GenBank accession number AF253420) were first computer

codon-optimized to conform to the preferential codon usage in the human genome, and to eliminate nucleotide sequence repeats longer than 8 nt to ensure high genetic stability of inserts (if determined to be necessary, further shortening of nt sequence repeats can be performed). The genes were chemically synthesized and cloned into plasmids for PIV-  
5 WN and YF/JE LAV, in place of corresponding prM-E genes. Resulting plasmids were in vitro transcribed and appropriate cells (Vero for chimeric viruses, and helper BHK cells for PIV) were transfected with RNA transcripts to generate virus/PIV samples.

#### *YF/TBE LAV constructs*

10 In YF/TBE constructs containing either the TBE Hypr (plasmids p42, p45, and p59) or LGT E5 (plasmid P43) prM-E genes, two different types of the C/prM junction were first examined (see in Fig. 6; C/prM junctions only are shown in Sequence Appendix 1, and complete 5'-terminal sequences covering the 5'UTR-C-prM-E-  
beginning of NS1 region are shown in Sequence Appendix 2). The p42-derived  
15 YF17D/Hypr chimera contained a hybrid YF17D/Hypr signal peptide for the prM protein, while the p45-derived YF17D/Hypr chimera contained a hybrid YF17D/WN signal peptide for prM (Sequence Appendix 1). The former chimeric virus produced very high titers at both P0 (immediately after transfection) and P1 (the next passage in Vero cells), up to 7.9 log<sub>10</sub> PFU/ml, which were 0.5 log<sub>10</sub> times higher, compared to the latter virus; in  
20 addition it formed significantly larger plaques in Vero cells (Fig. 6). Thus, use of TBE-specific residues in the signal peptide for prM conferred a significant growth advantage over the signal containing WN-specific residues. The p43-derived YF17D/LGT chimera had the same prM signal as the p42-derived virus; it also produced very high titers at P0 and P1 passages (up to 8.1 log<sub>10</sub> PFU/ml) and formed large plaques. A derivative of the  
25 p42-derived virus was also produced from plasmid p59, which contained a strong attenuating mutation characterized previously in the context of a YF/WN LAV vaccine virus, specifically, a 3-a.a. deletion in the YF17D-specific C protein (PSR, residues 40-42 in the beginning of  $\alpha$ -Helix I; WO 2006/116182). As expected, the p59 virus grew to lower titers (5.6 and 6.5 log<sub>10</sub> PFU/ml at P0 and P1, respectively), and formed small  
30 plaques (determined in a separate titration experiment and thus not shown in Fig. 6),

compared to the parent p42-derived chimera. These initial observations of growth properties of YF/TBE LAV prototypes, and correlation of replication in vitro with plaque morphologies, have been confirmed in growth curve experiments (Fig. 8).

#### 5 *PIV-TBE constructs*

PIV-WN/TBE variants were constructed, and packaged PIV samples were derived from plasmids p39 and p40 (Fig. 7; Sequence Appendix 1 for C/prM junction sequences, and Sequence Appendix 3 for complete 5'UTR-ΔC-prM-E-beginning of NS1 sequences). These contained complete Hypr or WN prM signals, respectively. Both PIVs were  
10 successfully recovered and propagated in BHK-CprME(WN) or BHK-C(WN) helper cells (Mason et al., Virology 351:432-443, 2006; Widman et al., Vaccine 26:2762-2771, 2008). The P0 and P1 sample titers of the p39 variant were 0.2 – 1.0 log<sub>10</sub> times, higher than p40 variant. In addition, Vero cells infected with p39 variant were stained brighter in immunofluorescence assay using a polyclonal TBE-specific antibody, compared to  
15 p40, indicative of more efficient replication (Fig. 7). The higher rate of replication of the p39 candidate than p40 candidate was confirmed in a growth curve experiment (Fig. 8). In the latter experiment, both candidates appeared to grow better in the BHK-C(WN) helper cells compared to BHK-CprME(WN), with the p39 variant reaching titer of ~ 7 log<sub>10</sub> PFU/ml on day 5 (note that peak titers have not been reached). The discovery of the  
20 effect of prM signal on replication rates of both PIV and chimeric LAV vaccine candidates, and head-to-head comparison of different signals to generate the most efficiently replicating and immunogenic (see above) construct, are a distinguishing feature of our approach. As discussed above, the invention also includes the use of other flavivirus signals, including with appropriate mutations, the uncoupling the viral protease and signalase cleavages at the C/prM junction, e.g., by mutating or deleting the viral  
25 protease cleavage site at the C-terminus of C preceding the prM signal, the use of strong non-flavivirus signals (e.g., tPA signal, etc.) in place of prM signal, as well as optimization of sequences downstream from the signalase cleavage site.

Other PIV-TBE variants based entirely on wt TBE (Hypr strain) and LGT virus  
30 (TP21 wild type strain or attenuated E5 strain), and chimeric YF 17D backbone/prM-E

(TBE or LGT) sequences are also included in the invention. Helper cells providing appropriate C, C-prM-E, etc., proteins (e.g., TBE-specific) for trans-complementation can be constructed by means of stable DNA transfection or through the use of an appropriate vector, e.g., an alphavirus replicon, such as based on VEE strain TC-83, with antibiotic  
5 selection of replicon-containing cells. Vero and BHK21 cells can be used in practice of the invention. The former are an approved substrate for human vaccine manufacture; any other cell line acceptable for human and/or veterinary vaccine manufacturing can be also used. In addition to s-PIV constructs, d-PIV constructs can also be assembled. To additionally ascertain safety for vaccinees and the environment, appropriate modifications  
10 can be employed, including the use of degenerate codons and complementary mutations in the 5' and 3' CS elements, to minimize chances of recombination that theoretically could result in viable virus. Following construction, all vaccine candidates can be evaluated in vitro for manufacturability/stability, and in vivo for attenuation and immunogenicity/efficacy, in available pre-clinical animal models, such as those used in  
15 development and quality control of TBE and YF vaccines.

*Neurovirulence and neuroinvasiveness in mice of PIV-TBE and YF/TBE LAV constructs*

Young adult ICR mice (~ 3.5 week-old), were inoculated with graded doses of PIV-TBE and YF/TBE LAV candidates by the IC route to measure neurovirulence, or IP  
20 route to measure neuroinvasiveness (and later immunogenicity/efficacy). Animals that received 5 log<sub>10</sub> FFU of PIV-Hypr (p39 and p40) variants by both routes survived and showed no signs of sickness, similar to mock-inoculated animals (Table 7), and thus PIV-TBE vaccines are completely avirulent. Mice inoculated IC with YF 17D control (1 – 3 log<sub>10</sub> PFU) showed dose-dependent mortality, while all animals inoculated IP (5 log<sub>10</sub>  
25 PFU) survived, in accord with the knowledge that YF 17D virus is not neuroinvasive. All animals that received graded IC doses (2 – 4 log<sub>10</sub> PFU) of YF/TBE LAV prototypes p42, p45, p43, and p59 died (moribund animals were humanely euthanized). These variants appear to be less attenuated than YF 17D, e.g., as evidenced by complete mortality and shorter AST at the 2 log<sub>10</sub> PFU dose, the lowest dose tested for YF/TBE LAV candidates.  
30 The non-neurovirulent phenotype of PIV-TBE, virulent phenotype of YF/TBE LAV and

intermediate-virulence phenotype of YF 17D are also illustrated in Fig. 9, showing survival curves of mice after IC inoculation. It should be noted that the p43 (LGT prM-E genes) and p59 (the dC2 deletion variant of YF/Hypr LAV) were less neurovirulent than p42 and p45 YF/Hypr LAV constructs as evidenced by larger AST values for corresponding doses (Table 7). In addition, p43 and p59 candidates were non-neuroinvasive, while p42 and p45 caused partial mortality after IP inoculation (5 log<sub>10</sub> PFU/dose) (Table 7; Fig. 10). It should be noted however that all the YF/TBE LAV constructs were significantly attenuated as compared to wt TBE viruses, e.g., compared to wt TBE Hypr virus, which is uniformly highly virulent for mice, both at very low IC (LD<sub>50</sub> ~ 0.1 PFU) and IP (LD<sub>50</sub> ≤ 10 PFU) doses (Wallner et al., J. Gen. Virol. 77:1035-1042, 1996; Mandl et al., J. Virol. 72:2132-2140, 1998; Mandl et al., J. Gen. Virol. 78:1049-1057, 1997).

#### *Immunogenicity/efficacy of PIV-TBE and YF/TBE LAV constructs in mice*

TBE-specific neutralizing antibody responses in mice immunized IP with one or two doses of the PIV-TBE or YF/TBE LAV variants described above, or a human formalin-inactivated TBE vaccine control (1:30 of human dose) are being measured. Animals have been challenged with a high IP dose (500 PFU) of wt Hypr TBE virus; morbidity (e.g., weight loss), and mortality after challenge are monitored.

#### *Immunogenicity/efficacy of PIV-TBE and YF/TBE LAV constructs in mice*

TBE-specific neutralizing antibody responses in mice immunized IP with one or two doses of the PIV-TBE or YF/TBE LAV variants described above (from experiment in Table 7), or a human formalin-inactivated TBE vaccine control (1:20 of human dose; one or two doses), or YF 17D and mock controls, were measured on day 20 by PRNT<sub>50</sub> against wt TBE Hypr virus (Table 8; second dose of indicated test articles was given on day 14). [Titers were determined in individual sera, or pooled sera from two animals in most cases, or pooled sera from 4 animals for the YF17D and Mock negative controls]. Titers in individual test samples as well as GMTs for each group are provided in Table 8. Titers in test samples were similar within each group, e.g., in groups immunized with

PIVs, indicating high uniformity of immune response in animals. As expected, no TBE-specific neutralizing antibodies were detected in negative control groups (YF 17D and Mock; GMTs < 1:10); accordingly, animals in these groups were not protected from challenge on day 21 post-immunization with a high IP dose (500 PFU) of wt Hypr TBE virus. Mortalities from partial observation (on day 9 post-challenge; observation being continued) are provided in Table 8, and dynamics of average post-challenge body weights indicative of morbidity are shown in Fig. 11. Neutralizing antibodies were detected in killed vaccine controls, which were particularly high after two doses (GMT 1:1,496); animals in the 2-dose group were completely protected in that there was no mortality or body weight loss (but not animals in the 1-dose group). Animals that received both one and two doses of PIV-Hypr p39 had very high antibody titers (GMTs 1:665 and 1:10,584) and were solidly protected, demonstrating that robust protective immunity can be induced by s-PIV-TBE defective vaccine. The two animals that survived immunization with YF/Hypr p42 chimera (see in Table 7) also had high antibody titers (GMT 1:6,085) and were protected (Table 8; Fig. 11). Interestingly, PIV-Hypr p40 and YF/Hypr p45 were poorly immunogenic (GMTs 1:15 and 1:153 for one and two doses, and 1:68, respectively). As discussed above, these contained WN-specific sequences in the signal for prM, while the highly immunogenic PIV-Hypr p39 and YF/Hypr p42 constructs contained TBE-specific signal sequences. In agreement with discussion above, this result demonstrates the importance of choosing the right prM signal, e.g., the TBE-specific signal, to achieve high-level replication/VLP secretion, which in this experiment in vivo resulted in drastically different immune responses. Immunogenicity of YF/LGT p43 and YF/Hypr dC2 p59 chimeras was relatively low which could be expected, because of the use of a heterologous envelope (LGT, different from challenge TBE virus) and high attenuating effect of the dC2 deletion, respectively.

### Example 3. Foreign gene expression

In the examples of recombinant PIV constructs described below, genes of interest were codon optimized (e.g., for efficient expression in a target vaccination host) and to eliminate long nt sequence repeats to increase insert stability ( $\geq 8$  nt long;

additional shortening of repeats can be performed if necessary), and then chemically synthesized. The genes were cloned into PIV-WN vector plasmids using standard methods of molecular biology well known in the art, and packaged PIVs were recovered following in vitro transcription and transfection of appropriate helper (for s-PIVs) or regular (for d-PIVs) cells.

#### *Expression of rabies virus G protein in WN s-PIV and d-PIV*

Rabies virus, *Rhabdoviridae* family, is a significant human and veterinary pathogen. Despite the availability of several (killed) vaccines, improved vaccines are still needed for both veterinary and human use (e.g. as an inexpensive pre-exposure prophylactic vaccines). Rabies virus glycoprotein G mediates entry of the virus into cells and is the main immunogen. It has been expressed in other vectors with the purpose of developing veterinary vaccines (e.g., Pastoret and Brochier, *Epidemiol. Infect.* 116:235-240, 1996; Li et al., *Virology* 356:147-154, 2006).

Full length rabies virus G protein (original Pasteur virus isolate, GenBank accession number NC\_001542) was codon-optimized, chemically synthesized, and inserted adjacent to the  $\Delta C$ ,  $\Delta prM-E$  and  $\Delta C-prM-E$  deletions in PIV-WN vectors (Fig. 12). The sequences of constructs are provided in Sequence Appendix 4. General designs of the constructs are illustrated in Fig. 13. The entire G protein containing its own signal peptide was inserted in-frame downstream from the WN C protein either with the  $\Delta C$  deletion ( $\Delta C$  and  $\Delta C-prM-E$  constructs) or without ( $\Delta prM-E$ ) and a few residues from the prM signal. Foot and mouth disease virus (FMDV) 2A autoprotease was placed downstream from the transmembrane C-terminal anchor of G to provide cleavage of C-terminus of G from the viral polyprotein during translation. The FMDV 2A element is followed by WN-specific signal for prM and prM-E-NS1-5 genes in the  $\Delta C$  construct, or signal for NS1 and NS1-5 genes in  $\Delta prM-E$  and  $\Delta C-prM-E$  constructs.

Packaged WN( $\Delta C$ )-rabiesG, WN( $\Delta prME$ )-rabiesG, and WN( $\Delta CprME$ )-rabiesG PIVs were produced by transfection of helper BHK cells complementing the PIV vector deletion [containing a Venezuelan equine encephalitis virus (strain TC-83) replicon expressing WN virus structural proteins for trans-complementation]. Efficient replication and expression of

rabies G protein was demonstrated for the three constructs by transfection/infection of BHK-C(WN) and/or BHK-C-prM-E(WN) helper cells, as well as regular BHK cells, by immunostaining and immunofluorescence assay (IFA) using anti-Rabies G monoclonal antibody (RabG-Mab) (Fig. 14). Titers were determined in Vero cells by immunostaining with the Mab or an anti-WN virus polyclonal antibody. Growth curves of the constructs in BHK-CprME(WN) cells after transfection with in vitro RNA transcripts are shown in Fig. 14, bottom panels. The PIVs grew efficiently to titers ~ 6 to >7 log<sub>10</sub> FFU/ml. Importantly, nearly identical titers were detected by both RabG-Mab and WN-antibody staining, which was the first evidence of genetic stability of the insert. In PIV-infected Vero cells, which were fixed but not permeabilized, strong membrane staining was observed by RabG-Mab staining, demonstrating that the product was efficiently delivered to the cell surface (Fig. 15). The latter is known to be the main prerequisite for high immunogenicity of expressed G. Individual packaged PIVs can spread following infection of helper BHK cells, but cannot spread in regular cells as illustrated for WN( $\Delta$ C)-rabiesG PIV in Fig. 16. The fact that there is no spread in naïve BHK cells demonstrates that the recombinant RNA genomes cannot be non-specifically packaged into membrane vesicles containing the G protein, if produced by PIV infected cells. An identical result was obtained with the G protein of another rhabdovirus, Vesicular stomatitis virus (VSV), contrary to previous observations of non-specific packaging of Semliki Forest virus (SFV) replicon expressing VSV G protein (Rolls et al., Cell 79:497- 506, 1994). The latter is a desired safety feature. [Alternatively, some non-specific packaging could result in a limited spread of PIV in vivo, potentially enhancing anti-rabies immune response. The latter could be also a beneficial feature, given that such PIV is demonstrated to be safe]. The stability of the rabies G insert in the three PIVs was demonstrated by serial passages in helper BHK-CprME(WN) cells at high or low MOI (0.1 or 0.001 FFU/cell). At each passage, cell supernatants were harvested and titrated in regular cells (e.g., Vero cells) using immunostaining with an anti-WN polyclonal antibody to determine total PIV titer, or anti-rabies G monoclonal antibody to determine titer of particles containing the G gene (illustrated for MOI 0.1 in Fig. 17; similar results were obtained at MOI 0.001). The WN( $\Delta$ C)-rabiesG PIV was stable for 5 passages, while the titer of insert-containing PIV started declining at passage 6, indicative of insert instability. This could be

expected, because in this construct, large G gene insert (~ 1500 nt) is combined with a small  $\Delta$ C deletion (~ 200 nt), significantly increasing the overall size of the recombinant RNA genome. In contrast, in WN( $\Delta$ prME)-rabiesG, and WN( $\Delta$ CprME)-rabiesG PIVs, the insert is combined with a much larger deletion (~ 2000 nt). Therefore, these constructs stably  
5 maintained the insert for all 10 passages examined (Fig. 17). Further, it can be seen in Fig. 17 that at some passages, titers as high as 8 log<sub>10</sub> FFU/ml, or higher, were attained for all three PIVs, additionally demonstrating that PIVs can be easily propagated to high yields.

Following inoculation in vivo individually, the WN( $\Delta$ C)-rabiesG s-PIV is expected to induce strong neutralizing antibody immune responses against both rabies and WN viruses,  
10 as well as T-cell responses. The WN( $\Delta$ prME)-rabiesG and WN( $\Delta$ CprME)-rabiesG PIVs will induce humoral immune response only against rabies because they do not encode the WN prM-E genes. WN( $\Delta$ C)-rabiesG s-PIV construct can be also co-inoculated with WN( $\Delta$ prME)-rabiesG construct in a d-PIV formulation (see in Fig. 12), increasing the dose of expressed G protein, and with enhanced immunity against both pathogens due to limited  
15 spread. As an example of spread, titration results in Vero cells of a s-PIV sample, WN( $\Delta$ prME)-rabiesG, and a d-PIV sample, WN( $\Delta$ prME)-rabiesG + WN( $\Delta$ C) PIV (the latter did not encode rabies G protein), are shown in Fig. 18. Infection of naïve Vero cells with s-PIV gave only individual cells stainable with RabG-Mab (or small clusters formed due to division of cells). In contrast, large foci were observed following infection with the d-PIV  
20 sample (Fig. 18, right panel) that were products of coinfection with the two PIV types.

The WN( $\Delta$ CprME)-rabiesG construct can be also used in a d-PIV formulation, if it is co-inoculated with a helper genome providing C-prM-E in trans (see in Fig. 12). For example it can be a WN virus genome containing a deletion of one of the NS proteins, e.g., NS1, NS3, or NS5, which are known to be trans-complementable (Khromykh et al., J. Virol.  
25 73:10272-10280, 1999; Khromykh et al., J. Virol. 74:3253-3263, 2000). We have constructed a WN- $\Delta$ NS1 genome (sequence provided in Sequence Appendix 4) and obtained evidence of co-infection with WN( $\Delta$ prME)-rabiesG or WN( $\Delta$ CprME)-rabiesG constructs, and spread in vitro, by immunostaining. In the case of such d-PIVs, rabies G protein can be also inserted and expressed in helper genome, e.g., WN- $\Delta$ NS1 genome, to increase the  
30 amount of expressed rabies G protein resulting in an increased anti-rabies immune response.

As with any dPIV versions, one immunogen can be from one pathogen (e.g., rabies G) and the other from a second pathogen, resulting in three antigenic specificities of vaccine. As discussed above,  $\Delta$ NS1 deletions can be replaced with or used in combination with  $\Delta$ NS3 and/or  $\Delta$ NS5 deletions/mutations, in other examples.

5

#### *Expression of RSV F protein in WN s-PIV and d-PIV*

Respiratory syncytial virus (RSV), member of *Paramyxoviridae* family, is the leading cause of severe respiratory tract disease in young children worldwide (Collins and Crowe, Respiratory Syncytial Virus and Metapneumovirus, In: Knipe et al. Eds., Fields Virology, 5<sup>th</sup> ed., Philadelphia: Wolters Kluwer/Lippincott Williams and Wilkins, 2007:1601-1646).  
10 Fusion protein F of the virus is a lead viral antigen for developing a safe and effective vaccine. To avoid post-vaccination exacerbation of RSV infection observed previously with a formalin-inactivated vaccine candidate, a balanced Th1/Th2 response to F is required which can be achieved by better TLR stimulation, a prerequisite for induction of high-affinity  
15 antibodies (Delgado et al., Nat. Med. 15:34-41, 2009), which should be achievable through delivering F in a robust virus-based vector. We have previously demonstrated the capacity of yellow fever virus-based chimeric LAV vectors to induce a strong, balanced Th1/Th2 response in vivo against an influenza antigen (WO 2008/036146). In the present invention, both yellow fever virus-based chimeric LAVs and PIV vectors are used for delivering RSV F  
20 to induce optimal immune response profile. Other LAVs and PIV vectors described herein can also be used for this purpose.

Full-length RSV F protein of A2 strain of the virus (GenBank accession number P03420) was codon optimized as described above, synthesized, and cloned into plasmids for PIV-WN s-PIV and d-PIV, using the insertion schemes shown in Fig. 12 and 13 for rabies G  
25 protein, by applying standard methods of molecular biology. Exact sequences of the insertions and surrounding genetic elements are provided in Sequence Appendix 5. In vitro RNA transcripts of resulting WN( $\Delta$ C)-RSV F, WN( $\Delta$ prME)- RSV F, and WN( $\Delta$ CprME)- RSV F PIV constructs were used to transfect helper BHK-CprME(WN) cells. Efficient replication and expression of RSV F protein was first demonstrated by immunostaining of  
30 transfected cells with an anti-RSV F Mab, as illustrated for the WN( $\Delta$ prME)- RSV F

construct in Fig. 19. The presence of packaged PIVs in the supernatants from transfected cells (titer as high as 7 log<sub>10</sub> FFU/ml) was determined by titration in Vero cells with immunostaining. Additionally, similar constructs can be used that contain a modified full length F protein gene. Specifically, the N-terminal native signal peptide of F is replaced in modified F protein with the one from rabies virus G protein. The modification is intended to elucidate whether the use of a heterologous signal can increase the rate of F protein synthesis and/or replication of PIVs.

Table 1. PIV prototype constructs used in platform development studies

| Construct | Genetic composition                                    | Packaged in                                                                                                                             |
|-----------|--------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|
| PIV-WN    | wt NY99 WN virus                                       | WN envelope; BHK-CprME(WN) or BHK-C(WN) helper cells (Mason et al., Virology 2006, 351:432-43; Widman et al., Vaccine 2008, 26:2762-71) |
| PIV-YF/WN | Envelope (VLP): wt WN NY99<br>Backbone: YF 17D         | YF 17D envelope; BHK-CprME(YF) helper cells (Widman et al., Adv Virus Res. 2008, 72:77-126)                                             |
| PIV-WN/JE | Envelope (VLP): wt JE Nakayama<br>Backbone: wt WN NY99 | JE or WN envelope; BHK-C(WN) or BHK-CprME(WN) helper cells (Ishikawa et al., Vaccine 2008, 26:2772-8)                                   |
| PIV-YF    | YF 17D                                                 | YF 17D envelope; BHK-CprME(YF) or BHK-C(YF) helper cells (Mason et al., Virology 2006, 351:432-43)                                      |

Table 2. Safety: Suckling mouse neurovirulence<sup>1</sup>

| Construct | Doses (log <sub>10</sub> ) | Mortality (%) | AST (days) <sup>2</sup> |
|-----------|----------------------------|---------------|-------------------------|
| PIV-YF    | 1 - 4                      | 0/10 (0%)     | na                      |
| PIV-WN    | 2 - 5                      | 0/10 (0%)     | na                      |
| PIV-WN/JE | 1 - 4                      | 0/11 (0%)     | na                      |
| PIV-YF/WN | 1 - 4                      | 0/10-11 (0%)  | na                      |
| WN d-PIV  | 1 - 4                      | 0/10-11 (0%)  | na                      |
| YF d-PIV  | 1 - 4                      | 0/10 (0%)     | na                      |
| YF17D     | 2                          | 10/10 (100%)  | 7.6                     |
|           | 1                          | 10/10 (100%)  | 9.3                     |
|           | 0                          | 9/10 (90%)    | 9.9                     |
|           | -1                         | 3/10 (30%)    | 9.6                     |

|       |     |              |      |
|-------|-----|--------------|------|
| YF/JE | 4   | 9/11 (82%)   | 9.7  |
|       | 3   | 7/10 (70%)   | 12.3 |
|       | 2   | 3/11 (27%)   | 12   |
|       | 1   | 0/11 (0%)    | na   |
| YF/WN | 3   | 2/11 (18%)   | 12.5 |
|       | 0-2 | 0/10-11 (0%) | na   |

<sup>1</sup>Single dose, IC inoculation, ICR 5-day old mice, graded log doses administered.

<sup>2</sup>AST for mice that died; na, not applicable.

5

10 Table 3. PIV highly immunogenic and efficacious in mice<sup>1</sup>

| Group                       | Dose                             | PRNT<br>Day 20 | PRNT<br>Day 34 | Post-challenge<br>mortality (%) |
|-----------------------------|----------------------------------|----------------|----------------|---------------------------------|
| PIV-WN                      | 10 <sup>5</sup>                  | 640            | 1280           | 0/8 (0%)                        |
|                             | 10 <sup>6</sup>                  | 1280           | 2560           | 1/8 (12.5%)                     |
|                             | 10 <sup>6</sup> +10 <sup>5</sup> | 2560           | 2560           | 0/6 (0%)                        |
| YF/WN control               | 10 <sup>4</sup>                  | 1280           | 2560           | 1/8 (12.5%)                     |
| PIV-WN/JE                   | 10 <sup>4</sup>                  | 10             | 20             | N/D                             |
|                             | 10 <sup>5</sup>                  | 20             | 20             | N/D                             |
|                             | 10 <sup>5</sup> +10 <sup>5</sup> | 20             | 160            | N/D                             |
| YF/JE control               | 10 <sup>4</sup>                  | 160            | 320            | N/D                             |
| PIV-YF                      | 10 <sup>4</sup>                  | <10            | <10            | 8/8 (100%)                      |
|                             | 10 <sup>5</sup>                  | <10            | <10            | 5/7 (71%)                       |
|                             | 10 <sup>5</sup> +10 <sup>5</sup> | 10             | 10             | 2/5 (40%)                       |
| YF17D control               | 10 <sup>4</sup>                  | 640            | 1280           | 0/7 (0%)                        |
| Mock control – WN challenge | Diluent                          | N/D            | 0              | 7/7 (100%)                      |
| – YF challenge              | Diluent                          | N/D            | 0              | 8/8 (100%)                      |

<sup>1</sup>IP immunization (d0 prime, and d21 boost in select groups); challenge on d35: wt WN NY99, 3 log<sub>10</sub> PFU IP, 270 LD50; wt YF Asibi, 3 log<sub>10</sub> PFU IC, 500 LD50; N/D, not determined.

15

Table 4. PIV are immunogenic in hamsters and protect against challenge<sup>1</sup>

| Group                       | Dose(s)                          | PRNT<br>Day 38 | POST-CHALLENGE |            |                       |
|-----------------------------|----------------------------------|----------------|----------------|------------|-----------------------|
|                             |                                  |                | Mortality      | Morbidity  | Peak viremia<br>(log) |
| PIV-WN                      | 10 <sup>5</sup>                  | 320            | 0/5 (0%)       | 0/5 (0%)   | 2.3                   |
|                             | 10 <sup>6</sup>                  | 640            | 0/5 (0%)       | 0/5 (0%)   | 1.8                   |
|                             | 10 <sup>6</sup> +10 <sup>5</sup> | 1280           | 0/5 (0%)       | 0/5 (0%)   | <1.3                  |
| YF/WN control               | 10 <sup>4</sup>                  | ≥2560          | 0/5 (0%)       | 0/5 (0%)   | <1.3                  |
| PIV-WN/JE                   | 10 <sup>4</sup>                  | 20             | 2/5 (40%)      | 2/5 (40%)  | 2.2                   |
|                             | 10 <sup>5</sup> +10 <sup>5</sup> | 40             | 0/5 (0%)       | 0/5 (0%)   | <1.3                  |
| YF/JE control               | 10 <sup>4</sup>                  | 2560           | 0/5 (0%)       | 0/5 (0%)   | 1.3                   |
| PIV-YF                      | 10 <sup>4</sup>                  | <10            | 1/3 (33%)      | 3/3 (100%) | 8.3                   |
|                             | 10 <sup>5</sup>                  | <10            | 1/5 (20%)      | 4/5 (80%)  | 8.3                   |
|                             | 10 <sup>5</sup> +10 <sup>5</sup> | 20             | 0/4 (0%)       | 0/4 (0%)   | 2.5                   |
| YF17D control               | 10 <sup>4</sup>                  | ≥2560          | 0/4 (0%)       | 0/4 (0%)   | <1.3                  |
| Mock control – WN challenge | Diluent                          | <10            | 3/4 (75%)      | 4/4 (100%) | 4.0                   |
| – YF challenge              | Diluent                          | <10            | 1/4 (25%)      | 4/4 (100%) | 8.4                   |
| – JE challenge              | Diluent                          | <10            | 2/5 (40%)      | 2/5 (40%)  | 3.0                   |

<sup>1</sup>Syrian hamsters, SC inoculation (d0, and d21 in select groups); challenge (d39): wt WN NY385/99 6 log<sub>10</sub>

5 PFU IP, wt JE Nakayama 5.8 log<sub>10</sub> PFU IC, or hamster-adapted YF Asibi 7 log<sub>10</sub> PFU IP (McArthur et al., J. Virol. 77:1462-1468, 2003; McArthur et al., Virus Res. 110:65-71, 2005).

5 Table 5. Immunization of hamsters with PIV: comparison of SC and IP routes

| Inoculums | PRNT Day 20-21 |     | Boost<br>(log <sub>10</sub> ) | PRNT Day 34-38 |      |
|-----------|----------------|-----|-------------------------------|----------------|------|
|           | SC             | IP  |                               | SC             | IP   |
| PIV-WN    | 40             | 320 | 5                             | 1280           | 1280 |
| PIV-YF/WN | 10             | 320 | 5                             | 160            | 1280 |
| PIV-WN/JE | 10             | 80  | 5                             | 40             | 640  |
| PIV-YF    | <10            | 10  | 5                             | 20             | 80   |

10

Table 6. Immune responses to PIV cocktails (mice)<sup>1</sup>

| Group             | Dose                              | PRNT Day 20 |         | PRNT Day 34 |         |
|-------------------|-----------------------------------|-------------|---------|-------------|---------|
|                   |                                   | Anti-JE     | Anti-WN | Anti-JE     | Anti-WN |
| PIV-WN/JE + RV-WN | 10 <sup>5</sup> + 10 <sup>5</sup> | 20          | 320     | 640         | 5120    |
| PIV-WN/JE alone   | 10 <sup>5</sup>                   | 80          | <10     | 160         | 20      |
| PIV-WN alone      | 10 <sup>5</sup>                   | <10         | 640     | <10         | 5120    |
| Mock              | -                                 | <10         | <10     | <10         | <10     |

<sup>1</sup> C57/BL6 mice, IP inoculations on days 0 and 21; pooled serum PRNT titers.

15

Table 7. Neurovirulence (IC inoculation) and neuroinvasiveness (IP inoculation) of PIV-TBE and YF/TBE vaccine constructs in adult ICR mice

| Construct       | Neurovirulence (IC route)       |                  |                           | Neuroinvasiveness (IP route)    |                  |                           |
|-----------------|---------------------------------|------------------|---------------------------|---------------------------------|------------------|---------------------------|
|                 | Dose(s)<br>(log <sub>10</sub> ) | Mortality<br>(%) | AST,<br>days <sup>1</sup> | Dose(s)<br>(log <sub>10</sub> ) | Mortality<br>(%) | AST,<br>days <sup>1</sup> |
| PIV-Hypr p39    | 5                               | 0/7 (0%)         | na                        | 5                               | 0/16 (0%)        | na                        |
| PIV-Hypr p40    | 5                               | 0/6 (0%)         | na                        | 5                               | 0/16 (0%)        | na                        |
| YF/Hypr p42     | 4                               | 8/8 (100%)       | 6.3                       | 5                               | 6/8 (75%)        | 13.3                      |
|                 | 3                               | 8/8 (100%)       | 6.4                       |                                 |                  |                           |
|                 | 2                               | 8/8 (100%)       | 7.4                       |                                 |                  |                           |
| YF/LGT p43      | 4                               | 8/8 (100%)       | 7.9                       | 5                               | 0/8 (0%)         | na                        |
|                 | 3                               | 8/8 (100%)       | 7.6                       |                                 |                  |                           |
|                 | 2                               | 8/8 (100%)       | 8.4                       |                                 |                  |                           |
| YF/Hypr p45     | 4                               | 8/8 (100%)       | 6.1                       | 5                               | 5/8 (62.5%)      | 11.2                      |
|                 | 3                               | 8/8 (100%)       | 6.6                       |                                 |                  |                           |
|                 | 2                               | 8/8 (100%)       | 6.8                       |                                 |                  |                           |
| YF/Hypr dC2 p59 | 4                               | 8/8 (100%)       | 6.6                       | 5                               | 0/8 (0%)         | na                        |
|                 | 3                               | 8/8 (100%)       | 7.4                       |                                 |                  |                           |
|                 | 2                               | 8/8 (100%)       | 8.1                       |                                 |                  |                           |
| YF 17D          | 3                               | 8/8 (100%)       | 9                         | 5                               | 0/8 (0%)         | na                        |
|                 | 2                               | 7/8 (87.5%)      | 9.6                       |                                 |                  |                           |
|                 | 1                               | 4/8 (50%)        | 10                        |                                 |                  |                           |
| Mock (diluent)  | none                            | 0/8 (0%)         | na                        | none                            | 0/8 (0%)         | na                        |

<sup>1</sup>AST for mice that died.

5

Table 8. Neutralizing antibody titers (PRNT<sub>50</sub>) in mice immunized IP (determined against wt TBE virus Hypr), and protection from challenge (postchallenge observation, day 9)

| Immunogen             | Dose(s),<br>log <sub>10</sub> | PRNT <sub>50</sub> titer,<br>individ. samples <sup>1</sup> | PRNT <sub>50</sub> GMT | Postchallenge<br>mortality (%)<br>on day 9 <sup>2</sup> |
|-----------------------|-------------------------------|------------------------------------------------------------|------------------------|---------------------------------------------------------|
| PIV-Hypr p39, 1 dose  | 5                             | 1746 (2)<br>1187 (2)<br>164 (2)<br>574 (2)                 | 665                    | 0/8 (0%)                                                |
| PIV-Hypr p39, 2 doses | 5+5                           | 16229 (2)<br>12928 (2)<br>12927 (2)<br>4627 (2)            | 10,584                 | 0/8 (0%)                                                |
| PIV-Hypr p40, 1 dose  | 5                             | <10 (2)<br><10 (2)<br>18 (2)<br>33 (2)                     | 15                     | 6/8 (75%)                                               |
| PIV-Hypr p40, 2 doses | 5+5                           | 169 (2)<br>638 (2)<br>26 (2)<br>192 (2)                    | 153                    | 1/8 (12.5%)                                             |
| YF/Hypr p42           | 5                             | 9210 (1)<br>4020 (1)                                       | 6,085                  | 0/2 (0%)                                                |
| YF/LGT p43            | 5                             | 123 (2)                                                    | 64                     | 1/8 (12.5%)                                             |

|                                                                |           |          |       |             |
|----------------------------------------------------------------|-----------|----------|-------|-------------|
|                                                                |           | 32 (2)   |       |             |
|                                                                |           | 96 (2)   |       |             |
|                                                                |           | 45 (2)   |       |             |
| YF/Hypr p45                                                    | 5         | 292 (2)  | 68    | 0/3 (0%)    |
|                                                                |           | 16 (1)   |       |             |
| YF/Hypr dC2 p59                                                | 5         | 194 (2)  | 68    | 0/8 (0%)    |
|                                                                |           | 93 (2)   |       |             |
|                                                                |           | 45 (2)   |       |             |
|                                                                |           | 26 (2)   |       |             |
| Killed human TBE vaccine, 1 dose (at 1/20 of human dose)       | 1/20      | 19 (2)   | 12    | 1/8 (12.5%) |
|                                                                |           | <10 (2)  |       |             |
|                                                                |           | 13 (2)   |       |             |
|                                                                |           | <10 (2)  |       |             |
| Killed human TBE vaccine, 2 doses (each at 1/20 of human dose) | 1/20+1/20 | 3435 (2) | 1,496 | 0/6 (0%)    |
|                                                                |           | 1267 (2) |       |             |
|                                                                |           | 770 (2)  |       |             |
| YF 17D control                                                 | 5         | <10 (4)  | <10   | 5/8 (62.5%) |
|                                                                |           | 11 (4)   |       |             |
| Mock                                                           | none      | <10 (4)  | <10   | 4/8 (50%)   |
|                                                                |           | <10 (4)  |       |             |

<sup>1</sup>Numbers in parenthesis correspond to number of mice in each pooled serum sample tested.

<sup>2</sup>Mortalities on day 9 are shown.

5

Table 9. Examples of published attenuating E protein mutations that can be used for attenuation of chimeric TBE LAV candidates

| Residue  | Domain | Comments                                                                                                                                   | Attenuation in | Reference                                                                            |
|----------|--------|--------------------------------------------------------------------------------------------------------------------------------------------|----------------|--------------------------------------------------------------------------------------|
| N52R     | II     | DI- DII hinge, possibly involved in hinge motion required for fusion activation                                                            | JE, YF         | Hasegawa et al, 1992, Schlesinger et al, 1996                                        |
| E84K     | II     | conserved, E in TBE, K/R in others, attenuated by passage in <i>Ixodes ricinus</i> ticks, DII contains flavivirus cross reactive epitopes  | TBE            | Labuda et al, 1994                                                                   |
| E85K     | II     | conserved, E in TBE, K/R in others, attenuation obtained as plaque variants in Vero cells, DII contains flavivirus cross reactive epitopes | JE             | Wu et al, 1997                                                                       |
| H104K    | II     | within highly conserved fusion peptide (aa 98-113), H in TBE, G in others                                                                  | TBE            | Rey et al, 1995                                                                      |
| L107F    | II     | within highly conserved fusion peptide (aa 98-113), L in all flaviviruses, F in attenuated JE                                              | TBE, JE, WNV   | Rey et al, 1995, Arroyo et al, 1999, 2004                                            |
| T123K    | II     | DI- DII hinge, T in TBE, A in KFD                                                                                                          | TBE            | Holzmann et al, 1997                                                                 |
| K126E    | II     | DI- DII hinge, K in TBE, E in D-2                                                                                                          | DEN2           | Bray, 98                                                                             |
| K136E    | II     | DI- DII hinge, K in TBE and JE, E in D-2                                                                                                   | JE             |                                                                                      |
| N154L(Y) | I      | glycosylation site, packed with conserved H 104, involved in fusion.                                                                       | DEN2, DEN4, YF | Guirakhoo et al, 1993, Pletnev et al, 1993, Kawano et al, 1993, Jennings et al, 1994 |
| K171E    | I      | external edge of DI, involved in fusion                                                                                                    | TBE            | Mandi, 1989, Holzmann, 1997                                                          |
| I173T    |        | external edge of DI, involved in fusion                                                                                                    | YF             | Chambers and Nickells 2001                                                           |
| D181Y    |        | DI- DII hinge                                                                                                                              | TBE            | Holzmann et al, 1997                                                                 |
| K204R    |        | Lining Hydrophobic pocket, involve in                                                                                                      | DEN1, DEN3     | Guirakhoo et al, 2004                                                                |

|       |      |                                                                                                                                                         |          |                                                    |
|-------|------|---------------------------------------------------------------------------------------------------------------------------------------------------------|----------|----------------------------------------------------|
|       |      | fusion                                                                                                                                                  |          |                                                    |
| P272S | II   | highly conserved, junction of one the of 2 alpha helices                                                                                                | JE       | Cecilia et al, 1991                                |
| G308N | III  | cell attachment, DKT in TBE, EGS in KFD, T-X in others, change to N produced glycosylation site in LI and reduced virulence N-X-T/S glycosylation motif | LI       | Jiang et al, 1993, Gao et al, 1994                 |
| S310K | III  | putative cell attachment, change from E to G in JE reduced virulence                                                                                    | JE       | Jiang et al, 1993, Gao et al, 1994, Wu et al, 1997 |
| K311E | III  | highly conserved, putative cell attachment                                                                                                              | TBE, YF  | Rey et al, 1995, Jennings, 1994                    |
| T333L | III  | putative cell attachment                                                                                                                                | YF, LGT  | Raynman et al, 1998                                |
| G334K | III  | putative cell attachment                                                                                                                                | YF       | Chambers and Nickells, 2001                        |
| S335K | III  | putative cell attachment                                                                                                                                | JE       | Wu et al, 1997                                     |
| K338D | III  | putative cell attachment                                                                                                                                | JE       | Cecilia and Gould, 1991                            |
| P337D | III  | putative cell attachment                                                                                                                                | JE       | Cecilia and Gould, 1991                            |
| G368R | III  | putative cell attachment                                                                                                                                | TBE, JE  | Holzman et al 1997, Hasegawa et al 1992            |
| Y384H | III  | change to H attenuated TBE, putative cell attachment, -3 position to deleted RGD in TBE                                                                 | TBE      | Holzmann et al, 1990                               |
| V385R | III  | conserved, -2 position to deleted RGD in TBE, putative cell attachment                                                                                  | D2       | Hiramatsu et al, 1996, Lobigs, 1990                |
| G386R | III  | highly conserved, -1 position to deleted RGD in TBE, putative cell attachment                                                                           | D2, MVE  | Hiramatsu, 1996, Lobigs et al, 1990                |
| E387R | III  | conserved, +2 position to deleted RGD in TBE, putative cell attachment                                                                                  | D2, MVE  | Hiramatsu, 1996, Lobigs et al, 1990                |
| F403K | none | highly conserved, C-terminal region not included in crystal structure sE                                                                                | D-2, D-4 | Kawano et al, 1993, Bray et al, 1998               |
| H438Y | None | highly conserved, C-terminal region not included in crystal structure sE                                                                                | LGT      | Campbell and Pletnev 2000                          |
| H496R | none | highly conserved, C-terminal region not included in crystal structure sE                                                                                | TBE      | Gritsun et al, 2001                                |

References: Hasegawa et al., Virology 191(1):158-165; Schlesinger et al., J. Gen. Virol. 1996, 77 ( Pt 6):1277-1285, 1996; Labuda et al., Virus Res. 31(3):305-315, 1994; Wu et al., Virus Res. 51(2):173-181, 1997; Holzmann et al., J. Gen. Virol. 78 (Pt 1):31-37, 1997; Bray et al., J. Virol. 72(2):1647-1651, 1998; Guirakhoo et al., Virology 194(1):219-223, 1993; Pletnev et al., J. Virol. 67(8):4956-4963, 1993; Kawano et al., J. Virol. 67(11):6567-6575, 1993; Jennings et al., J. Infect. Dis. 169(3):512-518, 1994; Mandl et al., J. Virol. 63(2):564-571, 1989; Chambers et al., J. Virol. 75(22):10912-10922, 2001; Cecilia et al., Virology 181(1):70-77, 1991; Jiang et al., J. Gen. Virol. 74 (Pt 5):931-935, 1993; Gao et al., J. Gen. Virol. 75 (Pt 3):609-614, 1994; Holzmann et al., J. Virol. 64(10):5156-5159, 1990; Hiramatsu et al., Virology 224(2):437-445, 1996; Lobigs et al., Virology 176(2):587-595, 1990; Campbell et al., Virology 269(1):225-237, 2000; Gritsun et al., J. Gen. Virol. 82(Pt 7):1667-1675, 2001.

## Sequence Appendix 1

### CV-TBEV Hypr or CV-LGT E5 with YFV/TBEV chimeric signal (p42, p59, and p43 constructs)

YF17D partial signal  
-----  
TBEV partial signal  
-----  
C protein YF17D  
-----  
Hypr or LGT E5 prM protein  
-----

```

401  R K R R S H D V L T V Q F L I L G M L G M T I A A T V R
    A GGAAACGCCG TTCCCATGAT GTTCTGACTG TGCAATTCCT AATTTTGGGC ATGCTGGGCA TGACAATCGC AGCTACGGTT CGC
    T CCTTTGCCGC AAGGGTACTA CAAGACTGAC ACGTTAAGGA TTAAAACCCG TACGACCCGT ACTGTTAGCG TCGATGCCAA GCG
  
```

### CV-TBEV Hypr with YFV/WNV chimeric signal (p45)

C protein YF17D  
-----  
YF 17D partial signal  
-----  
WNV partial signal  
-----  
Hypr prM protein  
-----

```

401  R K R R S H D V L T V Q F L I L G M L A C V G A A T V R
    A GGAAACGCCG TTCCCATGAT GTTCTGACTG TGCAATTCCT AATTTTGGGC ATGCTGGCTT GTGTCCGAGC AGCTACCGTG CGA
    T CCTTTGCCGC AAGGGTACTA CAAGACTGAC ACGTTAAGGA TTAAAACCCG TRCGACCGAA CACAGCCTCG TCGATGGCAC GCT
  
```

### RV-WNV/TBEV Hypr with TBEV signal (p39)

TBEV signal  
-----  
WNV C protein  
-----  
Hypr prM protein  
-----

```

201  Q K K R G G T D W N S W L L V I G M L G M T I A A T V R
    CAAAAGAAA CGGGGGGGAA CAGACTGGAT GAGCTGGCTG CTCGTAATCG GCATGTGTGG CATGACAATC GCAGCTACGG TTCGC
    GTTTCTTT GCCCCCCCTT GTCTGACCTA CTCGACCGAC GAGCATTAGC CGTACGACCC GTACTGTTAG CGTCGATGCC AAGCG
  
```

### RV-WNV/TBEV Hypr with WNV signal (p40)

WNV signal  
-----  
WNV C protein  
-----  
Hypr prM protein  
-----

```

201  Q K K R G G K T G I A V M I G M L A C V G A A T V R
    CAAAAGAAA CGGGGGGGAA AGACAGGCAT AGCTGTGATG ATAGGCATCG TGGCTTGTGT CGGAGCAGCT ACCGTGCCA
    GTTTCTTT GCGCCCCCTT TCTGTCCGTA TCGACACTAC TATCCGTACG ACCGAACACA GCCTCGTCGA TGGCACGCT
  
```

## Sequence Appendix 2

### CV-TBEV Hypr with YFV/TBEV chimeric signal (p42)

#### 5' UTR

1 AGTAAATCCT GTGTGCTAAT TGAGGTGCAT TGGTCTGCAA ATCCAGTTGC TAGGCAATAA ACACATTTCG ATTAATTTTA ATCGTTTCGT GAGCGATTAG  
TCATTTAGGA CACACGATTA ACTCCACGTA ACCAGACGTT TAGCTCAACG ATCCGTTATT TGTGTAAACC TAATTAAAT TAGCAAGCAA CTCGCTAATC  
5' UTR

#### C protein

101 CAGAGAACTG ACCAGAACAT GTCTGGTTCG AAAGCTCAGG GAAARACCCCT GGGCGTCAAT ATGGTACGAC GAGGAGTTCG CTCCTTGTC AACAATAA  
GTCTCTGAC TGGTCTTGTA CAGACCAGCA TTCGAGTCC CTTTGGGA CCGCAGTTA TACCATGCTG CTCCTCAAGC GAGGAACAGT TTGTTTATT  
C protein

201 Q K T K Q I G N R P G P S R G V Q G F I F F F L F N I L T G K K I  
AACAAAAAC AAACAATTT GGAACAGAC CTGGACCTTC AAGAGGTGTT CAAGGATTTA TCTTTTCTT TTTGTTCAAC ATTTTGACTG GAAAAAGAT  
TTGTTTTTG TTTGTTTAA CCTTGTCTG GACCTGGAAG TTCTCCACAA GTTCTTAAAT AGAAAAAGAA AAACAAGTTG TAAACTGAC CTTTTTCTA  
C protein

301 T A H L K R L W K M L D P R Q G L A V L R K V K R V V A S L M R G  
CACAGCCAC CTAAAGAGGT TGTGGAAAT GCTGGACCCA AGACAGGCT TGGCTGTTCT AAGGAAAGTC AAGAGAGTGG TGGCCAGTTT GATGAGAGGA  
GTGTGGGTG GATTCTCCA ACACCTTTA CGACCTGGGT TCTGTCCGA ACCGACAGA TTCCTTTCAG TTCTCTCACC ACCGGTCAA CTACTCTCCT  
YF17D partial signal

#### TBEV partial signal

#### C protein

401 L S S R K R R S H D V L T V Q F L I L G M L G M T I A A T V R K E R  
TTGTCTCAA GGAACGCCG TTCCCATGAT GTTCTGACTG TGCAATTCCT AATTTTGGGC ATGCTGGGCA TGACAATCGC AGCTACGGTT CGCAAGGAAA  
AACAGGAGTT CCTTGCGCC AAGGGTACTA CAAGACTGAC ACGTTAAGGA TTAAACCCG TACGACCCGT ACTGTTAGCG TCGATGCCAA GCGTTCCTTT  
prM protein

501 D G S T V I R A E G K D A A T Q V R V E N G T C V I L A T D M G S  
GAGACGGCAG TACGGTCATA CGCGCGGAAG GTAAGGATGC CGTACCCAA GTGAGAGTGG AAAATGGTAC CTGCGTCATT CTGGCCACCG ACATGGGCTC  
CTCTGCCGTC ATGCCAGTAT GCGCGCCTTC CATTCTACG GCGATGGGTT CACTCTCACC TTTTACCATG GACGCAGTAA GACCGGTGGC TGTACCCGAG  
prM protein

601 W C D D S L S Y E C V T I D Q G E E P V D V D C F C R N V D G V Y  
TTGGTGTGAT GATAGCCTTT CTATAGAGTG CGTAACCATA GATCAAGGTG AGGAACCTGT TGACGTTGAT TGCTTCTGCC GAAACGTGGA TGGGGTGTAT  
AACCACACTA CTATCGGAAA GAATACTCAC GCATGGTAT CTAGTTCCAC TCCTTGGACA ACTGCACTA ACGAAGACGG CTTTGCACCT ACCCCACATA  
prM protein

701 L B Y G R C G K Q E G S R T R R S V L I P S H A Q G E L T G R G H K  
CTCGAATATG GACGGTGTGG TAAACAAGAA GGAAGCAGAA CCAGACGCTC AGTGCTTATA CCTCCACG CTCAAGGAGA GCTGACCGGA CGGGACATA  
GAGCTTATAC CTGCCACACC ATTTGTTCTT CCTCGTCTT GGTCTGCGAG TCACGAATAT GGGAGGGTGC GAGTTCCTCT CGACTGGCCT GCCCCTGTAT

#### prM protein

prM protein

801 W L E G D S L R T H L T R V E G W V W K N R L L A L A M V T V V W  
AATGGTTGGA GGGCGACTCA CTCGGAACAC ATTGACCCG CGTCGAGGGC TGGGTCTGGA AAAATCGGCT GTTGGCCCTC GCTATGGTGA CAGTCGTTTG  
TTACCAACCT CCCGCTGAGT GAGGCTTGTC TAAACTGGGC GCAGCTCCCG ACCCAGACCT TTTTAGCCGA CAACCGGGAG CGATACCACT GTCAGCAAAAC

Hypr E protein

prM protein

901 L T L E S V V T R V A V L V V L L C L A P V Y A S R C T H L E N R  
GCTCAGCTG GAGTCTGTGG TTACTCGCGT GGCAGTGCTG GTGGTGCTCC TCTGTCTGCG CCCTGTCTAC GCGTCCAGGT GTACTCATTT GGAAACAGA  
CGAGTGGCAG CTCAGACACC AATGAGCGCA CCGTCACGAC CACCACGAGG AGACAGAACG GGGACAGATG CGCAGGTCCA CATGAGTAAA CCTTTTGTCT

Hypr E protein

1001 D F V T G T Q G T T R V T L V L E L G G C V T I T A E G K P S M D V  
GATTTTGICA CCGGCACCCA GGGGACGACT CGGGTAACCC TGGTGCTTGA ACTGGGTGGT TGGGTACTA TTACCGCTGA GGGCAAACCC TCTATGGATG  
CTAAAACAGT GGCCGTGGGT CCCCTGCTGA GCCCATTGGG ACCACGAACCT TGACCCACCA ACGCAATGAT AATGGCGACT CCGGTTTGGG AGATACCTAC

Hypr E protein

1101 W L D A I Y Q E N P A Q T R E Y C L H A K L S D T K V A A R C P T  
TGTGGCTGGA TGCAATCTAT CAGGAGAATC CCGCACAAAC CAGGGAATAT TGCCTTCACG CAAAGCTGTC CGATCAAAAG GTGCGGGCTA GGTGCCCCAAC  
ACACCGACCT ACGTTAGATA GTCCTCTTAG GCGGTGTTTG GTCCCTTATA ACGGAAGTGC GTTTCGACAG GCTATGTTTC CAGCGCCGAT CCACGGGTTG

Hypr E protein

1201 M G P A T L A E E H Q G G T V C K R D Q S D R G W G N H C G L F G  
AATGGGACCG GCCACCCTGG CGGAGGAACA TCAGGGAGGT ACAGTGTGCA AACGGGACCA GAGTGATAGA GGCTGGGGTA ATCACTGCGG CCTGTTCCGG  
TTACCCCTGGC CGGTGGGACC GCCTCCTTGT AGTCCCTCCA TGTACACAGT TTGCCCTGGT CTCATATCT CCGACCCCAT TAGTGACGCC GGACAAGCCG

Hypr E protein

1301 K G S I V A C V K A A C E A K K K A T G H V Y D A N K I V Y T V K V  
AAAGGAAGTA TTGTGCTTG CGTCAAGGCA GCCTGTGAGG CCAAAAAGAA GGCTACTGGG CACGTCTATG ACGCCAACAA GATCGTTTAT ACACTGAAG  
TTTCCTTCAT AACAGCGAAC GCAGTCCGT CGGACACTCC GGTTTTCTT CCGATGACCC GTGCAGATAC TGCGGTTGTT CTAGCAAATA TGTCACITTC

Hypr E protein

1401 E P H T G D Y V A A N E T H S G R K T A S F T V S S E K T I L T M  
TGGAACCACA CACAGGGGAT TACGTGGCGG CCAACGAGAC TCATTCCCGT CGCAAAACGG CCAGCTTCAC CGTGTCTATC GAAAAGACCA TCCTCACTAT  
ACCTTGGTGT GTGTCCCTTA ATGCACCGCC GGTGCTCTG AGTAAGGCCA GCGTTTTCGC GGTGGAAGTG GCACAGTAGG CTTTTCTGGT AGGAGTGATA

Hypr E protein

1501 G E Y G D V S L L C R V A S G V D L A Q T V I L E L D K T V E H L  
GGGGGAGTAT GGCAGCTTT CTCTGCTCTG CCGGGTGGCT AGCGGAGTCG ACCTGGCCCA GACAGTCATC CTGGAAGTGG ATAAAACAGT TGAGCATCTG  
CCCCCTCATA CCGCTGCAAA GAGACGAGAC GGCCCAACCGA TCGCCTCAGC TGGACCGGGT CTGTCACTAG GACCTTGACC TATTTTGTCA ACTCGTAGAC

Hypr E protein

1601 P T A W Q V H R D W F N D L A L P W K H E G A R N W N N A E R L V E  
CCTACCGCTT GGCAGGTGCA CAGGATTGG TTAAACGACC TTGCCCTGCC ATGGAAACAT GAAGGAGCGA GAAACTGGAA TAATGCAGAG CGACTCGTAG  
GGATGGCGAA CCGTCCACGT GTCCCTAACC AAATTGCTGG AACGGGACGG TACCTTTGTA CTTCCTCGCT CTTTGACCTT ATTACGTCTC GCTGAGCATC

Hypr E protein

1701 F G A P H A V K M D V Y N L G D Q T G V L L K A L A G V P V A H I  
AATTGGGTGC CCCTCATGCC GTGAAGATGG ACGTCTACAA TCTGGGTGAT CAGACCGGCG TTCTCTTAA AGCTCTCGCT GCGGTACCAG TTGCCACAT  
TTAAGCCACG GGGAGTACGG CACTTCTACC TGCAGATGTT AGACCCACTA GTCTGGCCG AAGAGGAATT TCGAGAGCGA CCGCATGGTC AACGGGTGTA

Hypr E protein

1801 · E G T K Y H L K S G H V T C E V G L E K L K M K G L T Y T M C D K  
 CGAAGGAACG AAGTACCACC TGAAGTCAGG CCATGTAAC TGCAGGTTGG CCCTGGAGAA GTTGAAATG AAAGGTCTTA CGTACACAAT GTGTGACAAG  
 GCTTCCTTGC TTCATGGTGG ACTTCAGTCC GGTACATTGA ACGCTCCACC CGGACCTCTT CAACCTTTAC TTTCCAGAAAT GCATGTGTTA CACACTGTTT  
 Hypr E protein

1901 T K F T W K R A P T D S G H D T V V M E V T F S G T K P C R I P V R ·  
 ACCAAGTTCA CATGGAAGAG GGGCCCCACA GATAGCGGCC ACGATACTGT GGTGATGGAG GTGACCTTTT CTGGAACAAA ACCCTGCAGA ATACCCGTGC  
 TGGTTCAGGT GTACCTTCTC CCGGGGGTGT CTATCGCCGG TGCTATGACA CCACTACCTC CACTGGAAAA GACCTTGTTT TGGGACGTCT TATGGGCACG  
 Hypr E protein

2001 · A V A H G S F D V N V A M L I T P N P T I E N N G G G F I E M Q L ·  
 GGGCTGTAGC TCACGGATCT CCCGATGTCA ATGTTGCTAT GCTGATTACA CCTAACCCCTA CCATCGAGAA TAACGGTGGT GGTTTTATTG AGATGCAGCT  
 CCCGACATCG AGTGCTTAGA GGGCTACAGT TACAACGATA CGACTAATGT GGATTGGGAT GGTAGCTCTT ATTGCCACCA CCAAAATAAC TCTACGTCSA  
 Hypr E protein

2101 · P P G D N I I Y V G E L S Y Q W F Q K G S S I G R V F Q K T K K G ·  
 TCCGCCAGGC GATAACATCA TCTACGTGGG CGAAGTCTCT TACCAGTGGT TCCAGAAAGG GAGTTCRAAT GGGCGGTCT TCCAAAAAC GAAGAAGGGA  
 AGGCGGTCCG CTATTGTAGT AGATGCACCC GCTTGAGAGA ATGGTCACCA AAGTCTTTCC CTCAAGTTAA CCCGCCAGA AGGTTTTTTG CTCTCTCCCT  
 Hypr E protein

2201 I E R L T V I G E H A W D F G S A G G F L S S I G K A L H T V L G G ·  
 ATCGAAGCAT TGACGGTTAT CGGCGAGCAC GCATGGGAT TTGGTTCCGC AGGGGGATTC CTGTCTTCTA TTGGTAAGGC ACTGCATAAC GTGCTGGGG  
 TAGCTTGCTA ACTGCCAATA GCCGCTCGTG CGTACCCTAA AACCAAGGCG TCCCCTTAAG GACAGAAGAT AACCATTCGG TGACGTATGG CACGACCCCC  
 Hypr E protein

2301 · A F N S I F G G V G F L P K L L L G V A L A W L G L N M R N P T M ·  
 GCGCATTCAA TTCTATTTTC GGGGGCGTGG GGTTCCTGCC TAAACTCCTG CTGGGAGTAG CCCTGGCCCTG GTTGGGACTG AATATGCGGA ATCCGACGAT  
 CGCGTAAGTT AAGATAAAG CCCCCGACCC CCAAGGACGG ATTTGAGGAC GACCCCTCATC GGGACCGGAC CAACCTGAC TTATACGCCT TAGGCTGCTA  
 Hypr E protein

NS1 gene of YF17D

2401 · S M S F L L A G V L V L A M T L G V G A D Q G C A I N F G K R E L ·  
 GTCCATGTCA TTCCTCTTGG CCGGCGTGCT TGTACTGGCC ATGACACTGG GCGTTGGCGC CGATCARGGA TGCGCCATCA ACTTTGGCAA GAGAGAGCTC  
 CAGGTACAGT AAGGAGAACC GGGCCACGA ACATGACCGG TACTGTGACC CGCAACCGCG GCTAGTTCCT ACGCGGTAGT TGAACCGTT CTCTCTCGAG

## CV-TBEV Hypr with YFV/WNV chimeric signal (p45)

### 5' UTR

1 AGTAAATCCT GTGTGCTAAT TGAGGTGCAT TGGTCTGCAA ATCGAGTTGC TAGGCAATAA ACACATTTGG ATTAATTTTA ATCGTTCGTT GAGCGATTAG  
 TCATTTAGGA CACACGATTA ACTCCACGTA ACCAGACGTT TAGCTCAACG ATCCGTTATT TGTGTAAACC TAATTAAAT TAGCAAGCAA CTCGCTAATC  
 5' UTR

### C protein YF17D

101 M S G R K A Q G K T L G V N M V R R G V R S L S N K I K ·  
 CAGAGAACTG ACCAGAACAT GTCTGGTCTG AAAGCTCAGG GAAAAACCTT GGGCGTCAAT ATGGTACGAC GAGGAGTTCT CTCCTGTCA AACAAAATAA  
 GTCTCTTGAC TGGTCTTGTA CAGACCAGCA TTTCTGAGTCC CTTTTGGGA CCCGCGATTA TACCATGCTG CTCCTCAAGC GAGGAACAGT TTGTTTATT  
 C protein YF17D

· Q K T K Q I G N R P G P S R G V Q G F I F F F L P N I L T G K K I ·

201 AACAAAAAAC AAAACAAATT GGAACACGAC CTGGACCTTC AAGAGGTGTT CAAGGATTTA TCTTTTCTT TTTGTTCAAC ATTTTGACTG GAAAAAAGAT  
 TTGTTTTTTG TTTTGTITAA CCTTTGTCIG GACCTGGAAG TTCTCCACAA GTTCCTAAAT AGAAAAAGAA AAACAAGTTG TAAACTGAC CTTTTTCTA  
 C protein YF17D

301 T A H L K R L W K M L D P R Q G L A V L R K V K R V V A S L M R G  
 CACAGCCAC CTAAAGAGT TGTGAAAAAT GCTGGACCCA AGACAAGGCT TGGCTGTCT AAGGAAAGTC AAGAGAGTGG TGGCCAGTTT GATGAGAGGA  
 GTGTGGGTG GATTTCTCCA ACACCTTTTA CGACCTGGGT TCTGTCCGA ACCGACAGA TTCCTTTCAG TTCTCTCACC ACCGGTCAAA CTACTCTCCT  
 C protein YF17D WNV partial signal

YF 17D partial signal Hypr prM protein  
 401 L S S R K R R S H D V L T V Q F L I L G M L A C V G A A T V R K E R  
 TTGTCCTCAA GGAACGCCG TTCCCATGAT GTTCTGACTG TGCAATTCCT AATTTTGGGC ATGCTGGCTT GTGTCGGAGC AGCTACCGTG CGAAAAGAAC  
 AACAGGAGTT CCTTTGCGGC AAGGGTACTA CAAGACTGAC ACGTTAAGGA TTAAACCCG TACGACCGAA CACAGCCTCG TCGATGGCAC GCTTTTCTTG  
 Hypr prM protein

501 D G S T V I R A E G K D A A T Q V R V E N G T C V I L A T D M G S  
 GCGACGAAG CACCGTGATA AGGGCTGAGG GTAAGGATGC GGCTACGCGAG GTGAGAGTAG AGAATGGCAC TTGCGTAATA CTCGCGACTG ATATGGGATC  
 CGCTGCCTTC GTGGCACTAT TCCCGACTCC CATTCTACG CCGATCGCTC CACTCTCATC TCTTACCGTG AACGCATTAT GAGCGCTGAC TATACCTTAG  
 Hypr prM protein

601 W C D D S L S Y E C V T I D Q G E E P V D V D C F C R N V D G V Y  
 CTGGTGTGAC GATAGCCTCA GTTATGAATG CGTAACAATA GACCAGGGCG AAGAACCTGT GGACGTTGAC TGTTCCTGTA GAAATGTGGA TGGCGTTTAT  
 GACCACACTG CTATCGGAGT CAATACCTAC GCATTGTTAT CTGGTCCCGC TTCTTGGACA CCTGCAACTG ACAAGACAT CTTTACACCT ACCGCAATA  
 Hypr prM protein

701 L E Y G R C G K Q E G S R T R R S V L I P S H A Q G E L T G R G H K  
 CTGGAGTACG GCGCTGTGG AAAACAGSAG GGCTCAGCA CCGAAGATC TGTCTGATT CCAAGTCAG CCGAAGGAGA GTTGACCGGT AGAGGCCACA  
 GACCTCATGC CCGCGACACC TTTGTCTC CCGAGTGCTT GAGCTTCTAG ACACGACTAA GGTTCAGTGC GCGTCTCTCT CAACGCGCA TCTCCGTGT  
 Hypr prM protein

801 W L E G D S L R T H L T R V E G W V W K N R L L A L A M V T V V W  
 AGTGGCTTGA AGGGGACTCA TTGAGGACCC ACCTGACTAG GGTGGAGGGT TGGGTTTGA AGAATCGGTT GCTCGCGCTC GCTATGCTCA CCGTCGTGTG  
 TCACCGAACT TCCCTGAGT AACTCCTGGG TGGACTGATC CCACCTCCCA ACCCAAACCT TCTTAGCAA CGAGCGCGAG CGATACCACT GGCAGCACAC  
 Hypr prM protein

Hypr E protein  
 901 L T L E S V V T R V A V L V V L L C L A P V Y A S R C T H L E N R  
 GCTGACACTG GAGAGTGTG TGACTCGGGT TGCTGTGTTG GTTGTCTCTC TCTGTTTGGC CCCAGTGTAC GCGTCCAGGT GTACTCATTT GGAAACAGA  
 CGACTGTGAC CTCTACAGC ACTGAGCCCA ACGACACAAC CAACAGGAGG AGACAAACCG GGGTCACATG CGCAGGTCCA CATGAGTAAA CTTTTTGTCT  
 Hypr E protein

1001 D F V T G T Q G T T R V T L V L E L G G C V T I T A E G K P S M D V  
 GATTTGTCA CCGGCACCCA GGGGACGACT CCGGTAACCC TGGTGTCTGA ACTGGTGGT TGGCTTACTA TTACCGCTGA GGGCAACCC TGTATGGATG  
 CTAAACAGT GCGCGTGGT CCCCTGCTGA GCGCATTTGG ACCACGAAT TGACCCACCA ACGCAATGAT AATGCGGACT CCCGTTGGG AGATACCTAC  
 Hypr E protein

1101 W L D A I Y Q E N P A Q T R E Y C L H A K L S D T K V A A R C P T  
 TGTGGCTGGA TGCAATCTAT CAGGAGAATC CCGCACAAAC CAGGGAATAT TGCCITCAGC CAAAGCTGTC CGATACAAAG GTCGCGGCTA GGTGCCCAAC  
 ACACCGACCT ACGTTAGATA GTCTCTTAG GCGGTGTTTG GTCCCTTATA ACGGAAGTGC GTTTCGACAG GCTATGTTTC CAGCGCCGAT CCACGGGTG  
 Hypr E protein

M G P A T L A E E H Q G G T V C K R D Q S D R G W G N H C G L F G

1201 AATGGGACCG GCCACCTCG CGGAGGAACA TCAGGGAGGT ACAGTGTGCA AACGGGACCA GAGTGATAGA GGCTGGGGTA ATCACTGCGG CCTGTTCCGC  
 TTACCTGGC CGGTGGGACC GCCTCCTTGT AGTCCCTCCA TGTCACACGT TTGCCCTGGT CTCACTATCT CCGACCCCAT TAGTGACGCC GGACAAGCCG  
 Hypr E protein

1301 K G S I V A C V K A A C R A K K K A T G H V Y D A N K I V Y T V K V  
 AAAGGAAGTA TTGTGCTTG CGTCAAGGCA GCCTGTGAGG CCAAAAAGAA GGCTACTGGG CACGTCTATG ACGCCAACAA GATCGITTAT ACAGTGAAG  
 TTTCCTTCAT AACAGCGAAC GCAGTTCGGT CGGACACTCC GGTTTTCTT CCGATGACCC GTGCAGATAC TCGGGTTGTT CTAGCAATA TGTCACITTC  
 Hypr E protein

1401 E F H T G D Y V A A N E T H S G R K T A S F T V S S E K T I L T M  
 TGGACCACA CACAGGGGAT TACGTGGCG CCAACGAGAC TCATTCCGGT CGCAAAACGG CCAGCTTCAC CGTGTCTACC GAAAAGACCA TCCTCACTAT  
 ACCTTGGTGT GTGTCCCTTA ATGCACCGCC GGTTGCTCTG AGTAAGGCCA GCGTTTGGC GGTGGAAGTG GCACAGTAGG CTTTCTGGT AGGAGTGATA  
 Hypr E protein

1501 G E Y G D V S L L C R V A S G V D L A Q T V I L E L D K T V E H L  
 GGGGAGTAT GCGACGTTT CTCTGCTCTG CCGGGTGGCT AGCGAGTTCG ACCTGGCCCA GACAGTCATC CTGGAAGTGG ATAAAACAGT TGAGCATCTG  
 CCCCCATA CCGCTGCAAA GAGACGAGAC GGCCACCGA TCGCCTCAGC TGGACCGGGT CTCTCAGTAG GACCTTGACC TATTTGTCA ACTCGTAGAC  
 Hypr E protein

1601 P T A W Q V H R D W F N D L A L P W K H E G A R N W N N A E R L V E  
 CCTACCGCTT GGCAGGTGCA CAGGGATTGG TTTAACGACC TTGCCCTGCC ATGGAAACAT GAAGGAGCGA GAAACTGGAA TAATGCAGAG CGACTCGTAG  
 GGATGGCGAA CCGTCCACGT GTCCCTAACC AAATTGCTGG AACGGGACGG TACCTTTGTA CTTCCTCGCT CTTTGACCTT ATTACGCTC GCTGAGCATC  
 Hypr E protein

1701 F G A P H A V K M D V Y N L G D Q T G V L L K A L A G V P V A H I  
 AATTGGTGC CCCTCATGCC GTGAAGATGG ACGTCTACAA TCTGGGTGAT CAGACCGGCG TTCTCCTTAA AGCTCTCGCT GCGGTACCAG TTGCCACAT  
 TTAAGCCACG GGGAGTACGG CACTTCTACC TGAGATGTT AGACCCACTA GTCTGGCCGC AAGAGGAATT TCGAGAGCGA CCGCATGGTC AACGGGTGTA  
 Hypr E protein

1801 E G T K Y H L K S G H V T C E V G L E K L K M K G L T Y T M C D K  
 CGAAGGAACG AAGTACCACC TGAAGTCAGG CCATGTAAC TCGAGGTGG GCCTGGAGAA GTTGAAAATG AAAGGTCTTA CGTACACAAT GTGTGACAAG  
 GCTTCCTTGC TTCATGGTGG ACTTCAGTCC GGTACATTGA ACGCTCCACC CGGACCTCTT CAACTTTTAC TTTCAGAAAT GCATGTGTTA CACACTGTTC  
 Hypr E protein

1901 T K F T W K R A P T D S G H D T V V M E V T F S G T K P C R I P V R  
 ACCAAGTTCA CATGAAGAG GCGCCCCACA GATAGCGGCC ACGATACTGT GGTGATGGAG GTGACCTITT CTGGAACAAA ACCCTGCAGA ATACCCGTGC  
 TGGTTCAAGT GTACCTTCTC CCGGGGGTGT CTATCGCCGG TGCTATGACA CCACTACCTC CACTGGAAAA GACCTTGTTF TGGGACGTCT TATGGGCACG  
 Hypr E protein

2001 A V A H G S P D V N V A M L I T P N P T I E N N G G G F I E M Q L  
 GGGCTGTAGC TCACGGATCT CCCGATGTCA ATGTTGCTAT GCTGATTACA CCTAACCTTA CCATCGAGAA TAACGGTGGT GGTTTATTG AGATGCAGCT  
 CCCGACATCG AGTGCTTAGA GGGCTACAGT TACAACGATA CGACTAATGT GGATTGGAT GGTAGCTCTT AITGCCACCA CCAAAATAAC TCTACGTGGA  
 Hypr E protein

2101 P P G D N I I Y V G E L S Y Q W F Q K G S S I G R V F Q K T K K G  
 TCCGCCAGGC GATAACATCA TCTACGTGG CGAAGTCTCT TACCAAGTGT TTCAGAAAGG GAGTTCAATT GGGCGGGTCT TCCAAAAAAC GAAGAAGGGA  
 AGGCGGTCCG CTATTGTAGT AGATGCACCC GCTTGAGAGA ATGGTCACCA AAGTCTTTCC CTCAAGTTAA CCGGCCGAGA AGGTTTTTTG CTTCTTCCCT  
 Hypr E protein

2201 I E R L T V I G E H A W D F G S A G G F L S S I G K A L H T V L G G  
 ATCGAACGAT TGACGGTAT CGGCGAGCAC GCATGGGATT TTGGTTCCGC AGGGGAGTTC CTGTCTTCTA TTGGTAAGGC ACTGCATACC GTGCTGGGGG  
 TAGCTTGCTA ACTGCCAATA GCCGCTCGTG CGTACCCTAA AACCAAGGCG TCCCCTAAG GACAGAAGAT AACCATTCG TGACGTATGG CACGACCCCC  
 Hypr E protein

2301 . A F N S I F G G V G F L F K L L L G V A L A W L G L N M R N P T M .  
 GCGATTCAA TTCTATTTTC GGGGGCGTGG GGTTCCTGCC TAAACTCCTG CTGGGAGTAG CCCTGGCCTG GTTGGGACTG AATATGCGGA ATCCGACGAT  
 CGCGTAAGTT AAGATAAAAG CCCCCGCACC CCAAGGACGG ATTTGAGGAC GACCCTCATC GGGACCGGAC CAACCTGAC TTATACGCT TAGGCTGCTA  
 Hypr E protein

NS1 gene of YF17D

2401 . S M S F L L A G V L V L A M T L G V G A D Q G C A I N F G K R E L  
 GTCCATGTCA TTCTCTTGG CCGGCGTGCT TGTACTGGCC ATGACACTGG GCGTTGGCGC CGATCAAGGA TCGGCCATCA ACITTTGGCAA GAGAGAGCTC  
 CAGGTACAGT AAGGAGAACC GGCCGCACGA ACATGACCGG TACTGTGACC CGCAACCGCG GCTAGTTCCT ACGCCGTAGT TGAACCGTT CTCTCTCGAG

## CV-LGTV E5 with YFV/TBEV chimeric signal (p43)

5' UTR

1 AGTAAATCCT GTGTGCTAAT TGAGGTGCAT TGGTCTGCAA ATCGAGTTGC TAGGCAATAA ACACATTTCG ATTAATTTTA ATCGTTCGTT GAGCGATTAG  
 TCATTTAGGA CACACGATTA ACTCCACGTA ACCAGACGTT TAGCTCAACG ATCCGTTATT TGTGTAAACC TAATTAAAAT TAGCAAGCAA CTCGCTAATC  
 5' UTR

C protein YF17D

101 M S G R K A Q G K T L G V N M V R R G V R S L S N K I K  
 CAGAGAACTG ACCAGAACAT GTCGTGTCGT AAAGCTCAGG GAAAAACCCT GGGCGTCAAT ATGGTACGAC GAGGAGTTTC CTCCTGTGCA AACAAATATA  
 GTCTCTTGAC TGGTCTTGTA CAGACCAGCA TTTCGAGTCC CTTTTTGGGA CCCGCACTTA TACCATGCTG CTCCTCAAGC GAGGAACAGT TTGTTTTATT  
 C protein YF17D

201 . Q K T K Q I G N R P G P S R G V Q G F I F F F L F N I L T G K K I  
 AACAAAAAC AAAACAAATT GGAACAGAC CTGACCTTC AAGAGGTGTT CAAGGATTA TCTTTTCTT TTTGTTCAAC ATTTTGACTG GAAAAAAGAT  
 TTGTTTTTGG TTTTGTITAA CCTTTGTCTG GACCTGGAG TTCTCCACAA GTTCCTAAAT AGAAAAAGAA AAACAAGTTG TAAACTGAC CTTTTTCTA  
 C protein YF17D

301 . T A H L K R L W K M L D P R Q G L A V L R K V K R V V A S L M R G  
 CACAGCCAC CTAAGAGGT TGTGGAAT GCTGGACCCA AGACAAGGCT TGGCTGTTCT AAGGAAAGTC AAGAGAGTGG TGGCCAGTTT GATGAGAGGA  
 GTGTGGGTG GATTTCTCCA ACACCTTTTA CGACCTGGGT TCTGTCCGA ACCGACAAGA TTCCTTTTCA TTCTCTCACC ACCGGTCAAA CTACTCTCCT  
 C protein YF17D TBEV partial signal

YF 17D partial signal

prM protein Langat E5

401 L S S R K R R S H D V L T V Q P L I L G M L G M T I A A T V R R E R  
 TTGTCTCAA GGAACGCCG TTCCATGAT GTTCGACTG TGCAATTCCT AATTTTGGGC ATGCTGGGGA TGACGATCGC AGCTACTGTG CGAAGGGAGA  
 AACAGGAGTT CCTTTGCGGC AAGGTACTA CAAGACTGAC ACGTAAAGGA TTAACACCCG TACGACCCCT ACTGCTAGCG TCGATGACAC GCTTCCTCT  
 prM protein Langat E5

501 . D G S M V I R A E G R D A A T Q V R V E N G T C V I L A T D M G S  
 GAGACGGCTC TATGGTGATC AGAGCCGAAG GTAGGGACGC TCGACCCAG GTGAGGGTCG AAAATGGCAC CTGTGTTATT CTGGGACCG ACATGGGCTC  
 CTCTGCGGAG ATACCACTAG TCTCGGCTTC CATCCCTGCG ACGCTGGGT CACTCCAGC TTTTACCGTG GACACAATAA GACCGCTGGC TGTACCCGAG  
 prM protein Langat E5

601 . W C D D S L A Y E C V T I D Q G S E P V D V D C F C R G V E K V T  
 CTGGTGTGAT GATTTCTGCG CTTATGAATG TGTTACTATT GATCAGGGTG AAGAGCTGT GGACGTGGAC TGTTCCTGTA GAGGCGTCGA GAAAGTGACC  
 GACCACACTA CTAAGAGACC GAATACCTAC ACAATGATAA CTAGTCCAC TTCTCGACA CTRGACCTG ACRAAGACAT CTCGCGAGCT CTTTCACTGG

prM protein Langat E5

701 L E Y G R C G R R E G S R S R R S V L I P S H A Q R D L T G R G H Q  
CTGGAATATG GACGATGTGG CCGGCGAGAA GGCTCCAGGA GTCCGAGATC CGTGTGTATC CCTTCACATG CGCAGCGCGA TCTGACAGGG AGGGGTCCACC  
GACCTTATAC CTGCTACACC GCGCGCTCTT CCGAGGTCTCT CAGCCTCTAG GCACAACAG GGAAGTGTAC GCGTCGCGCT AGACTGTCCC TCCCCAGTGG  
prM protein Langat E5

801 W L E G E A V K A H L T R V E G W V W K N K L F T L S L V M V A W  
AGTGGCTCGA AGGCGAAGCA GTCAAGGCC ATCTGACTCG CGTTGAGGC TGGGTGTGGA AAAACAACT CTTTACCCCT AGCCTGTGTA TGGTGCCTG  
TCACCGAGCT TCCGCTTCGT CAGTCCCGGG TAGACTGAGC GCAACTTCG ACCCACACCT TTTGTGTTGA GAAATGCGAA TCGGACCACT ACCAGCGCAC  
prM protein Langat E5

E protein Langat E5

901 L M V D G L L P R I L I V V V A L A L A P A Y A S R C T H L E N R  
GCTGATGGTA GACGACTCC TCCCCGCAT TCTCATGTGT GTGGTGGCTC TCGCGCTCGC CCGTGCATAC GCGTCCAGGT GTACGCACCT CGAAAATCGA  
CGACTACCAT CTGCTGAGG AAGGGGCGTA AGAGTAACAA CACCACCGAG AGCGCGAGCG GGGACGTATG CGCAGGTCCA CATGCGTGA GCTTTTAGCT  
E protein Langat E5

1001 D F V T G V Q G T T R L T L V L E L G G C V T V T A D G K P S L D V  
GATTTCTCA CAGGCGTCCA AGGTACTACC CGGCTCACCG TCGTGTGGA GCTGGGAGGC TGTGTCACTG TTACAGCCGA CGGAAAACCT AGTCTGATG  
CTAAAGCAGT GTCCGCGAGT TCCATGATGG GCCGAGTGGG AGCAGACCT CGACCTCCG ACACAGTGAC AATGTCGGCT GCCTTTTGA TCAGACCTAC  
E protein Langat E5

1101 W L D S I Y Q E S P A Q T R E Y C L H A K L T G T K V A A R C P T  
TGTGGCTGGA CTCATCTAT CAGGAGAGCC CGGCACAGAC CAGGGAGTAC TGCTCCACG CTAAGCTGAC TGGGACAAAG GTAGCCGCA GATGTCCAC  
ACACCGACCT GAGGTAGATA GTCTCTCGG GCCGTGTCTG GTCCCTCATG ACAGAGGTGC GATTGCACTG ACCCTGTTC CATCGCGGT CTACAGGGTG  
E protein Langat E5

1201 M G P A T L P E E H Q S G T V C K R D Q S D R G W G N H C G L F G  
AATGGGGCTT GCCACCTTGC CCGAGGAACA CCAATCCGGT ACGGTATGCA AGCGAGATCA GTCTGATCGC GGATGGGGGA ATCATTGCGG CCTCTTCGGT  
TTACCCCGGA CGGTGGAACG GGCTCCTTGT GGTTAGGCCA TGCCATACCT TCGCTCTAGT CAGACTAGCG CTTACCCCTT TAGTAACGCC GGAGAAGCCA  
E protein Langat E5

1301 K G S I V T C V K V T C E D K K K A T G H V Y D V N K I T Y T I K V  
AAAGGCAGCA TTGTCATG CGTGAAGGTG ACATGCGAGG ACAAGAAGAA GGCCACAGGT CATGTATATG ATGTGAACAA AATCAGATAT ACCATTAGG  
TTTCCGTCTT AACAGTGAAC GCATTCCAC TGTACGCTCC TGTCTTCTT CCGGTGTCCA GTACATATAC TACACTTGT TTAGTGTATA TGGTAATTCC  
E protein Langat E5

1401 E P H T G E F V A A N E T H S G R K S A S P T V S S E K T I L T L  
TAGRACCACA TACAGGGGAA TTCGTGGCAG CAAACGAGAC TCATAGCGGA CGAAAGTCCG CCTCCTTCAC CGTCTCCTCC GAGAAAACAA TCCTGACCCT  
ATCTTGGTGT ATGTCCCTT AAGCACCGTC GTTGTCTCTG AGTATCGCCT GCTTTACAGG GAGGGAAGTG GCAGAGGAGG CTCTTTTGT AGGACTGGGA  
E protein Langat E5

1501 G D Y G D V S L L C R V A S G V D L A Q T V V L A L D K T H E H L  
CGGAGACTAC GGCAGGTAT CTTTGTCTG CAGGTGTGCC AGCGCGGTGG ACCTTGCTCA GACAGTGTG TTGGCCCTGG ACAAGACACA TGAGCACTTG  
GCCTCTGATG CCGCTGCATA GAAACGACAC GTCCCAACCG TCGCGCACC TGAACAGAGT CTGTACGAC AACCGGGACC TGTCTGTGT ACTCGTGAAC  
E protein Langat E5

1601 P T A W Q V H R D W F N D L A L P W K H D G A E A W N E A G R L V E  
CCAACAGCCT GGCAGGTGCA CAGGACTG TTTAACGACC TGGCGCTCCC GTGGAACAT GACGGCGCTG AAGCATGGAA TGAGGCAGG AGACTGGTGG  
GGTGTCTGGA CCGTCCAGT GTCCCTGACC AATTTGTGG ACCGCGAGG CACCTTGTA CTGCCGCGAC TTCGTACCTT ACTCCGTCCC TCTGACCACC  
E protein Langat E5

1701 · F G T P H A V K M D V F N L G D Q T G V L L K S L A G V P V A S I ·  
 AATTTGGAAC CCCACACGCC GTAAAGATGG ACGTTTTCAA TCTTGGTGAC CAGACAGGGG TGCTCCTGAA ATCACTGGCG GCGGTGCCTG TAGCCAGCAT  
 TTAAACCTTG GGGTGTGCGG CATTCTAC TCACAAAAGTT AGAACCACTG GTCTGTCCCG ACGAGGACTT TAGTGACCGC CCGCACGGAC ATCGGTCTGA  
 E protein Langat E5

1801 · E G T K Y H L K S G H V T C E V G L E K L K M K G L T Y T V C D K ·  
 CGAGGGCACA AAGTATCACC TGAAGTCTGG GCATGTAACC TGGGAAGTGG GCCTGGAAAA GCTGAAGATG AAAGGACTTA CGTACACTGT TTGTGATAAG  
 GCTCCCGTGT TTCATAGTGG ACTTCAGACC CGTACATTGG ACGCTTCACC CGGACCTTTT CGACTTCTAC TTTCTGAAAT GCATGTGACA AACACTATTG  
 E protein Langat E5

1901 T K F T W K R A P T D S G H D T V V M E V G F S G T R P C R I P V R ·  
 ACCAAGTTTA CATGGAAGCG AGCCCCAACG GATTCCGGCC ATGATACCGT CGTGATGAG GTTGGTTTCT CCGGCACCAG ACCATGTAGA ATACCAGTGA  
 TGGTTCAAAT GTACCTTCGC TCGGGGTGCG CTAAGGCGCG TACTATGGCA GCACTACCTC CAACCAAAGA GGCCGTGGTC TGGTACATCT TATGGTCACT  
 E protein Langat E5

2001 · A V A R G V P E V N V A M L I T P N P T M E N N G G G F I E M Q L ·  
 GAGCTGTGCG CCACGGTGTA CCCGAGGTAA ACGTGGCCAT GCTGATTACA CCGAATCCCA CTATGGAGAA CAATGGCGGA GGGTTCATCG AAATGCAGCT  
 CTCGACAGCG GGTGCCACAT GGGCTCCATT TGCACCGTA CCACTAATGT GGCTTAGGCT GATACCTCTT GTTACCGCCT CCCAAGTAGC TTTACGTGCA  
 E protein Langat E5

2101 · P P G D N I I Y V G D L D H Q W F Q K G S S I G R V L Q K T R K G ·  
 GCCGCTGGA GACAACATCA TTTATGTCGG CGACCTCGAT CATCAATGGT TCCAGAAAGG GTCTTCCATC GGCCGCGTCC TTCAGAAGAC ACGAAAAGGC  
 CGCCGACCT CTGTTGTAGT AAATACAGCC GCTGGAGCTA GTAGTTACCA AGGTCTTTCC CAGAAGGTAG CCGGCGCAGG AAGTCTTCTG TGCTTTTCCG  
 E protein Langat E5

2201 I E R L T V L G E H A W D F G S V G G V M T S I G R A M H T V L G G ·  
 ATTGAAAGAC TTACAGTCCT GGGCGAACAT GCCTGGGACT TCGGGTCAGT TGGCGGGGTA ATGACAAGCA TAGGCAGAGC TATGCACACC GTTCTCGGTG  
 TAACTTTCTG AATGTGAGGA CCCGCTTGTA CGGACCTGTA AGCCCACTCA ACCGCCCCAT TACTGTTCGT ATCCGTCTCG ATACGTGTGG CAAGAGCCAC  
 E protein Langat E5

2301 · A F N T L L G G V G F L P K I L L G V A M A W L G L N M R N P T L ·  
 GGGCAATTAA TACTCTGTTG GGTGGCGTGG GTTTTCTTCC GAAAATCCTG CTCGGTGTG CAATGGCCTG GCTTGGACTG AATATGCGCA ATCCTACACT  
 CCCGTAATT ATGAGACAAC CCACCGCACC CAAAAGAAGG CTTTTAGGAC GAGCCACAGC GTTACCGGAC CGAACCTGAC TTATACGGCT TAGGATGTGA  
 E protein Langat E5

NS1 gene of YF17D

2401 · S M G F L L S G G L V L A M T L G V G A D Q G C A I N F G K R E L ·  
 GAGTATGGG TTTCTTCTGT CAGGAGGCCT GGTCTGGCA ATGACTCTGG GAGTGGGCGC CGATCAAGGA TGCCCATCA ACTTGGCAA GAGAGAGCTC  
 CTCATACCCC AAAGAAGACA GTCTCCGGA CCAGGACCGT TACTGAGACC CTCACCGCG GCTAGTTCCT ACGCGGTAGT TGAAACCGTT CTCCTCTGAG

### CV-TBEV Hypr with YFV/TBEV chimeric signal and dC2 deletion in C protein (p59)

#### 5' UTR

1 AGTAAATCCT GTGTGCTAAT TGAGGTGCAT TGGTCTGCAA ATCGAGTTCG TAGGCAATAA ACACATTTGG ATTAATTTTA ATCGTTCGTT GAGCGATTAG  
 TCATTAGGA CACACGATTA ACTCCACGTA ACCAGACGTT TAGCTCAACG ATCCGTTAAT TGTGTAAACC TAATTAAAT TAGCAAGCAA CTCGCTAATC  
 5' UTR

#### C protein

101 M S G R K A Q G K T L G V N M V R R G V R S L S N K I K .  
 CAGAGAACTG ACCAGAACAT GTCTGGTCTG AAAGCTCAGG GAAAAACCCT GGGCGTCAAT ATGGTACGAC GAGGAGTTCG CTCCTTGTC AACAATAA  
 GTCTCTTGAC TGGTCTTGTA CAGACCAGCA TTTCGAGTCC CTTTTGGGA CCCGAGTTA TACCATGCTG CTCCTCAAGC GAGGAACAGT TTGTTTATT  
 dc2 deletion (PSR)

C protein

201 Q K T K Q I G N R P G G V Q G F I F F F L F N I L T G K K I T A H .  
 AACAAAAAC AAAACAATT GGAAACAGAC CTGGAGGTGT TCAAGGATTT ATCTTTTCT TTTTGTCAA CATTITGACT GGAAAAAGA TCACAGCCCA  
 TTGTTTTTIG TTTTGTAA CCTTGTCTG GACCTCCACA AGTTCCTAAA TAGAAAAAGA AAAACAAGT GTAAACTGA CCTTTTCT AGTGTGGGT  
 C protein

301 L K R L W K M L D P R Q G L A V L R K V K R V V A S L M R G L S S .  
 CCTAAAGAGG TTGTGGAAAA TGCTGGACCC AAGACAAGGC TTGGCTGTTT TAAGGAAAGT CAAGAGAGTG GTGGCCAGTT TGATGAGAGG ATTGTCCTCA  
 GGATTCTCC AACACCTTTT ACGACCTGGG TTCTGTCCG AACCGACAG ATTCTTTTCA GTTCTCTCAC CACCGGTCAA ACTACTCTCC TAACAGGAGT  
 YF17D partial signal

TBEV partial signal

C protein

Hypr prM protein

401 R K R R S H D V L T V Q F L I L G M L G M T I A A T V R K E R D G S .  
 AGGAAACGCC GTTCCCATGA TGTTCTGACT GTGCAATTCC TAAITTTGGG CATGCTGGGC ATGACAATCG CAGCTACGGT TCGCAAGGAA AGAGACGGCA  
 TCTTTTGGG CAAGGGTACT ACAAGACTGA CAGCTTAAGG ATTAAACCC GTACGACCCG TACTGTTAGC GTCGATGCCA AGCGTTCTT TCTCTGCCGT  
 Hypr prM protein

501 T V I R A E G K D A A T Q V R V E N G T C V I L A T D M G S W C D .  
 GTACGGTCAT ACGCGCGGAA GGTAAAGATG CCGCTACCCA AGTGAGAGTG GAAATGGTA CCTGCGTCAT TCTGGCCACC GACATGGGCT CTTGGTGTGA  
 CATGCCAGTA TCGCGCGCTT CCATTCTTAC GCGATGGGT TCACTCTCAC CTFTTACCAT GGACGCAGTA AGACCGGTGG CTGTACCCGA GAACCACACT  
 Hypr prM protein

601 D S L S Y E C V T I D Q G E E P V D V D C F C R N V D G V Y L E Y .  
 TGATAGCCTT TCTTATGAGT GCGTAACCAT AGATCAAGGT GAGGAACCTG TTGACGTGA TTGCTTCTGC CGAAACGTGG ATGGGGTGTA TCTCGAATAT  
 ACTATCGGAA AGAATACTCA CGCATTGGTA TCTAGTTCCA CTCCTTGGAC AACTGCAACT AACGAAGAGC GCTTTCACCC TACCCCATAT AGAGCTTATA  
 Hypr prM protein

701 G R C G K Q E G S R T R R S V L I P S H A Q G E L T G R G H K W L E .  
 GCACGGTGTG GTAACAAGA AGGAAGCAGA ACCAGAGGCT CAGTGTCTAT ACCCTCCAC GCTCAAGGAG AGCTGACCGG ACGGGGACAT AAATGGTGG  
 CCTGCCACAC CATTGTCTT TCTTCTGCTT TGGTCTGGA GTCACGAATA TGGGAGGGTG CGAGTTCTC TCGACTGGCC TGCCCCGTA TTTACCAACC  
 Hypr prM protein

801 G D S L R T R L T R V E G W V W R N R L L A L A M V T V V W L T L .  
 AGGGCGACTC ACTCCGAACA CATTGACCC GCGTCGAGG CTGGGTCTGG AAAAATCGGC TGTTGGCCCT CGCTATGGTG ACAGTGGTTT GGCTCAGCT  
 TCCGCTGAG TGAGGCTGT GTAACCTGGG CGCAGCTCC GACCCAGACC TTTTAGCCG ACAACCGGGA CGGATACCAC TCTCAGCAA CCGAGTGGCA  
 Hypr E protein

Hypr prM protein

901 E S V V T R V A V L V V L L C L A P V Y A S R C T H L E N R D F V .  
 GGAGTCTGTG GTTACTCGCG TGGCAGTGCT GGTGGTGCTC CTCGTCTTGG CCCCTGTCTA CGCGTCCAGG TGTACTCAT TGGAAAAAG AGATTTTGTC  
 CCTCAGACAC CAATGAGCGC ACCGTCACGA CCACCAAGAG GAGACAGAAC GGGGACAGAT GCGCAGGTCC ACATGAGTAA ACCTTTTGTC TCTAAACAG  
 Hypr E protein

T G T Q G T T R V T L V L E L G G C V T I T A E G K P S M D V W L D .

1001 ACCGGCACCC AGGGGACGAC TCGGGTAACC CTGGTGCTTG AACTGGGTGG TTGCGTTACT ATTACCGCTG AGGGCAAACC CTCTATGGAT GTGTGGCTGG  
TGGCCGTGGG TCCCTGCTG AGCCCATTTG GACCACGAAC TTGACCCACC AACGCAATGA TAATGGCGAC TCCCGTTTGG GAGATACCTA CACACCGACC  
Hypr E protein

1101 A I Y Q E N P A Q T R E Y C L H A K L S D T K V A A R C P T M G P  
ATGCAATCTA TCAGGAGAAT CCCGCACAAA CCAGGGAATA TTGCTTCTAC GCAAGCTGT CCGATACAAA GGTGCGGGCT AGGTGCCCAA CAATGGGACC  
TACGTTAGAT AGTCTCTCTA GGGCGTGT TTGCTCCCTT AACGGAAGTG CGTTTCGACA GGCTATGTTT CCAGCGCCGA TCCACGGGTT GTTACCCTGG  
Hypr E protein

1201 A T L A E E H Q G G T V C K R D Q S D R G W G N H C G L F G K G S  
GGCCACCTG GCGGAGGAAC ATCAGGGAGG TACAGTGTC AAACGGGACC AGAGTGATAG AGGCTGGGGT AATCACTGCG GCCTGTTCGG CAAGGAAGT  
CCGGTGGGAC CGCTCCTTG TAGTCCCTCC ATGTCACAG TTGCTCCCTG TCTCACTATC TCCGACCCCA TTAGTGACGC CGGACAAGCC GTTTCCTTCA  
Hypr E protein

1301 I V A C V K A A C E A K K K A T G H V Y D A N K I V Y T V K V E P H  
ATTGTCGCTT GCGTCAAGGC AGCCTGTGAG GCCAAAAGA AGGCTACTGG GCAGTCTAT GACGCCAACA AGATCGTTTA TACAGTGAAA GTGGAACCAC  
TAACAGCGAA CGCAGTTCG TCGACACTC CGGTTTTCT TCCGATGACC CGTCAGATA CTGCGGTGT TCTAGCAAT ATGCACTTT CACCTTGGTG  
Hypr E protein

1401 T G D Y V A A N E T H S G R K T A S F T V S S E K T I L T M G E Y  
ACACAGGGGA TTACGTGGCG GCCAACGAGA CTCATTCCGG TCGCAAAACG GCCAGCTTCA CCGTGTCTC CGAAAAGACC ATCCTCACTA TGGGGGAGTA  
TGTGTCCCTT AATGCACCG CGGTGCTCT GAGTAAGGCC AGCGTTTTG CGGTGGAAGT GGCACAGTAG GCTTTTCTGG TAGGAGTGAT ACCCCCTCAT  
Hypr E protein

1501 G D V S L L C R V A S G V D L A Q T V I L E L D K T V E H L P T A  
TGGCGACGTT TCTCTGCTCT GCCGGGTGGC TAGCGGAGTC GACCTGGCCC AGACAGTCAT CCTGGAAGTG GATAAAACAG TTGAGCATCT GCCTACCGCT  
ACCGCTGCAA AGAGACGAGA CGGCCACCG ATCGCCTCAG CTGGACCGGG TCTGTCACTA GGACCTTGAC CTATTTTGTC AACTCGTAGA CGGATGGCGA  
Hypr E protein

1601 W Q V H R D W F N D L A L P W K H E G A R N W N N A E R L V E F G A  
TGGCAGGTGC ACAGGGATTG GTTAAACGAC CTGCGCTGCG CATGGAAACA TGAAGGAGCG AGAACTGGA ATAATGCAGA GCGACTCGTA GAATTCGGTG  
ACCGTCCACG TGTCCCTAAC CAATTTGCTG GAACGGGACG GTACCTTTGT ACTTCCTCGC TCTTTGACCT TATTACGTCT CGCTGAGCAT CTTAAGCCAC  
Hypr E protein

1701 P H A V K M D V Y N L G D Q T G V L L K A L A G V P V A H I E G T  
CCCCTCATGC CGTGAAGATG GACGTCTACA ATCTGGGTGA TCAGACCGGC GTTCTCTTAA AAGCTCTCGC TGGCGTACCA GTTGGCCACA TCGAAGGAAC  
GGGGAGTAGC GCACCTCTAC CTGCAGATGT TAGACCCACT AGTCTGGCCG CAAGAGGAAT TTCGAGAGCG ACCGCAATGT CAACGGGTGT AGCTTCTCTG  
Hypr E protein

1801 K Y H L K S G H V T C E V G L E K L K M K G L T Y T M C D K T K F  
GAAGTACCAC CTGAAGTCAG GCCATGTAAC TTGCGAGGTG GGCCTGGAGA AGTTGAAAAT GAAAGGTCTT ACGTACACAA TGTTGACAA GACCAAGTTC  
CTTCATGGTG GACTTCAGTC CGGTACATTG AACGCTCCAC CCGGACCTCT TCAACTTTTA CTTTCCAGAA TGCATGTGTT ACACACTGTT CTGGTTCAAG  
Hypr E protein

1901 T W K R A P T D S G H D T V V M E V T F S G T K P C R I P V R A V A  
ACATGGAAGA GGGCCCCAC AGATAGCGGC CACGATACTG TGGTGATGGA GGTGACCTTT TCTGGAACAA AACCTGCGAG AATACCGTG CGGGCTGTAG  
TGTACCTTCT CCCGGGGTG TCTATCGCCG GTGCTATGAC ACCACTACCT CCACCTGAAA AGACCTTGTT TTGGGACGTC TTATGGGCAC GCCCGACATC  
Hypr E protein

2001 H G S P D V N V A M L I T P N P T I E N N G G G F I E M Q L P P G  
CTCACGGATC TCCCGATGTC AATGTTGCTA TGCTGATTAC ACCTAACCTT ACCATCGAGA ATAACGGTGG TGGTTTTAIT GAGATGCAGC TTCCGCCAGG  
GAGTGCCTAG AGGGCTACAG TTACAACGAT ACGACTAATG TGGATTGGGA TGGTAGCTCT TATTGCCACC ACCAAAATAA CTCTACGTG AAGGCGGTCC  
Hypr E protein

2101 · D N I I Y V G E L S Y Q W F Q K G S S I G R V F Q K T K K G I E R  
 CGATAACATC ATCTACGTGG GCGAACTCTC TTACCACTGG TTTCAGAAAG GGAGTTCAAT TGGGCGGGTC TTCCAAAAA CGAAGAAGGG AATCGAACGA  
 GCTATTGTAG TAGATGCACC CGCTTGAGAG AATGGTCACC AAAGTCTTTC CCTCAAGITA ACCGCGCCAG AAGGTTTTTT GCTTCTTCCC TTAGCTTGCT  
 Hypr E protein  
 -----  
 2201 L T V I G E H A W D F G S A G G F L S S I G K A L K T V L G G A E N  
 TTGACGGTTA TCGGCGAGCA CGCATGGGAT TTGGTTCCG CAGGGGGATT CCTGTCTTCT ATTGTAAGG CACTGCATAC CGTGCTGGGG GCGCGATTCA  
 AACTGCCAAT AGCCGCTCGT GCGTACCCTA AAACCAAGGC GTCCCCCTAA GGACAGAAGA TAACCATTCG GTGACGTATG GCACGACCCC CCGCGTAAGT  
 Hypr E protein  
 -----  
 2301 · S I F G G V G F L P K L L L G V A L A W L G L N M R N P T M S M S  
 ATTCTATTTT CCGGGGCGTG GGGTTCTGTC CTAACTCCT GCTGGGAGTA GCCCTGGCCT GGTTGGGACT GAATATGCGG AATCCGACGA TGTCCATGTC  
 TAAGATAAAA GCGCCCGCAC CCAAGGACG GATTTGAGGA CGACCCCTCAT CCGGACCGGA CCAACCCCTGA CTTATACGCC TTAGGCTGCT ACAGGTACAG  
 Hypr E protein  
 -----  
 NSI gene of YF17D  
 -----  
 2401 · F L L A G V L V L A M T L G V G A D Q G C A I N F G K R E L  
 ATTCTCTTGG GCGGCGTGC TTGTACTGGC CATGACACTG GCGGTGGCG CCGATCAAGG ATGCGCCATC AACTTTGGCA AGAGAGAGCT C  
 TAAGGAGAAC CGGCCGACG AACATGACCG GTACTGTGAC CCGCAACCGG GCGTAGITCC TACGCGGTAG TTGAACCGT TCTCTCTCGA G

## Sequence Appendix 3

### PIV-WN/TBEV Hypr with TBEV signal (p39)

deleted C

-----  
 S' UTR  
 -----  
 1 AGTAGTTGCG CTGTGTGAGC TGACAACTT AGTAGTGT TTGTGAGGATTA ACAACAATTA ACACAGTGCG ACCTGTTTCT TAGCAGGAAG ATCTCGATGT  
 TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCACAAA CACTCTTAAT TGTGTTAAT TGTGTCACCG TCGACAAAGA ATCGTGCTTC TAGAGCTACA  
 WN deleted C protein  
 -----  
 101 · K K P G G P G K S R A V Y L L K R G M P R V L S L I G L K R S S K  
 CTAAGAAACC AGGAGGGGCC GCGAAGAGCC GGGCTGTCTA TTGCTAAAA CCGGGAATGC CCGCGTGT GTCTTGATT GGACTTAAGC GGAGCTCCAA  
 GATTCTTGG TCCTCCCGGG CCGTCTCGG CCGACAGAT AAACGATTTT GCGCCTACG GGGCGCACAA CAGGAAGTAA CCTGAATTCG CCTCGAGGTT  
 TBEV signal  
 -----  
 deleted C prM Hypr  
 -----  
 201 · Q K K R G G T D W M S W L L V I G M L G M T I A A T V R K E R D G  
 ACAAAGAAA CCGGGGGGAA CAGACTGGAT GAGCTGGCTG CTCGTAATCG GCATGCTGGG CATGACAATC GCAGCTACGG TTGCAAGGA AAGAGACGGC  
 TGTITICTTT GCCCCCCCTT GTCTGACCTA CTCGACCGAC GAGCATTAGC CGTACGACCC GTACTGTAG CGTCCGATGC AAGCGTTCCT TTCTCTGCCG  
 prM Hypr  
 -----  
 301 S T V I R A E G K D A A T Q V R V E N G T C V I L A T D M G S W C D  
 AGTACGGTCA TACGCGCGGA AGGTAAGGAT GCCGCTACCC AAGTGAGAGT GGAAATGGT ACCTGCGTCA TTCTGGCCAC CGACATGGGC TCTTGGTGTG

TCATGCCAGT ATGCGCGCCT TCCATTCCCTA CGGCGATGGG TTCACTCTCA CCTTTTACCA TGGACGCAGT AAGACCGGTG GCTGTACCCG AGAACCACAC  
prM Hypr

401 D S L S Y E C V T I D Q G E E P V D V D C F C R N V D G V Y L E Y  
ATGATAGCCT TTCTTATGAG TGCCTAACCA TAGATCAAGG TGAGGAACCT GTTGACGTTG ATTGCTTCTG CCGAAACGTG GATGGGGTGT ATCTCGAATA  
TACTATCGGA AAGAATACTC ACGCATTGGT ATCTAGTTCC ACTCCTTGGG CAACCTGCAAC TAACGAAGAC GGCITTGCAC CTACCCACCA TAGAGCTTAT  
prM Hypr

501 G R C G K Q E G S R T R R S V L I P S H A Q G E L T G R G H K W L  
TGGACGGTGT GGTAAACAAG AAGGAAGCAG AACGAGACGC TCAGTGCCTA TACCCCTCCA CGCTCAAGGA GAGCTGACCG GACGGGGACA TAAATGGTTG  
ACCTGCCACA CCATTGTTC TTCTTCGTC TTGGTCTGCG AGTCACGAAT ATGGGAGGCT GCGAGTTCCT CTCGACTGGC CTGCCCCGTG ATTTACCAAC  
prM Hypr

601 E G D S L R T H L T R V E G W V W K N R L L A L A M V T V V W L T L  
GAGGGGCGACT CACTCCGAAC ACATTGACC CGGCTCGAGG GCTGGGTCTG GAAAANTCGG CTGTTGGCCC TCGCTATGGT GACAGTGGT TGGCTACCGC  
CTCCCGCTGA GTGAGGCTG TGTAACATGG GCGCAGCTCC CGACCCAGAC CTTTTTAGCC GACAACCGGG AGCGATACCA CTGTACAGAA ACCGAGTGGC  
E Hypr

prM Hypr

701 E S V V T R V A V L V V L L C L A P V Y A S R C T H L E N R D F V  
TGGAGTCTGT GGTACTCGC GTGGCAGTGC TGGTGGTGT CTTCTGTCTT GCCCTGTCT ACCTGCTCCAG GTGTACTCAT TTGGAAAACA GAGATTTTGT  
ACCTCAGACA CCAATGAGCG CACCGTCAGC ACCACCAGCA GGAGACAGAA CGGGGACAGA TGCGCAGGTC CACATGAGTA AACCTTTTGT CTCTAAAACA  
E Hypr

801 T G T Q G T T R V T L V L E L G G C V T I T A E G K P S M D V W L  
CACCGGCACC CAGGGGACGA CTGGGTAAC CTTGGTGTCT GAATGGGCTG GTTGGGTTAC TATTACCGCT GAGGGCAAAC CCTCTATGGA TGTGTGGCTG  
GTGGCCGTGG GTCCCTGCT GAGCCCATTG GGACCACGAA CTTGACCCAC CAACGCAATG ATAATGGCGA CTCCCGTTTG GGAGATACCT ACACACCGAC  
E Hypr

901 D A I Y Q E N P A Q T R E Y C L H A K L S D T K V A A R C P T M G P  
GATGCAATCT ATCAGGAGAA TCCCGCACAA ACCAGGGAAT ATTGCTTCA CGCAAGCTG TCGATACAA AGGTGCGGCG TAGGTGCCCA ACAATGGGAC  
CTACGTTAGA TAGTCTCTT AGGCGTGTG TGTCCCTTA TAACGGAAGT GCGTTTCGAC AGGCTATGTT TCCAGCGCGG ATCCACGGGT TGTACCCTG  
E Hypr

1001 A T L A E E H Q G G T V C K R D Q S D R G W G N H C G L F G K G S  
CGGCCACCTT GGCGGAGGAA CATCAGGAGG GTACAGTGTG CAAACGGGAC CAGAGTGATA GAGGCTGGGG TAATCACTGC GGCTGTTCG GCAAAGGAAG  
GCCGGTGGGA CCGCTCCTT GTAGTCCCTC CATGTCACAC GTTTGCCCTG GTCTCACTAT CTCCGACCCC ATTAGTGACG CCGGACAGG CGTTCCTTC  
E Hypr

1101 I V A C V K A A C E A K K K A T G H V Y D A N K I V Y T V K V E P  
TATTGTGCTT TGGTCAAGG CAGCCTGTGA GGCCAAAAG AAGGCTACTG GGCAGTCTA TGACGCCAAC AGATCGTTT ATACAGTGAA AGTGGGAACCA  
ATAACAGGGA ACGCAGTTC GTGGACACT CCGGTTTTT TTCCGATGAC CCGTGACAGT ACTGCGGTTG TTCTAGCAAA TATGTCATT TCACCTTGGT  
E Hypr

1201 H T G D Y V A A N E T H S G R K T A S F T V S S E K T I L T M G E Y  
CACACAGGGG ATTACGTGGC GGCCACGAG ACTCATTCCG GTCCGAAAAC GGCAGCTTC ACCGTGTCT CCGAAAAGAC CATCCTCACT ATGGGGGAGT  
GTGTGTCCCC TAATGCACCG CCGGTGCTC TGAGTAAGGC CAGGTTTTG CCGGTGGAAG TGGCACAGTA GGCTTTTCTG GTAGGAGTGA TACCCCTCA  
E Hypr

1301 G D V S L L C R V A S G V D L A Q T V I L E L D K T V E H L P T A  
ATGGCGACGT TTCCTGCTC TGGCGGTGG CTAGCGAGT CGACCTGGCC CAGACAGTCA TCCTGGAAC GGATAAACA GTTGAGCATC TGCCTACCGC  
TACCGCTGCA AAGAGACGAG ACGGCCACC GATCGCTCA GCTGGACCG GTCTGTGAGT AGGACCTGA CCTATTGTGT CAACCTGTAG ACGGATGGCG  
E Hypr

1401 W Q V H R D W F N D L A L P W K H E G A R N W N N A E R L V E F G  
 TTGGCAGGTG CACAGGGATT GGTTTAACGA CTTGCGCTG CCATGGAAC ATGAAGGAGC GAGAACTGG AATAATGCAG AGCGACTCGT AGAATTCGGT  
 AACCGTCCAC GTGTCCCTAA CCAAATGCT GGAACGGGAC GGTACCTTTG TACTTCCTCG CTCTTTGACC TTATTACGTC TCGCTGAGCA TCTTAAGCCA

E Hypr

1501 A P H A V K M D V Y N L G D Q T G V L L K A L A G V P V A H I E G T  
 GCCCCTCATG CCGTGAAGAT GGACGTCTAC AATCTGGGTG ATCAGACCGG CGTTCTCCTT AAAGCTCTCG CTGGCGTACC AGTTGCCAC ATCGAAGGAA  
 CGGGGAGTAC GGCACCTTCTA CCTGCAGATG TTAGACCCAC TAGTCTGGCC GCAAGAGGAA TTTCGAGAGC GACCGCATGG TCAACGGGTG TAGCTTCCTT

E Hypr

1601 K Y H L K S G H V T C E V G L E K L K M K G L T Y T M C D K T K F  
 CGAAGTACCA CCTGAAGTCA GGCCATGTAA CTGCGAGGT GGGCCTGGAG AAGTTGAAAA TGAAAGGTCT TACGTACACA ATGTGTGACA AGACCAAGTT  
 GGTTCATGGT GGACTTCAGT CCGGTACATT GAACGCTCCA CCGGACCTC TTCACTTTT ACTTTCCAGA ATGCATGTGT TACACACTGT TCTGGTTCAA

E Hypr

1701 T W K R A P T D S G H D T V V M E V T F S G T K P C R I P V R A V  
 CACATGGAAG AGGGCCCCCA CAGATAGCGG CCACGATACT GTGGTGTATG AGGTGACCTT TTCTGGAACA AAACCTTGCA GAATACCCGT GCGGGCTGTA  
 GTGTACCTTC TCCCGGGGT GTCTATCGCC GGTGCTATGA CACCACTACC TCCACTGGAA AAGACCTTGT TTTGGGACGT CTTATGGGCA CGCCCGACAT

E Hypr

1801 A H G S P D V N V A M L I T P N P T I E N N G G G F I E M Q L P P G  
 GCTCAGGAT CTCCGATGT CAATGTTGCT ATGCTGATTA CACCTAACCC TACCATCGAG AATAACGGTG GTGGTTTTAT TGAGATGCAG CTTCCGCCAG  
 CGAGTGCCTA GAGGCTTACA GTTACAACGA TACGACTAAT GTGGATTGGG ATGGTAGCTC TTATTGCCAC CACCAAATA ACTCTACGTC GAAGGCGGTC

E Hypr

1901 D N I I Y V G E L S Y Q W F Q K G S S I G R V F Q K T K K G I E R  
 GCGATAACAT CATCTACGTG GCGGAACCTCT CTTACCAAGT GTTTCAGAAA GGGAGTTCAA TTGGGCGGGT CTTCCAAAAA ACGAAGAAGG GAATCGAACG  
 CGCTATTGTA GTAGATGCAC CCGCTTGAGA GAATGGTCAC CAAAGTCTTT CCCTCAAGTT AACCCGCCCA GAAGGTTTTT TGCTTCTTCC CTTAGCTTGC

E Hypr

2001 L T V I G E H A W D F G S A G G F L S S I G K A L H T V L G G A P  
 ATTGACGGTT ATCGGCGAGC ACGCATGGGA TTTTGGTTCC GCAGGGGGAT TCCTGTCTTC TATTGGTAAG GCACTGCATA CGTGCTGGG GGGCGCATTC  
 TAACTGCCAA TAGCGCTCG TCGGTACCTT AAAACCAAGG CGTCCCTTA AGGACAGAAG ATAACCATC CGTGACGTAT GGCACGACCC CCGCGTAAG

E Hypr

2101 N S I F G G V G F L P K L L L G V A L A W L G L N M R N P T M S M S  
 AATTCATTT TCGGGGGCGT GGGGTTCTG CCTAACTCC TGCTGGGAST AGCCCTGGCC TGGTGGGAC TGAATATGCG GAATCCGACG ATGTCCATGT  
 TTAAGATAAA AGCCCCGCA CCCCAGGAC GGATTTGAGG ACGACCCTCA TCGGACCGG ACCAACCTG ACTTATACGC CTTAGGCTGC TACAGGTACA

E Hypr

WNV NS1 protein

2201 F L L A G V L V L A M T L G V G A D T G C A I D I S R Q  
 CATTCTCTT GCGCGGCGTG CTTGTACTGG CCATGACACT GGGCGTTGGC CCGGACACTG GGTGTGCCAT AGACATCAGC CGGCAA  
 GTAAGGAGAA CCGGCCGCAC GAACATGACC GGTACTGTGA CCGCAACCG CGGCTGTGAC CCACACGGTA TCTGTAGTCG GCCGTT

# PIV-WN/TBEV Hypr with WNV signal (p40)

deleted C

5' UTR

1 AGTAGTTCGC CTGTGTGAGC TGACAAACTT AGTAGTGTTC GTGAGGATTA ACAACAATTA ACACAGTGGC AGCTGTTTCT TAGCACGAAG ATCTCGATGT  
TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCACAAA CACTCCTAAT TGTTGTTAAT TGTGTCACGC TCGACAAAGA ATCGTGCTTC TAGAGCTACA  
WNV deleted C

101 K K P G G P G K S R A V Y L L K R G M P R V L S L I G L K R S S K  
CTAAGAAACC AGGAGGGGCC GGCAAGAGCC GGGCTGTCTA TTGCTATAAA CGCGGAATGC CCCGCGTGT GTCCCTTGATT GGACTTAAGC GGAGCTCCAA  
GATTCTTTGG TCCTCCCGGG CCGTTCTCGG CCGACAGAT AAACGATTTT GCGCTTACG GGGCGCACAA CAGGAACATA CCTGAATTCG CCTCGAGGTT  
WNV signal

WNV deleted C

prM Hypr

201 Q K K R G G K T G I A V M I G M L A C V G A A T V R K E R D G S T  
GCAAAAGAAA CGCGGGGGAA AGACAGGCAT AGCTGTGATG ATAGGCATGC TGGCTTGTTG CCGAGCAGCT ACCGTGCGAA AAGAACGCGA CGGAAGCACC  
CGTTTTCTTT GCGCCCCCTT TCTGTCCGTA TCGACACTAC TATCCGTACG ACCGAACACA GCCTCGTCCA TGGCAGCGCT TTCTTGCGCT GCCTTCGTGG  
prM Hypr

301 V I R A E G K D A A T Q V R V E N G T C V I L A T D M G S W C D D S  
GTGATAAGGG CTGAGGGTAA GGATGCGGCT ACGCAGGTGA GAGTAGAGAA TGGCACTTGC GTAATACTCG CGACTGATAT GGGATCCTGG TGTGACGATA  
CACTATTCCC GACTCCCAT CTACGCGGA TGGCTCCACT CTCATCTCTT ACCGTGAACG CATTATGAGC GCTGACTATA CCTAGGACC AACTGCTAT  
prM Hypr

401 L S Y E C V T I D Q G E E P V D V D C F C R N V D G V Y L E Y G R  
GCCTCAGTTA TGAATGCGTA ACAATAGACC AGGGCGAAGA ACCTGTGGAC GTTGACTGTT TCTGTAGAAA TGTGGATGGC GTTATCTGG AGTACGGCCG  
CGGAGTCAAT ACTTACGCAT TGTATCTGG TCCCGCTTCT TGGACACCTG CAATGACAAA AGACATCTTT ACACCTACCG CAAATAGACC TCATGCGGCG  
prM Hypr

501 C G K Q E G S R T R R S V L I P S H A Q G E L T G R G H K W L E G  
CTGTGGAAAA CAGGAGGGCT CACGAACCTG AAGATCTGTG CTGATTCCAA GTCACGCGCA AGGAGAGTTG ACCGGTAGAG GCCACAAAGT GCTTGAAGCG  
GACACCTTTT GTCTCCCGA GTGCTTGAGC TTCTAGACAC GACTAAGGTT CAGTGCAGGT TCCTCTCAAC TGGCCATCTC CGGTGTTTAC CGAATCTCCC  
prM Hypr

601 D S L R T H L T R V E G W V W K N R L L A L A M V T V V W L T L E S  
GACTCATTTA GGACCCACCT GACTAGGGTG GAGGGTTGGG TTTGGGAAGAA TCGGTTGCTC GCGCTCGCTA TGGTCACCGT CGTGTGGCTG AACTGGGAGA  
CTGAGTAACT CCTGGGTGGA CTGATCCAC CTCCCAACCC AAACCTTCTT AGCCAACGAG CGCGAGCGAT ACCAGTGGCA GCACACCGAC TGTGACCTCT  
E Hypr

prM Hypr

701 V V T R V A V L V V L L C L A P V Y A S R C T H L E N R D F V T G  
GTGTCGTGAC TCGGGTTGCT GTGTTGGTTG TCCTCTCTG TTTGGCCCCA GTGTACCGT CCAGGTGTAC TCATTGGAA AACAGAGATT TTGTCACCGG  
CACAGCACTG AGCCCAACGA CACAACCAAC AGGAGGAGAC AAACCGGGT CACATGCGCA GGTCCCATG AGTAAACCTT TTGCTCTAA AACAGTGGCC  
E Hypr

801 · T Q G T T R V T L V L E L G G C V T I T A E G K P S M D V W L D A  
 CACCCAGGGG ACGACTCGGG TAACCTGGT GCTTGAACG GGTGGTGGC TTACTATTAC CGCTGAGGGG AAACCTCTA TGGATGTGTG GCTGGATGCA  
 GTGGGTCCCC TGCTGAGCCC ATTTGGGACCA CGAACTTGAC CCACCAACGC AATGATAATG GCGACTCCCG TTGCGGAGAT ACCTACACAC CGACCTACGT  
 E Hypr

901 I Y Q E N P A Q T R E Y C L H A K L S D T K V A A R C P T M G P A T  
 ATCTATCAGG AGAATCCCCG ACAACCAGG GAATATTGCC TTCACGCAAA GCTGTCCGAT ACAAGGTCG CGGCTAGGTG CCCAACAATG GGACCGGCCA  
 TAGATAGTCC TCITAGGGCG TGTITGGTCC CTATATAACG AAGTGCCTTT CGACAGGCTA TGTTCACAG GCCGATCCAC GGGTTGTITAC CTTGGCCGGT  
 E Hypr

1001 · L A E E H Q G G T V C K R D Q S D R G W G N H C G L F G K G S I V  
 CCCTGGCCGA GGAACATCAG GGAGGTACAG TGTGCAACG GGACCAAGT GATAGAGGCT GGGGTAAATCA CTCCGGCCCTG TTCGGCAAG GAAGTATTGT  
 GGGACCGCCT CCTTGTAGTC CCTCCATGTC ACACGTTTGC CTTGGTCTCA CTATCTCCGA CCCCATTAGT GACGCGGAC AAGCCGTTTC CTTCAATACA  
 E Hypr

1101 · A C V K A A C E A K K K A T G H V Y D A N K I V Y T V K V E P H T  
 CGCTTGGCTC AAGGCAGCCT GTGAGGCCAA AAAGAAGGCT ACTGGGCACG TCTATGACGC CAACAAGATC GTTTATACAG TGAAAGTGA ACCACACACA  
 GCGAACCGAG TTCCGTCGGA CACTCCGGT TTTCTTCCGA TGACCCGTGC AGATACTGCG GTTGTCTAG CAAATATGTC ACITTCACCT TGGTGTGTGT  
 E Hypr

1201 G D Y V A A N E T H S G R K T A S F T V S S E K T I L T M G E Y G D  
 GGGGATTACG TGGCGGCCAA CGAGACTCAT TCCGGTCGCA AAACCGCCAG CTTCACCGTG TCATCCGAAA AGACCATCCT CACTATGGGG GAGTATGGCG  
 CCCCTAATGC ACCGCCGGT GCTCTGAGTA AGGCCAGCGT TTTGCCGGTC GAAGTGGCAC AGTAGGCTTT TCTGGTAGGA GTGATACCCC CTCATACCCG  
 E Hypr

1301 · V S L L C R V A S G V D L A Q T V I L E L D K T V E H L P T A W Q  
 ACGTTTCTCT GCTCTGCCGG GTGGCTAGCG GAGTCGACCT GGCCGAGACA GTCATCTGG AACTGGATAA AACAGTTGAG CATCTGCCTA CCGCTTGGCA  
 TGCAAGAGA CGAGACGGCC CACCGATCGC CTCAGCTGGA CCGGGTCTGT CAGTAGGACC TTGACCTATT TTGTCAACTC GTAGACGGAT GGCGAACCGT  
 E Hypr

1401 · V H R D W F N D L A L P W K H E G A R N W N N A E R L V E F G A P  
 GGTGCACAGG GATTGGTTTA ACGACCTTGC CTGCCATGG AAACATGAAG GAGCGAGAAA CTGGAATAAT GCAGAGCGAC TCGTAGAATT CGGTGCCCTT  
 CCACGTGTCC CTAACCAAT TGCTGGAACG GGACGGTACC TTTGTACTTC CTCGCTCTTT GACCTTATTA CGTCTCGCTG AGCATCTTAA GCCACGGGGA  
 E Hypr

1501 H A V K M D V Y N L G D Q T G V L L K A L A G V P V A H I E G T K Y  
 CATGCCGTGA AGATGGACGT CTACAATCTG GGTGATCAGA CCGCGCTTCT CTTAAAGCT CTCGCTGGCG TACCACTTGC CCACATCGAA GGAACGAAGT  
 GTACGGCACT TCTACTGCA GATGTTAGAC CCACTAGTCT GGCCGCAAGA GGAATTTGGA GAGCGACCGC ATGGTCAACG GGTGTAGCTT CTTGCTTCA  
 E Hypr

1601 · H L K S G H V T C E V G L E K L K M K G L T Y T M C D K T K P T W  
 ACCACCTGAA GTCAGGCCAT GTAACCTGCG AGGTGGGCCT GGAGAAGTTG AAAATGAAG GTCTTACGTA CACAATGTGT GACAAGACCA AGTTCACATG  
 TGGTGGACTT CAGTCCGTA CATGAACGC TCCACCCGGA CCTCTTCAAC TTTTACTTTC CAGAATGCAT GTGTACACA CTGTTCTGGT TCAAGTATC  
 E Hypr

1701 · K R A P T D S G H D T V V M E V T F S G T K P C R I P V R A V A H  
 GAAGAGGGCC CCCACAGATA GCGGCCACCA TACTGTGGTG ATGGAGGTGA CCTTTTCTGG AACAAAACCC TGCAGAATAC CCGTGCGGGC TGTAGCTCAC  
 CTTCTCCCGG GGGTGTCTAT CGCCGGTGT ATGACACCAC TACCTCCACT GGAAAAGACC TTGTTTGGG ACGTCTTATG GGCACGCCCG ACATCGAGTG  
 E Hypr

1801 G S P D V N V A M L I T P N P T I E N N G G G F I E M Q L P P G D N  
 GGATCTCCCG ATGTCAATGT TGCTATGCTG ATTACACCTA ACCCTACCAT CGAGAATAAC GGTGGTGGT TTATTGAGAT GCAGCTTCCG CCAGGCCATA  
 CCTAGAGGGC TACAGTTACA ACGATACGAC TAATGTGAT TGGGATGGTA GCTCTTATTG CCACCACCA AATAACTCTA CGTCGAAGGC GGTCCGCTAT  
 E Hypr

.....  
 · I I Y V G E L S Y Q W F Q K G S S I G R V F Q K T K K G I E R L T ·  
 1901 ACATCATCTA CGTGGGCGAA CTCCTTACC AGTSGTTTC GAAAGGGAGT TCAATTGGGC GGGTCTTCCA AAAAACGAAG AAGGGAATCG AACGATTGAC  
 TGTAGTAGAT GCACCCGCTT GAGAGAATGG TCACCAAAGT CTTCCCTCA AGTTAACCCG CCCAGAAGGT TTTTGTCTTC TTCCCTTAGC TTGCTAAGT  
 E Hypr

.....  
 · V I G E H A W D F G S A G G F L S S I G K A L H T V L G G A F N S  
 2001 GGTATCGGC GAGCAGCAT GGGATTTGG TTCCGCAGGG GGATTCCTGT CTTCTATTGG TAAGGCACTG CATACCGTGC TGGGGGGCGC ATTCAATTCT  
 CCAATAGCCG CTCGTCCGTA CCCTAAAACC AAGGCGTCCC CCTAAGGACA GAAGATAACC ATTCCGTGAC GTATGGCAGC ACCCCCCGCG TAAGTTAAGA  
 E Hypr

.....  
 I F G G V G F L P K L L L G V A L A W L G L N M R N P T M S M S F L ·  
 2101 ATTTTCGGGG GCGTGGGGT CCTGCCTAAA CTCCTGCTGG GAGTAGCCCT GGCCTGGTGG GGAAGAATA TGCGGAATCC GACGATGTCC ATGTCATTCC  
 TAAAGCCCC CGCACCCCAA GGACGGATTG GAGGACGACC CTCATCGGGA CCGGACCAAC CCTGACTTAT ACGCCTTAGG CTGCTACAGG TACAGTAAGG  
 E Hypr

WNV NS1 protein

.....  
 · L A G V L V L A M T L G V G A D T G C A I D I S R Q  
 2201 TCTTGGCCGG CGTGCTTGTA CTGGCCATGA CACTGGGCGT TGGCCCGGAC ACTGGGTGTG CCATAGACAT CAGCCGGCAA  
 AGAACCGGCC GCACGAACAT GACCGGTACT GTGACCCGCA ACCCGGCTG TGACCCACAC GGTATCTGTA GTCGGCGGT

# Sequence Appendix 4. WN PIV constructs expressing rabies virus G protein.

## WN ( $\Delta$ CprME)-Rabies PIV sequence (partial)

N-terminus of C  
-----

5' UTR

M S

1 AGTAGTTCGC CTGTGTGAGC TGACAAACTT AGTAGTGTTC GTGAGGATTA ACAACAATTA ACACAGTGGC AGCTGTTTCT TAGCAGCAAG ATCTCGATGT  
TCATCAGCG GACACACTCG ACTGTTTGA TCTATCAAAA CACTCCCTAAT TGTGTTTAA TGTGTCACGC TCGACAAAGA ATCGTGCTTC TAGAGCTACA  
N-terminus of C

-----

101 CTAAGAAACC AGGAGGGGCC GSCAAGAGCC GGGCTGTCTA TTTGCTAAAA CGCGGAATGC CCCGGGTGTT GTCCCTGATT GSACTTACG AAAAGAAGCG  
GATTCCTTGG TCCCTCCCGG CCGTCTCGG CCGACAGAT AAGCGATTTT GCGCCTTACG GGGCGCACAA CAGGAACATA CCTGAAATCG TTTTCTTCGC  
N-terminus of C Rabies-G signal

-----

partial C signal

Rabies-G protein

-----

201 AGGGGGCAAG ACTGGTATAG CTGTGATCGT TCCTCAGGCT CTTTGTGTTG TACCTTGGCT GGTATTTCCT CTTGCTTTG GTAAATTTC TATCTATACC  
TCCCCCGTTC TGACCATATC GACACTAGCA AGGAGTCGSA GAAACAAC ATGGGAACGA CCATTAAGGG GAAACGAAAC CATTAAAGG ATAGATATGG  
Rabies-G protein

-----

301 ATCCCTGATA AGCTCGGGCC TTGGAGTCCC ATTGATATTC ACCATTGAG CTGCCCAAC AACCTGTCG TTGAGGATGA AGGGTGCAC TATCTTCTG  
TAGGGACTAT TCGAGCCCGG AACCTCAGG TAACATATAAG TGTAAACT GACCGGTTG TTGGAGCAGC AACTCCTACT TCCACGTGA TTAGAAAGAC  
Rabies-G protein

-----

401 GATTTCTCTA CATGGAGTGG AAGTGGGCT ATATTTCAGC CATTAAGATG AACGCTTTA CTGTACAGG AGTGTGAGC GAAGCGAGA CATATACAA  
CTAAAGGAT GTACCTCAAC TTTACCCGA TATAAGTGC GTAATTCTAC TTGCCGAAT GAACATGTCC TCAGCACTCG CTTCCGCTCT GTATATGTTT  
Rabies-G protein

-----

501 TTTCTGGGA TACGTACCA CCACTTCAA GAGAAACAC TTCGCGCAA CGCTGACGC TTGTCGGGC GTTACAACT GGAAGATGGC AGGAGATCCT  
AAGCACCTC ATGCAGTGGT GGTGGAAGTT CTCTTTTGTG AAGCGGGTT GCGACTGCG AACAGCCCGG CGAATGTGA CTTCTACCG TCTCTAGGA  
Rabies-G protein

-----

601 CGATATGAAG AATCTCTGCA CAACCCGTAT CCGATTACC ATTGGCTGCG GACAGTCAAG ACTACCAAGG AGAGTCTGGT CATTATATCA CCAAGCGTGG  
GCTATACTTC TTAGAGACGT GTTGGGCATA GGAATAATGG TAACCGACGC CTGTCACTTC TGATGGTTCC TCTCAGACCA GTATATAGT GGTTCGCACC  
Rabies-G protein

-----

701 CCGATCTTGA TCCTTATGAT AGATCCCTGC ACAGTAGGGT TTTCTCTGGC GGAATTTGA GCGTGTTCG AGTATCAAGT ACCTACTGCT CCACTAACCA  
GGCTAGAACT AGGAATACTA TCTAGGAGCG TGTATCCCA AAAAGGACCG CCTTAACAT CGCCCAACG TCATAGTTCA TGGATGACGA GGTGATTGGT  
Rabies-G protein

-----

801 CGACTACACT ATATGGATGC CTGAGAACC TCGACTCGGT ATGAGTTTGG ACATTTTTAC GAATCAACG GSCAAGCGGG CATCTAAGGG GTCTGAACA  
GCTGATGTGA TATACCTACG GACTCTTGGG AGCTGAGCCA TACTCAACGC TGTAAATATG CTGTAGTSCC CCGTTCGCC GTAGATTGCC CAGACTTTGT  
Rabies-G protein

-----

901 TGCGGGTTTG TTGATGAGC GGGGTTGTAT AAATCTCTTA AAGCGCGCTG TAAGCTGAAA CTCTGTGGCG TACTGGGGCT GCGCTGATG GACGGACAT  
ACGCCCAAC AACTACTGCG CCGCACATA TTTAGAGAA TTTCCGCGAC ATTGACTTT GAGACACCG ATGACCCGA CCGGACTAC CTGCGGTGA  
Rabies-G protein

-----

1001 CGGTGGCTAT GCGACAACG AATGAACAA AGTGGTGTC CCGTGGTCG CTGGTTAATC TGCACGACTT TAGGTCTGAC GAAATCGAGC ACCTTGTGGT  
CCCACCGATA CGTCTGTTCG TACTTTGTT TCACCACAGG GGGACAGTC GACCAATTAG ACGTGCTGAA ATCCAGACTG CTTAGCTCG TGAACACCA  
Rabies-G protein

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- E E L V K K R E E C L D A L E S I M T T K S V S P R R L S H L R K
1101 GGAGGAAC TGTAAGAAC GCGAAGAGT CCGGAGCGA CTGAGAGTA TTATGACCAC CAAATCCGTT TCCTTCAGAA GACTGAGCCA CCGCGAAAG
CCCTCTGAC CACTTCTTG CGCTTCTCAC GGACCTGGT GAATCTCAT AATACTGGT GTTAGGCCA AGGAAGTCTT CTGACTGGT GGAGCTTTC
Rabies-G protein
- L V P G F G K A Y T I P N K T L M E A D A H Y K S V R T W N E I I P
1201 CTGGTGCCAG GGTTCGGGA GGCCTTACT ATTTCAACA AGACTCTTAT GGAGGCGGAT GCCATTATA AGTCAGTTAS GACTTGGAA GAGATAATTC
GACCACGGTC CCAAGCCCTT CCGAATATGA TAAAGTTGT TCTGAGAATA CCTCCGCCA CGGGTAATAT TCAGTCAATC CTGAACCTTA CTCTATTAAG
Rabies-G protein
- S K G C L R V G G R C H P H V N G V P F N G I I L G P D G N V L I
1301 CCTCCAAAGG ATGTCGAGA GTCGGTGGG GATGCCACCC CCATGTCAAT GGGGTGTTCT TTAACGGANT CATCCGGA CCGACGGGA AGTGCTGAT
GGAGGTTTC TACGACTCT CAGCCACCT CTACGGTGG GGTACAGTTA CCCCACAAGA AATTGCCCTA GTAGGACCTT GGACTGCCCT TGCAGACTA
Rabies-G protein
- F E M Q S S L L Q Q H M E L L V S S V I P L M H P L A D P S T V F
1401 TCCCGAGATG CAATCTTCC TTCTGCAGCA ACACATGGA CTCTGGTGT CTTCAGTAT ACCCTGATG CACCCACTGG CCGACCCAG CACTGTGTT
AGGGCTCTAC GTTAGAAGG AAGACGTCGT TGTGTACCTT GAGGACCACA GAATCACTA TGGGACTAC GTGGTGACC GGCTGGGTC GTGACACAAG
Rabies-G protein
- K N G D E A E D P V E V H L P D V H E R I S G V D L G L P M N G K Y
1501 AAAAATGGG ATGAGSCCA AGACTTTGT GAAGTTCACC TGCCCGATGT ACACGAAAG ATATCAGGAG TAGACCTGG CCTTCCTAAT TGGGTAAAT
TTTTACCC TACTCCGCT TCTGAACAC CTCAAGTGG ACCGGCTACA TGTCTTTC TATAGACCTC ATCTGGACC GGAAGGATTA ACCCCTTCA
Rabies-G protein
- V L L S A G A L T A L M L I I P L M T C W R R V N R S E P T Q H N
1601 ACGTCTCTCT GAGTGGGGT GCCITGACG CTTTGATGCT GATCATTTTT CTGATGACCT GCTGGGCGAG GGTGAATCG TCCGAGCCGA CACAGCACA
TGACAGGGA CTCACGCCA CGAAGTGGC GAACTACGA CTAGTAAAA GACTACTGGA GAGCCGCTC CCAGTACGG AGGCTCGGCT GTGTGTGTT
Rabies-G protein
- L R G T G R E V S V T P Q S G K I I S S W E S Y K S G G E T G L N
1701 TCTCAGAGG ACAGGCCGG AGTAAGTGT GACTCCGCA TCTGGCAGA TTATTAGTAG TTGGGAGAGT TACAAGTCTG GAGGAGAGAC TGGTTGAAT
AGAGTCTCC TGTCCGCC TCAATCACA CTGAGGCGTT AGACCGTCT AATAATCAT AACCTCTCA ATGTTACAG CTCTCTCTG ACCCAACTTA
preNS1 signal
FMDV 2A NS1 signal
- F D L L K L A G D V E S N P G P A R D R S I A L T F L A V G G V L L
1801 TTGATCTGC TCAAACTGC AGGCGATGA GAATCAATC CTGGACCCG CCGGACAGG TCCATACCTC TCAGTCTCT CCGAGTTGGA GCAGTCTGC
AACTAGAGG AGTTGAAG TCCGCTACAT CTAGTTFAG GACCTGGGG GSCCTGTCC AGGTATCGAG AGTGCAAGA GGTCAACCT CCAAGAGG
NS1 signal
- F L S V N V H A D T G C A I D I S R Q E L R C G S G V F I H N D V
1901 TCTCTCTCT CGTGAACGT CACGCTGCA CTGGGTGTC CATAGACATC AGCCGGCAAG AGCTGAGATG TGGAGTGA GTGTTCAATC ACAATGATG
AGAAGGAGG GCAGTTGAC GTGCGACTGT GACCCACAG GTATCTGTAG TCGGCGTTC TCGACTCTAC ACCTTCACCT CACAAGTATG TGTACTACA

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# WN ( $\Delta$ C)-Rabies G PIV sequence (partial).

5'UTR

terminus of C N-

M S

1 AGTAGTTGCG CTGTGTGAGC TGACAAACTT AGTAGTGTTC GTGAGGATTA ACAACAATTA ACACAGTGGC AGCTGTTTCT TAGCAGGAAG ATCTCGATGT  
TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCAGAAA CACTCCTAAT TGTGTTAAT TGTGTACGC TCGACAAAGA ATCTGCTTC TAGAGCTACA  
N-terminus of C

· K K P G G P G K S R A V N M L K R G M P R V L S L I G L K Q K K R ·

101 CTAAGAAACC AGGAGGCGCC GSCAAGAGCC GGGCTGTCAA TATGCTAATA CGCGGAATGC CCCGCTGTT GTCTTGATT GACCTAAGC AAAAGAGCG  
GATTCTTGG TCTCCCGGG CGGTTCTGG CCCGACAGT ATACGATTTT GCGCTTACG GGGCGCACA CAGGAATTA CTTGAATTG TTTTCTTGC  
N-terminus of C Rabies-G protein

partial C signal Rabies-G signal

· G G K T G I A V I V P Q A L L F V P L L V F P L C F G K F P I Y T ·

201 AGGGGSCAAG ACTGGTATAG CTGTGATCGT TCTCAGGCT CTTTGTGTTG TACCTTGGT GGTATTCCC CTTGCTTGG GTAAATTTCC TATCTATACC  
TCCCCGTTT TGACCATATC GACACTAGCA AGGAGTCCGA GAAACAAAC ATGGGAACGA CCATAAAGG GAAAGAAAC CATTTAAAG ATAGATATGG  
Rabies-G protein

· I P D K L G P W S P I D I H H L S C P N N L V V E D E G C T N L S G ·

301 ATCCCTGATA AGCTCGGGCC TTGAGTCCC ATTGATATTC ACCATTGAG CTGCCCAAAC AACCTCGTCG TTGAGGATGA AGGGTGCAT AATCTTTCTG  
TAGGGACTAT TCGAGCCCGG AACCTCAGG TAACTATAAG TGGTAAACTC GACGGGTTTG TTGGAGCAGC AACTCTACT TCCCAGTGA TTAGAAAGAC  
Rabies-G protein

· P S Y M E L K V G Y I S A I K M N G P T C T G V V T E A E T Y T N ·

401 GATTTTCTTA CATGGAGTTG AAAGTGGCT ATATTTGAGC CATTAGATG AACGGCTTFA CTTGTACAGG AGTCTGACC GAAGCCGAGA CATATACAAA  
CTAAAAGGAT GTACCTCAAC TTTCACCGCA TATAAAGTGS GTAATTCTAC TTGCCGAAAT GAACATGTCC TCAGCACTGG CTTGCGCTCT GTATATGTTT  
Rabies-G protein

· F V G Y V T T T P K R K H F R P T P D A C R A A Y N W K M A G D P ·

501 TTTCTGGGA TACGTACCA CCACCTCAA GAGAAACAC TTCCGCCCAA CGCTGACGC TTGTGGGGCC GCTTACACT GGAAGATGC AGGAGATCCT  
AAAGCACCT ATCAGTGGT GTTGGAAGTT CTCTTTGTG AAGGGGGGTT GGGAGCTGCG AACAGCCCGG CGAATGTGA CCTTCTACCG TCTCTAGGA  
Rabies-G protein

· R Y E E S L H N P Y P D Y H W L R T V K T T K E S L V I I S P S V A ·

601 CGATATGAAG AATCTCTGCA CAACCCGTAT CTTGATTACC ATTGGCTGCG GACAGTCAAG ACTACCAAGG AGAGTCTGGT CATTATATCA CCAAGCGTGG  
GCTATACITC TTAGAGACGT GTTGGGCATA GGACTAATGG TAACCGAGCG CTGTCACTTC TGATGGTTC TCTCAGACCA GTAAATATAG GTTTCGCACC  
Rabies-G protein

· D L D P Y D R S L H S R V F P G G N C S G V A V S S T Y C S T N H ·

701 CGATCTTGA TCCCTATGAT AGATCCCTGC ACAGTAGGCT TTTTCTGCG GGAATTTGTA GGGGTGTTGC AGTATCAAGT ACCTACTGCT CCACATAACA  
GGCTAGAACT AGGAATACTA TCTAGGGAGC TGTATCCCA AAAAGGAGCG CCTTACATAT CCCCACAACG TCATAGTTCA TGGATGACGA GGTGATTGGT  
Rabies-G protein

· D Y T I W M P E N P R L G M S C D I F T N S R G K R A S K G S E T ·

801 CGACTACACT ATATGGATGC CTGAGAACCC TCGACTCGGT ATGAGTTGCG ACATTTTAC GAACACAGG GCGAAGCGGG CATCTAAGGG GTCTGAACA  
GCTGATGTA TATACCTAGC GACTCTTGGG AGCTGAGCCA TACTCAACGC TGTAAAAATG CTTGAGTGCC CGGTCGCCC GTAGATTCCC CAGACTTTGT  
Rabies-G protein

· C G P V D E R G L Y K S L K G A C K L K L C G V L G L R L M D G T W ·

901 TGCGGGTTTG TTGATGAGCG GGGGTTGTAT AATCTCTTA AAGGCGCTG TAAGCTGAAA CTCTGTGCGG TACTGGGGCT GCGCTGTATG GACGGACAT  
ACGCCAAAC AACTACTCGC CCCCAACATA TTTAGAGAAAT TTCCGCGGAC ATTCGACTTT GAGACACCGC ATGACCCCGA CCGCGACTAC CTGCGGTGA  
Rabies-G protein

· V A M Q T S N E T K W C P P G Q L V N L H D P R S D E I E H L V V ·

1001 GGGTGGCTAT SCAGACAAGC AATGAAACAA AGTGGTGTCC CCTGTGTAG CTGGTTAATC TGACAGACTT TAGGTCTGAC GAAATCGAGC ACCTGTGTGT  
CCCACCGATA CGTCTGTTCG TTAATTTGTT TCACCAACAG GGGACCACTC GACCAATTAG AGTGTCTGAA ATCCAGACTG CTTTGTCTCG TGGAAACCCA  
Rabies-G protein

· E E L V R K R E E C L D A L E S I M T T K S V S P R R L S H L R K ·

1101 GGAGGAAGTG GTGAAGAAC GCGAAGAGTG CTTGAGCGCA CTTGAGAGTA TTATGACCAC CAATCCGTT TCTTCAGAA GACTGAGCCA CTTGCGAAG  
CCTCTTGAC CACTCTTTCG CGCTTCTCAC GGAAGTGGT GAAGTCTCAT AATAGTGGT GTTTAGGCAA AGGAAGTCTT CTGACTGGT GGAAGCTTTC  
Rabies-G protein

L V P G F G K A Y T I P N K T L M E A D A H Y K S V R T W N E I I P .  
 1201 CTGTGTCAG GGTTCGGGAA GGCTTATCT ATTTTCAACA AGACTCTTAT GGAGGCGGAT GCCCATTATA AGTCAGTTAG GACTTGGAT GAGATAATTC  
 GACCAAGGTC CCAAGCCCTT CCGAATATGA TAAAGTTGT TCTGAGAATA CTCCGCCCTA GGGTAATAT TCAGTCAATC CTGAACCTTA CTCTATTAAG  
 Rabies-G protein  
 S K G C L R V G G R C H P H V N G V F P N G I I L G P D G N V L Y .  
 1301 CCTCCAAAGG ATGTCTGAGA GTCCGTGGGA GATGCCACCC CCATGTCAAT GGGTGTCTT TTAACGGAAT CATCTGGGA CTGACGGGA ACGTGTGAT  
 GGAGTTTCC TACAGACTCT CAGCCACCCCT CTACGGTGGG GGTACAGTTA CCCCACAAGA AATTGCCCTA GTAGGACCTT GGACTGCCCT TGCACGACTA  
 Rabies-G protein  
 P E M Q S S L L Q Q H M E L L V S S V I P L M H P L A D P S T V F  
 1401 TCCGAGATG CAATCTTCCC TTCTGCAGCA ACACATGGAA CTCCTGGTGT CTTCAGTGT ACCCTGATG CACCCACTGG CGAGCCCAAG CACTGTGTTT  
 AGGGCTCTAC GTTAGAAGG AAGACGTGCT TGTGTACCTT GAGGACCACA GAATCACTA TGGGGACTAC GTGGGTGACC GGCTGGGGTC GTGACACAAG  
 Rabies-G protein  
 K N G D E A E D F V E V H L P D V H E R I S G V D L G L P N W G K Y .  
 1501 AAAATGGCG ATGAGGCGCA AGACTTTGTG GAAGTTCAAC TGCCCGATGT ACACGAAAGG ATATCTGGAG TAGACCTGGG CCTTCTTAAT TGGGGTAAGT  
 TTTTACCGC TACTCCGCTT TCTGAACAC CTCAAGTGG ACGGGCTACA TGTGCTTTC TATAGACCTC ATCTGGACCC GGAAGGATTA ACCCATTCCTA  
 Rabies-G protein  
 V L L S A G A L T A L M L I I F L M T C W R R V N R S E P T Q H N .  
 1601 ACGTCTCTCT GAGTGGGGT GCCTTCACCG CTTGATGCT GATCATTTT CTGATGACCT GCTGGCGGAG GGTGAATGCG TCCGAGCCGA CACAGCACAA  
 TGCACGGA CTACGCCCA CGGAAGTGG GAAACTACGA CTAGTAAAA GACTACTGGA CGACCGCTC CCATTTAGCG AGGCTCGGCT GTGTGTGTT  
 Rabies-G protein  
 L R G T G R E V S V T P Q S G K I I S S W E S Y K S G G E T G L N  
 1701 TCTCAGAGG ACAGGCGGG AAGTAAGTGT GACTCCGCA TCTGCAAGA TTATTAGTAG TTGGGAGAGT TACAAGTCTG GAGGAGAGAC TGGGTGAAT  
 AGAGTCTCC TGTCCGGCC TTCAATCACA CTGAGGCGTT AGACGTTCT AATAATCCTC AACCTCTCA ATGTTACAGC CTCTCTCTG ACCCACTTA  
 C/prM signal  
 PMDV 2A  
 F D L L K L A G D V E S N P G P G G K T G I A V M I G L I A C V G A .  
 1801 TTGATCTGC TCAAACTTGC AGGCGATGTA GAATCAATC CTGGACCGGG AGGAAGAGCC GGTATTGCG TCATGATGG CCTGATCGCC TGCCTAGGAG  
 AAAGTAGAG AGTTTGAACG TCCGCTACAT CTAGTTTGG GACCTGGGCC TCCTTTCTGG CCATAAGCTC AGTACTAACC GGACTAGCGG ACGCATCTC  
 C/prM signal  
 prM  
 V T L S N P Q G K V M M T V N A T D V T D V I T I P T A A G K N L .  
 1901 CAGTTACCTT CTCTAATTC CAAGGGAAGG TGATGATGAC GGTAAATGCT ACTGACGTC CAGATGTCAT CACGATTCCT ACAGTCTCTG GAAAGAACCT  
 GTCAATGGGA GAGATGGAAG GTTCCCTTCC ACTACTACTG CCATTTACGA TGACTGCAGT GTCTACAGTA GTGCTAAGST TGTGAGCGAC CTCTCTTGG  
 prM  
 C I V R A M D V G Y M C D D T I T Y E C P V L S A G N D P E D I D  
 2001 ATGCATTGTC AGAGCAATGG ATGTGGGATA CATGTGGAT GATACTATCA CTATGAATG CCGAGTGTG TCGGCTGGTA ATGATCCAGA AGACATCGAC  
 TACGTAACAG TCTCGTTACC TACACCTTAT GTACACGCTA CTATGATAGT GAATACTTAC GGGTCACGAC AGCCGACCAT TACTAGGTCT TCTGTAGCTG  
 prM  
 C W C T K S A V Y V R Y G R C T K T R H S R R S R R S L T V Q T R G .  
 2101 TGTGTGTGCA CAAAGTCAGC AGTCTACGTC AGGTATGGAA GATGCRCCAA GACACGCCAC TCAGACGCA GTCCGGAGTC ACTGACAGTG CAGACACAG  
 ACAACCGGT GTTTCAGTGC TCAGATGCGA TCATACCTT CTACGCTGTT CTGTGGGGT AGTTCTGCT CAGCTCCAG TGAATGTAC GTCTGTGTG  
 prM  
 E S T L A N K K G A W M D S T K A T R Y L V K T E S W I L R N P G  
 2201 GAGAAAGCAC TCTAGCGAAC AAGAAGGGGG CTGATGGA CAGCACCAAG GCCACAGGT ATTGGTAAA AACAGATCA TGGATCTTGA GGAACCTTGG  
 CTCTTCTGTG AGATGCTGT TTCTTCCCTC GAACCTACCT GTCTGGGTC CAGTGTCCA TAAACCAATT TTGTCTTAGT ACCTAGAATC CCTTGGGACC  
 prM  
 Y A L V A A V I G W M L G S N T M Q R V V F V V L L L L V A P A Y  
 2301 ATATGCCCTG GTGGCAGCG TCAITGGTGG GATGCTTGG AGCAACACCA TGCAGAGAGT TGTGTTTGT GTGCTATTGC TTTTGGTGGC CCGACTTAC  
 TATACGGGAC CACCGTCGGC AGTAACACAC CTACGAACCC TGTGTGTGT ACGTCTCTCA ACACAAACAG CACGATAACG AAACACCGG GGTGCAATG  
 E  
 prM  
 S F N C L G M S N R D F L E G V S G A T W V D L V L E G D S C V T I .  
 2401 AGCTTTAAT GCCTTGGAT GAGCAACAGA GACTCTTGG AAGAGTGTG TGGAGCAACA TGGGTGGATT TGGTTCTGA AGGAGACAGC TGGGTGACTA  
 TCGAATTGA CGGAACCTTA CTGCTGTCT CTGAAGAACC TTCTCAGAC ACCTGCTGT ACCCACCTAA ACCAAGAGCT TCCGCTGTG ACGCACTGAT  
 E

. M S K D K P T I D V K M M N M E A A N L A E V R S Y C Y L A T V S .  
 2501 TCATGTCTAA GGACAAGCCT ACCATCGATG TGAAGATGAT GAATATGGAG GCGGCCAACC TGGCAGAGGT CCGCAGTTAT TGCTATTGGG CTACCGTCAG  
 AGTACAGATT CCGTGTGGGA TGGTAGCTAC ACTTCTACTA CTATACCTC GCGCGTGTGG ACCGTCTCCA GCGTCAATA ACCTATAACC GATGGCAGTC  
 E  
 . D L S T K A A C P A M G E A H N D K R A D P A F V C R Q Q V V D R .  
 2601 CGATCTCTCC ACCAAGCTG CGTGCCCGGC CATGGGAGAA GCTCAATG ACAAACGTGC TGACCCAGCT TTTGTGTGCA GACAAGGAGT GGTGGACAGG  
 GCTAGAGAGG TGGTTTGGAC GCACGGGCGG GTACCCCTCT CGAGTGTATC TGTTTGCAAG ACTGGGTGCA AAACACAGT CTGTCTCTCA CCACCTGTCC  
 E  
 . G W G N G C G L P G K G S I D T C A K F A C S T K A I G R T I L K E .  
 2701 GGCTGGGGCA ACGGCTGCGG ACTATTGGC AAAGGAAGCA TTGACACATG CGCCAAATTT GCCTGTCTCTA CCAAGGCAAT AGGAAGAACC ATTTTGAAG  
 CCGACCCCGT TGGCGACGCC TGATAAACCG TTTCCTTCTG AACTGTGTAC GCGGTTTAAA CCGACGAGAT GGTTCGGTTA TCTTCTGTGG TAAACCTTTC  
 E  
 . N I K Y E V A I P V H G P T T V E S H G N Y S T Q V G A T Q A G R .  
 2801 AGAATATCAA GTACGAAGTG GCCATTTTGG TCCATGGACC AACTACTGTG GAGTCGCAAG GAACTACTC CACACAGGTT GGAGCCACTC AGGACGGGAG  
 TCTTATAGTT CATGCTTCAC CGGTAAAAAC AGGTACCTGG TTGATGACAC CTCAGCGTGC CTTTGATGAG GTGTGTCCAA CCGTGGTGAG TCCGTCCCTC  
 E  
 . F S I T P A A P S Y T L K L G E Y G E V T V D C E P R S G I D T N .  
 2901 ATTGAGCATC ACTCTGCGG CGCCTTCATA CACACTAAAG CTGGAGGAAT ATGGAGAGGT GACAGTGGAC TGTGAACCA CCGTACGGAT TGACACCAAT  
 TAAGTGTAG TGAGGACGCC GCGGAAGTAT GTGTGATTC GAACCTCTTA TACCTCTCCA CTGTACCTG ACACCTGGTG CCGTCCCTA ACTGTGGTTA  
 E  
 . A Y Y V M T V G T K T P L V H R E W F M D L N L P W S S A G S T V W .  
 3001 GCATAGTACG TGATGACTGT TGGAACAAAG ACGTTCTTGG TCCATCTGTA GTGGTTCATG GACCTCAACC TCCCTTGGAG CAGTGTGGA AGTACTGTGT  
 CGTATGATGC ACTACTGACA ACCTTGTTC TGCAAGAACC AGGTAGCACT CACCAAGTAC CTGGAGTTGG AGGGAACCTC GTCACGACCT TCATGACACA  
 E  
 . R N R E T L M E P E E P H A T K Q S V I A L G S Q E G A L H Q A L .  
 3101 GGAGGAACAG AGAGACGTTA ATGGAGTTTG AGGAACCACA CGCCACGAAG CAGTCTGTGA TAGCATTGGG CTCACAAGAG GGAGCTCTGC ATCAAGCTTT  
 CCTCTGTGTC TCTCTGCAAT TACCTCAAAC TCCTTGGTGT GCGGTGCTTC GTACAGACT ATCTTAACCC GAGTGTCTC CCGTACAGC TAGTTCGAAA  
 E  
 . A G A I P V E F S S N T V K L T S G H L K C R V K M E K L Q L K G .  
 3201 GGCTGGAGCC ATCTCTGTGG AATTTTCAAG CAACACTGTC AAGTTGACGT CCGGTCAATT GAAGTGTAGA GTGAAGATGG AAAAATTGCA GTTGAAGGGA  
 CCGACCTCGG TAAGGACACC TTAAGATTC GTGTGACAG TTCAACTGCA GCCCAGTAAA CTTCACATCT CACTTCTACC TTTTAAAGT CACTTCCCT  
 E  
 . T T Y G V C S K A F K F L G T P A D T G H G T V V L E L Q Y T G T D .  
 3301 ACAAACCTAG GCGTCTGTTT AAGGCTTTC AAGTTCTTGG GAGTCTCCGC AGACACAGST CACCGCACTG TGGTGTGGA ATGTCAGTAC ACTGGGACGG  
 TGTGGATAC CCGAGACAAG TTTCCGAAG TTCAAGAAC CCGTGGGGCG TCTGTGTCCA GTGCGGTGAC ACCACAACCT TAACTCATG TGACCGTGGC  
 E  
 . G P C K V P I S S V A S L N D L T P V G R L V T V N P F V S V A T .  
 3401 ATGGACCTTG CAAAGTTCTT ATCTCTGTCAG TGGCTTCAT GAACGACCTA ACGCCAGTGG GCAGATTGGT CACTGTCAAC CTTTGTGTT CAGTGGCCAC  
 TACCTGGAAC GTTTCAGGA TAGAGCAGTC ACCGAAGTAA CTGCTGGAT TGCGGTCAAC CGTCTAACCA GTGACAGTGG GGAAACCAA GTACCGGTG  
 E  
 . A N A K V L I E L S P P P G D S Y I V V G R G E Q Q I N H H W H K .  
 3501 GGCCACGCT AAGGCTCTGA TTGAATTGGA ACCACCTTTT GGAGACTCAT ACATAGTGGT GGGCAGAGGA GAACAACAGA TCAATCAACA CTGGCACAAG  
 CCGGTGCGA TTCCAGGACT AACTTAACCT TGGTGGGAAA CCTCTGASTA TGTATCAACA CCGTCTCTCT CTGTGTGTCT AGTTAGTGGT GACCGTGTTC  
 E  
 . S G S S I G K A P T T T L K G A Q R L A A L G D T A W D F G S V G G .  
 3601 TCTGGAAGCA GCATTGGCRA AGCCTTTTACA ACCACCTTCA AAGGAGGCGA GAGACTAGCC GCTCTAGGAG ACACAGCTTG GCACTTTGGA TCAGTTGGAG  
 AGACCTTCGT CGTAACGTT TGGAAATGT TGGTGGGAGT TTCTCTCGGT CTCTGATGCG CGAGATCCTC TGTGTGCAAC CCGTAAACCT AGTCAACCTC  
 E  
 . V F T S V G K A V H Q V F G G A F R S L F G G M S W I T Q G L L G .  
 3701 GGGTGTTCAC CTCAGTTGGG AAGGCTGTCC ATCAAGTGTT CCGAGGAGCA TTCCGCTCAC TGTTCGAGG CATGTCTGGG ATAACGCAAG GATTGCTGGG  
 CCCCAAGTG GAGTCAACCC TTCCGACAGG TAGTTTCAAA GCCTCTCTGT AAGGCGAGTG ACAAGCCTCC GTACAGGACC TATTGCTTC CTACGACCC  
 E  
 . A L L L W M G I N A R D R S I A L T P L A V G G V L L P L S V N V .  
 3801 GGCTCTCTGT TGTGGATGG GCATCAATGC TCGTACAGG TCCATAGCTC TCAGTTTCT CCGAGTTGGA GGAGTTCTGC TCTTCTCTC CGTGAACGTG  
 CCGAGAGGAC AACACCTACC CGTAGTTAGG AGCACTGTCC AGGTATCGAG AGTGCAAGA GGTCAACCT CCTCAAGAG AGAAGGAGAG GCATTCGAC  
 E

H A D T G C A I D I S R Q E L R C G S G V F I H N D V E A W M D R Y .  
 3901 CACGCTGACA CTGGGTGTGC CATAGACATC AGCCGGCAAG AGCTGASATG TGGAAAGTGA GTGTTTCATAC ACAATGATGT GGAGGCTTGG ATGGACCGGT  
 TGCGACTGT GACCCACAGC GTATCTGTAG TCGGCGGTC TCGACTCTAC ACCTTCACCT CACAAGTATG TGTACTACA CCTCGAACC TACCTGSCCA

## WN ( $\Delta$ prME)-Rabies G PIV sequence (partial)

C protein  
 -----

5' UTR

M S

1 AGTAGTTCGC CTGTGTGAGC TGACAAACTT AGTAGTGTIT GTGAGGATTA ACAACAATTA ACACAGTGCG ASCTGTTTCT TAGCACGAAG ATCTOGATGT  
 TCATCAAGCG GACACACTCG ACTGTTTGA TATCACAATA CACTCCTAAT TGTGTTAAT TGTGTACGC TCGACAAAGA ATCGTGCTTC TAGAGCTACA  
 C protein

· K K P G G P G K S R A V Y L L K R G M P R V L S L I G L K R A M L ·

101 CTAAGAAACC AGGAGGCCCC GSCAAGAGCC GGGCTGTCTA TTTGCTAAA CCGGAATGC CCGCGTGTG GTCTTGATT GGACTTAAGA GGGCTATGTT  
 GATTCTTTGG TCCTCCCGGG CCGTTCCTGG CCGACAGAT AAAGATTTT GCGCTTACG GGGCGCACAA CAGGAATTA CCTGAATTCT CCGATACAA  
 C protein

· S L I D G K G P I R F V L A L L A F F R F T A I A P T R A V L D R ·

201 GAGCCTGATC GACGCGAAGG GGCCAATACG ATTGTGTGTG GCTCTCTTGG CGTCTTTCAG GTTCACAGCA ATTGCTCCGA CCGAGCAGT GCTGGATCGA  
 CTGCGACTAG CTGCGGTTCC CCGGTATGTC TAAACACAAC CGAGAGAACC GCAAGAAGTC CAAGTGTCTG TAACGAGGCT GGGCTCGTCA CGACCTAGCT  
 C protein

· W R G V N K Q T A M K H L L S F K K E L G T L T S A I N R R S S K Q ·

301 TGGAGAGGTG TGAACAAACA AACAGCGATG AAACACCTTC TGAGTTTCAA GAAGGAACCTA GGGACCTTGA CCAGTGTCTAT CAATCGGCGG AGCTCAAAGC  
 ACCTCTCCAC ACTGTGTTGT TTGTGCTAC TTTGTGGAAG ACTCAAAGTT CTTCCTTGAT CCCTGGAAT GGTACAGATA GTTAGCGCC TCGAGTTTCG  
 Rabies-G signal

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|                                                                       |                  |          |
|-----------------------------------------------------------------------|------------------|----------|
| C protein<br>protein                                                  | partial C signal | Rabies-G |
| -----                                                                 |                  |          |
| · K K R G G K T G I A V I V P Q A L L F V P L L V P P L C F G K F P · |                  |          |

401 AAAGAAGCG AGGCGGCAAG ACTGTATAG CTGTGATCT TCCTCAGGCT CTTTGTGTTG TACCCCTGCT GGTATTTCCC CTTGCTTGG GTAAATTTCC  
 TTTTCTTCGC TCCCGGTTTC TGACCATATC GACACTAGCA AGGAGTCCGA GAAACAAAC ATGGGAACGA CCATARAAGG GAAACGAAC CATTAAAGG  
 Rabies-G protein

· I Y T I P D K L G P W S P I D I H H L S C P N N L V V E D E G C T ·

501 TATCTATACC ATCCCTGATA AGCTCGGCCC TTGGAGTCCC ATTGATATTC ACCATTTGAG CTGCCCAAC AACCTCGTGC TTGAGGATGA AGGGTCACT  
 ATAGATATGG TAGGACTAT TCGAGCCCGG AACCTCAGG TAACATAAG TGTTAAAGTC GACGGGTTTG TTGAGGAGC AACTCTACT TCCACGTTGA  
 Rabies-G protein

· N L S G F S Y M E L K V G Y I S A I K M N G F T C T G V V T E A E T ·

601 AATCTTCTG GATTTTCTA CATGGAGTGG AAAGTGGGCT ATATTTGAGC CATTAAAGATG AACGGCTTGA CTTGTACAGG AGTCTGACC GAAGCCGAGA  
 TTAGAAAGAC CTAAAGGAT GTACCTCAAC TTTCACCCGA TATAAAGTGC GTAATTTCTAC TTGCGAAGAT GAACATGTCC TCAGCACTGG CTTCGCTCT  
 Rabies-G protein

· Y T N F V G Y V T T T P K R K H F R P T P D A C R A A Y N W K M A ·

701 CATATACAA TTTCTGGGA TACGTACCA CCACCTTCAA GAGAAACAC TTCCGCCCAA CGCTGACGC TTGTGGGGCC GCTTACAACT GGAAGATGGC  
 GTATATGTTT AAGCAACCT ATGCACTGGT GTTGGAAATG CTCCTTTGTG AAGCGGGGT GCGGACTGCC AACAGCCCG GAAATGTTGA CCTTCTACCG  
 Rabies-G protein

· G D P R Y E E S L H N P Y P D Y H W L R T V K T T K E S L V I I S ·

801 AGGAGATCTT CGATATGAAG AATCTCTGCA CAACCGTAT CCGATTACG ATTGGCTGCG GACAGTCAAG ACTACCAAGG AGAGTCTGGT CATTATATCA  
 TCCTCTAGGA GCTATCTTC TTAGAGAGCT GTTGGGCATA GGACTAATGG TAACGACGC CTGTGATTC TGATGGTTCC TCTCAGCA GFAATATAGT  
 Rabies-G protein

· P S V A D L D P Y D R S L H S R V F P G G N C S G V A V S S T Y C S ·

901 CCAAGCGTGG CCGATCTTGA TCCTATGAT AGATCCCTGC ACAGTAGGGT TTTTCTGCG GGGAAATTGA GCGGTGTGTC AGTATCAAGT ACCTACTGCT  
 GGTTCGACCG GCTAGAACT AGGAATACTA TCTAGGAGCG TGTATCCCA AAAAGGACCG CCCTTAACAT CCGCACAAAG TCATAGTTCA TGGATGACGA  
 Rabies-G protein

· T N R D Y T I W M P E N P R L G M S C D I F T N S R G K R A S K G ·

1001 CCACTAACCA CGACTACACT ATATGGATGC CTGAGAACCC TCGACTCGGT ATGAGTTGCG ACATTTTAC GAAGTCACGG GCGAAGCGGG CATCTAAGGG  
GGTGATTGGT GCTGATGTGA TATACCTACG GACTCTTGGG AGCTGAGCCA TACTCAACGC TGTAATAATG CTTGAGTGCC CCGTTCGCCC GTAGATTCCC  
Rabies-G protein  
S E T C G P V D E R G L Y K S L E G A C K L K L C G V L G L R L M

1101 GTCTGAACA TCGGGTTTG TTGATGAGCG GGGGTGTAT AAATCTCTTA AGGGCGCTG TAAGCTGAAA CTCGTGGCG TACTGGGCT GCGCTGATG  
CAGACTTTGT ACGCCCAAC AACTACTCGC CCCAACATA TTTAGAGAT TCCGCGGAC ATTCGACTTT GAGACACCGC ATGACCCCGA CCGGACTAC  
Rabies-G protein  
D G T W V A M Q T S N E T K W C P P G Q L V N L H D F R S D E I E H

1201 GACGGCACAT GGGTGGCTAT GCAGACAAGC AATGAARCAA AGTGGTGTCC CCGTGGTCAG CTGGTTAATC TGCAAGACTT TAGGTCTGAC GAAATCGAGC  
CTGCCGTGTA CCCACCGATA CGTCTGTTCG TTAATTTGTT TCACCAACAG GGGACCAATC GACCAATTAG ACGTGTGAA ATCCAGACTG CTTAGCTCG  
Rabies-G protein  
L V V E E L V K K R E E C L D A L E S I M T T K S V S F R R L S H

1301 ACCTTGTGGT GGAGGAACTG GTGAGAAAC GCGAAGAGTG CCGGACGCA CTTGAGAGTA TTATGACCAC CAAATCCGTT TCCTTCAGAA GACTGAGCCA  
TGGAACACCA CCTCCTGAC CACTTCTTTG CGCTTCTCAC GGACCTGGT GACTCTCAT AATACTGCTG GTTTAGGCAA AGGAAGTCTT CTGACTCGGT  
Rabies-G protein  
L R K L V P G P G K A Y T I F N K T L M E A D A H Y K S V R T W N

1401 CCTGCGAAAG CTGGTGCCAG GGTTCGGGAA GGCTTATACT ATTTTCARCA AGACTCTTAT GGAGGGGGAT GCCCATTATA AGTCAGTTAG GACTTGGAA  
GGACGCTTTC GACCAACGTC CCAAGCCCTT CGAATATGA TAAAGTTGT TCTGAGAATA CTTCCGCTA CCGGTAATAT TCAGTCAATC CTGAACCTTA  
Rabies-G protein  
E I I P S K G C L R V G G R C H P H V N G V P P N G I I L G P D G N

1501 GAGATAATTC CCTCCAAAGG ATGTCTGAGA GTGGTGGGA GATGCCACCC CCATGTCAAT GGGGTGTTCT TTAACGGAAT CATCTGGGA CTGACGGGA  
CTCTATTAG GGAGGTTTCC TACAGACTCT CAGCCACCT CTACGGTGGG GGTACAGTTA CCCACAAGA AATTGCCCTA GTAGGACCTT GGACTGCCCT  
Rabies-G protein  
V L I P E M Q S S L L Q Q H M E L L V S S V I P L M H P L A D P S

1601 ACGTCTGAT TCCGAGATG CAATCTTCCC TTCTGCAGCA ACACATGGA CTCTGGTGT CTTAGTGTAT ACCCTGATG CACCCACTGG CCGACCCAG  
TGACAGACTA AGGGCTCTAC GTTAGAAGGG AAGAGTGTGT TGTGTACCTT GAGGACACCA GAAGTCATA TGGGGACTAC GTGGGTGACC GGCTGGGTC  
Rabies-G protein  
T V F K N G D E A E D F V E V H L P D V H E R I S G V D L G L P N

1701 CACTGTGTTT AAAATGGCG ATGAGGCCGA AGACTTTGTG GAAGTTCACC TGCCCGATGT ACACGAAAGG ATATCTGGAG TAGACCTGGG CTTCTCTAAT  
GTGACACAG TTTTACCGC TACTCCGCT TCTGAACAC CTTCAAGTG ACGGCTACA TGTGCTTCC TATAGACCTC ATCTGGACCC GGAAGGATTA  
Rabies-G protein  
W G K Y V L L S A G A L T A L M L I I P L M T C W R R V N R S E P T

1801 TGGGGTAAGT ACGTCTCCCT GAGTGGGGT GCCTTGACCG CTTGATGCT GATCATTITT CTGATGACCT GCTGGCGGAG GGTGAATCSC TCCGAGCGGA  
ACCCCATTTA TGCACGAGG CTCACGCCCA CGAACTGGC GAAACTACGA CTAGTAAAAA GACTACTGGA CGACCGCCTC CCATTTAGCG AGGCTCGGCT  
Rabies-G protein  
Q H N L F G T G R E V S V T P Q S G K I I S S W E S Y K S G G E T

1901 CACAGCACAA TCTCAGAGGG ACAGGCCGGG AAGTAAGTGT GACTCCGCAA TCTGGCAAGA TTATTAGTAG TTGGGAGAGT TACAAGTCTG GAGGAGAGAC  
GTGTCTGTT AGAGTCTCCC TGTCCGGCCC TTCATTGACA CTGAGGCGTT AGACGCTTCT AATAATCATC AACCTCTCA ATGTTGAGAC CTCTCTCTG  
FMDV 2A NS1 signal  
Rabies-G protein preNS1 signal  
G L N F D L L K L A G D V E S N P G P A R D R S I A L T F L A V G

2001 TGGGTGAAT TTTGATCTGC TCRAACTGCG AGCGATGTA GAATCAATC CTGGACCCGC CCGGACAGG TCCATAGCTC TCACGTTTCT CGCAGTTGGA  
ACCCCACTTA AACTAGAGG AGTTGAAGG TCCGCTACAT CTTAGTTTAG GACCTGGGCG GCGCTGTCC AGGTATCGAG AGTGCAAGA GCGTCAACCT  
NS1  
NS1 signal  
G V L L F L S V N V H A D T G C A I D I S R Q E L R C G S G V F I H

2101 GGAGTTCTGC TCTTCTCTC CGTGAACGTG CACGCTGACA CTGGGTGTGC CATAGACATC AGCCGCAAG AGCTGAGATG TGAAGTGA GTGTTCTATC  
CCTCAAGAGC AGAAGGAGAG GCATTTGAC GTGCGACTGT GACCCACAG GTATCTGTAG TCGGCGTTC TCGACTCTAC ACCTTCACCT CACAAGTATG

# Sequence Appendix 4 (continued)

## PIV-WNV helper ΔNS1

C  
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5' UTR

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1 AGTAGTTCGC CTGTGTGAGC TGACAAACTT AGTAGTGTTC GTGAGGATTA ACAACAATTA ACACAGTGCG AGCTGTTTCT TAGCACGAAG ATCTCGATGT  
TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCACAAA CACTCCTAAT TGTGTGTAAT TGTGTACGCG TCGACAAAGA ATCGTGCTTC TAGAGCTACA  
C

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101 K K P G G P G K S R A V N M L K R G M P R V L S L I G L K R A M L  
CTAAGAAACC AGGAGGGCCC GGCAAGAGCC GGGCTGTCAA TATGCTAATA CCGGAATGC CCCGCGTGT GTCCTTGATT GGACTTAAGA GGGCTATGTT  
GATTCTTTGG TCCTCCCGGG CCGTTCTCGG CCGACAGTT ATACGATTTT GCGCCTTACG GGGCGCACAA CAGGAAGTAA CCTGAATTCT CCCGATACAA  
C

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201 S L I D G K G P I R F V L A L L A F F R F T A I A P T R A V L D R  
GAGCCTGATC GACGGCAAGG GGCCAATACG ATTTGTGTTC GCTCTCTTGG CGTCTTTCAG GTTCACAGCA ATTGCTTCCGA CCCGAGCAGT GCTGGATCGA  
CTCGGACTAG CTGCCGTTCC CCGGTTATGC TAAACACAAC CGAGAGAACC GCAAGAAGTC CAAGTGTCTG TAACGAGGCT GGGCTCGTCA CGACCTAGCT  
C

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301 W R G V N K Q T A M K H L L S F K K E L G T L T S A I N R R S S K Q  
TGGAGAGGTG TGAACAAACA AACAGCGATG AAACACCTTC TGAGTTTCAA GAAGGAACTA GGGACCTTGA CCAGTGTCTAT CAATCGGCGG AGCTCAAAAC  
ACCTCTCCAC ACTTGTTTGT TTGTGCTAC TTTGTGGAAG ACTCAAAGTT CTTCCTTGAT CCCTGGAAC TGGTACGATA GTTAGCCGCC TCGAGTTTTC  
Signal peptide

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C

prM

-----

401 K K R G G K T G I A V M I G L I A S V G A V T L S N F Q G K V M M  
AAAAGAAAAG AGGAGGAAAG ACCGGAATTG CAGTCATGAT TGGCCTGATC GCCAGCGTAG GAGCAGTTAC CCTCTCTAAC TTCCAAGGGA AGGTGATGAT  
TTTTCTTTTC TCCTCCTTTC TGGCCTTAAC GTCACTACTA ACCGGACTAG CCGTCCGATC CTCGTCAATG GGAGAGATTG AAGGTTCCCT TCCACTACTA  
prM

-----

501 T V N A T D V T D V I T I P T A A G K N L C I V R A M D V G Y M C  
GACGGTAAAT GCTACTGACG TCACAGATGT CATCAGGATT CCAACAGCTG CTGGAAGAA CCAATGCAAT GTACAGAGCA TGGATGTGGG ATACATGTGC  
CTGCCATTTA CGATGACTGC AGTGTCTACA GTAGTGCTAA GGTGTCTGAC GACCTTTCTT GGATACGTAA CAGTCTCGTT ACCTACACCC TATGTACAG  
prM

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601 D D T I T Y E C P V L S A G N D P E D I D C W C T K S A V Y V R Y G  
GATGATACTA TCACCTATGA ATGCCAGTG CTGTCCGCTG GTAATGATCC AGAAGACATC GACTGTTGGT GCACAAAGTC AGCAGTCTAC GTCAGGTATG  
CTACTATGAT AGTGAATACT TACGGGTCAC GACAGCCGAC CATTACTAGG TCTTCTGTAG CTGACAACCA CGTGTTTCAG TCGTCAGATG CAGTCCATAC  
prM

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701 R C T K T R H S R R S R R S L T V Q T H G E S T L A N K K G A W M  
GAAGATGCAC CAAGACACGC CACTCAAGAC GCAGTCGGAG GTCACTGACA GTGCAGACAC ACCGAGAAAG CACTCTAGCG AACAGAAGG GGGCTTGGAT  
CTTCTACGTG GTTCTGTGCG GTGAGTTCTG CGTCAGCCTC CAGTGACTGT CAGCTCTGTG TGCTCTTTC GTGAGATCGC TTGTTCTTCC CCCGAACCTA

prM

801 · D S T K A T R Y L V K T E S W I L R N P G Y A L V A A V I G W M L  
GGACAGCACC AAGGCCACAA GGTATTTGGT AAAACAGAA TCATGGATCT TGAGGAACCC TGGATATGCC CTGGTGGCAG CCGTCATTGG TTGGATGCTT  
CCTGTCGTGG TTCCGGTGT CCATAAACCA TTTTGTCTT AGTACCTAGA ACTCCTTGGG ACCTATACGG GACCACCGTC GGCAGTAACC AACCTACGAA  
E

prM

901 G S N T M Q R V V F V V L L L L V A P A Y S F N C L G M S N R D F L  
GGGAGCAACA CCATGCAGAG AGTTGTGTTT GTCGTGCTAT TGCTTTTGGT GGCCCCAGCT TACAGCTTTA ACTGCCTTGG AATGAGCAAC AGAGACTTCT  
CCCTCGTTGT GGTACGTCTC TCAACACAAA CAGCAGGATA ACGAAAACCA CCGGGGTGCA ATGTCGAAAT TGACCGAACC TTACTCGTTG TCTCTGAAGA  
E

1001 · E G V S G A T W V D L V L E G D S C V T I M S K D K P T I D V K M  
TGGAAGGAGT GTCTGGAGCA ACATGGGTGG ATTTGGTTCT CGAAGGCGAC AGCTGCGTGA CTATCATGTC TAAGGACAAG CCTACCATCG ATGTGAAGAT  
ACCTTCCTCA CAGACCTCGT TGTACCCACC TAAACCAAGA GCTTCGCTG TCGACGCACT GATAGTACAG ATTCTGTTC GGATGGTAGC TACACTTCTA  
E

1101 · M N M E A A N L A E V R S Y C Y L A T V S D L S T K A A C P A M G  
GATGAATATG GAGCGGCCA ACCTGGCAGA GGTCCGCACT TATTGCTATT TGGCTACCGT CAGCGATCTC TCCACCAAAG CTGCGTGCCC GGCCATGGGA  
CTACTTATAC CTCGCCGGT TGGACCGTCT CCAGGCGTCA ATAACGATAA ACCGATGGCA GTCGCTAGAG AGGTGGITTC GACGCACGGG CCGGTACCCCT  
E

1201 E A H N D K R A D P A F V C R Q G V V D R G W G N G C G L F G K G S  
GAAGCTCACA ATGACAAACG TGCTGACCCA GCTTTTGTGT GCAGACAAGG AGTGGTGGAC AGGGGCTGGG GCAACGGCTG CCGACTATTT GGCAAGGAA  
CTTCGAGTGT TACTGTTTGC ACGACTGGGT CGAAACACA CGTCTGTTCC TCACCACCTG TCCCCGACCC CGTTGCCGAC GCCTGATAAA CCGTTTCCTT  
E

1301 · I D T C A K F A C S T K A I G R T I L K E N I K Y E V A I F V H G  
GCATTGACAC ATGCGCCAAA TTTGCCTGCT CTACCAAGGC AATAGGAAGA ACCATTTTGA AAGAGAATAT CAAGTACGAA GTGGCCATTT TTGTCCATGG  
CGTAACCTGTG TACCGGGTTT AAACGGACGA GATGTTCCG TTATCCTTCT TGGTAAAACT TTCTCTTATA GTTCATGCTT CACCGGTAAA AACAGGTACC  
E

1401 · P T T V E S H G N Y S T Q V G A T Q A G R F S I T P A A P S Y T L  
ACCAACTACT GTGGAGTCGC ACGGAACTA CTCCACACAG GTTGGAGCCA CTCAGGCAGG GAGATTGAGC ATCACTCCTG CGGCGCCTTC ATACACACTA  
TGGTTGATGA CACCTCAGCG TGCCTTTGAT GAGGTGTGTC CACCTCGGT GAGTCCGTCC CTCTAAGTCG TAGTGAGGAC GCCGCGGAAG TATGTTGTAT  
E

1501 K L G E Y G E V T V D C E P R S G I D T N A Y Y V M T V G T K T F L  
AAGCTTGGAG AATATGGAGA GGTGACAGTG GACTGTGAAC CACGGTCAGG GATTGACACC AATGCATACT ACGTGATGAC TGTGGAAACA AAGACGTTCT  
TTGGAACCTC TTATACCTCT CCACTGTGAC CTGACACTTG GTGCCAGTCC CTAACGTGG TTACGTATGA TGCACTACTG ACAACCTTGT TTCTGCAAGA  
E

1601 · V H R E W F M D L N L P W S S A G S T V W R N R E T L M E F E E F  
TGGTCCATCG TGAGTGGTTC ATGGACCTCA ACCTCCCTTG GAGCAGTGTCT GGAAGTACTG TGTGGAGGAA CAGAGAGACG TTAATGGAGT TTGAGGAACC  
ACCAGGTAGC ACTACCAAG TACCTGGAGT TGGAGGGAAC CTCGTACGA CCTCATGAC ACACCTCCTT GTCTCTCTGC AATTACCTCA AACTCCTTGG  
E

1701 · H A T K Q S V I A L G S Q E G A L H Q A L A G A I P V E F S S N T  
ACACGCCACG AAGCAGTCTG TGATAGCATT GGGCTCACAA GAGGGAGCTC TGCATCAAGC TTTGGCTGGA GCCATTCTTG TGGAAATTTT AAGCAACACT  
TGTGCGGTGC TTCGTACAGC ACTATCGTAA CCCGAGTGT CTCCCTCGAG ACSTAGTTTC AAACCGACCT CGGTAAGGAC ACCTTAAAG TFCGTTGTGA  
E

V K L T S G H L K C R V K M E K L Q L K G T T Y G V C S K A F K F L  
 1801 GTCAAGTTGA CGTCGGGTCA TTGAAGTGT AGAGTGAAGA TGGAAAAATT GCAGTTGAAG GGAACAACCT ATGGCGTCTG TTCAAAGGCT TTCAAGTTTC  
 CAGTTCAACT GCAGCCCACT AAACCTCACA TCTCACTTCT ACCTTTTAA CGTCAACTTC CTTGTGTGGA TACCGCAGAC AAGTTTCCGA AAGTTCAAAG  
 E  
 G T P A D T G H G T V V L E L Q Y T G T D G P C K V P I S S V A S  
 1901 TTGGGACTCC CGCAGACACA GGTCAACGCA CTGTGGTGT GGAATTGCAG TACACTGGCA CGGATGGACC TTGCAAGATT CCTATCTCGT CAGTGGCTTC  
 AACCTGAGG GCGTCTGTGT CCAGTGCCGT GACACCACAA CCTTAACGTC ATGTGACCGT GCCTACCTGG AACGTTTCAA GGATAGAGCA GTCACCGAAG  
 E  
 L N D L T P V G R L V T V N E F V S V A T A N A K V L I E L E P P  
 2001 ATTGAACGAC CTAACGCCAG TGGGCAGATT GGTCACTGTC AACCTTTTG TTTCAGTGGC CACGGCCAAAC GCTAAGGTCC TGATTGAATT GGAACCAACC  
 TAACTTGCTG GATTGCGGTC ACCCGTCTAA CCAGTGACAG TTGGGAAAC AAAGTCACCG GTGCCGGTTC CGATTCCAGG ACTAAGTTAA CCTTGGTGGG  
 E  
 F G D S Y I V V G R G E Q Q I N H H W H K S G S S I G K A P T T T L  
 2101 TTGGGAGACT CATACTAGT GGTGGGCAGA GGAGAACAC AGATCAATCA CCACTGGCAC AAGTCTGGAA GCAGCATTGG CAAAGCCTTT ACAACCAACC  
 AACCTCTGA GTATGTATCA CCACCGTCT CCTCTGTG TCTAGTATG GTTGACCGTG TTCAGACCTT CGTCGTAACC GTTTCGGAAA TGTGGTGGG  
 E  
 K G A Q R L A A L G D T A W D F G S V G G V F T S V G K A V H Q V  
 2201 TCAAAGGAGC GCAGAGACTA GCCGCTCTAG GAGACACAGC TTGGGACTTT GGATCAGTTG GAGGGGTGTT CACCTCAGTT GGAAGGGCTG TCCATCAAGT  
 AGTTTCCTCG CGTCTCTGAT CGCGGAGATC CTCTGTGTG AACCTTGAAA CTTAGTCAAC CTCGCCACAA GTGGAGTCAA CCCTCCGAC AGGTAGTTCA  
 E  
 F G G A F R S L F G G M S W I T Q G L L G A L L L W M G I N A R D  
 2301 GTTCGGAGGA GCATTCCGCT CACTGTTCCG AGGCATGTCC TGGATAACGC AAGGATTGCT GGGGGCTCTC CTGTTGTGGA TGGGCATCAA TGCTCGTGAC  
 CAAGCCTCCT CGTAAGGCGA GTGACAAGCC TCCGTACAGG ACCTATTGCG TTCTAACGA CCCCCGAGG GACAACACCT ACCCGTAGTT ACGAGCACTG  
 deleted NS1  
 E  
 R S I A L T F L A V G G V L L F L S V N V H A D T G I H R G P A T R  
 2401 AGGTCCATAG CTCTACGTT TCTCGAGTT GGAGGAGTTC TGCTCTTCT CTCCGTGAAC GTGCACGCTG AACTGGGAT CCACCGTGA CCTGCCACTC  
 TCCAGGTATC GAGAGTGCAA AGAGCGTCAA CCTCCTCAAG ACGAGAGGA GAGGCACTTG CAGTGGCAG TGTGACCCTA GGTGGCACCT GGACGGTGAG  
 deleted NS1  
 T T T E S G K L I T D W C C R S C T L P P L R Y Q T D S G C W Y G  
 2501 GCACCACCAC AGAGAGCGGA AAGTTGATRA CAGATTGGTG CTGCAGGAGC TGCACCTTAC CACCACTGCG CTACCAACT GACAGCGGCT GTTGGTATGG  
 CGTGGTGGTG TCTCTCGCCT TTCAACTATT GTCTAACAC GACGTCTCC ACGTGAATG GTGGTGACGC GATGGTTTGA CTGTGCGCGA CAACCATACC  
 deleted NS1  
 NS2A  
 M E I R P Q R H D E K T L V Q S Q V N A Y N A D M I D P F Q L G L  
 2601 TATGGAGATC AGACCACAGA GACATGATGA AAAGACCCCT GTGCAGTCAC AAGTGAATGC TTATAATGCT GATATGATTG ACCCTTTTCA GTTGGGCTT  
 ATACCTCTAG TCTGGTGTCT CTGTACTACT TTTCTGGGAG CACGTCAAGT TTCACCTACG AATATTACGA CTATACTAAC TGGGAAAAGT CAACCCGGAA

# Sequence Appendix 5

## PIV-WNV(ΔprME)/RSV-F

C protein

5' UTR

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1 AGTAGTTCGC CTGTGTGAGC TGACAACTT AGTAGTGTTC GTGAGGATTA ACAACAATTA ACACAGTCCG AGCTGTTTCT TAGCACGAAG ATCTCGATGT
TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCACAAA CACTCCTAAT TGTTGTTAAT TGTCACGC TCGACAAAGA ATCTGCTTC TAGAGCTAGA
C protein
-----
. K K P G G P G K S R A V Y L L K R G M P R V L S L I G L K R A M L .
101 CTAAGAAACC AGGAGGGCCC GGCAAGAGCC GGGCTGTCTA TTTGCTAAAA CGCGGAATGC CCCGCGTGT GTCTTGATT GGACTTAAGA GGGCTATGTT
GATTCCTTGG TCCTCCCGGG CCGTTCCTCG CCCGACAGAT AAACGATTTT GCGCCTTACG GGGCGCACAA CAGGAATAA CCTGAATTCT CCCGATACAA
C protein
-----
. S L I D G K G P I R F V L A L L A F F R F T A I A P T R A V L D R .
201 GAGCCTGATC GACGGCAAGG GGCCAATACG ATTTGTGTGG GCTCTCTTGG CTTCTTCAG GTTCACAGCA ATTGCTCCGA CCCGAGCAGT GCTGGATGCA
CTCGGACTAG CTGCCGTTCC CCGGTTATGC TAAACACAAC CGAGAGAACC GCAAGAAGTC CAGTGTCTGT TAACGAGGCT GGGCTCGTCA CGACCTAGCT
NS3 cleavage
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C protein

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301 W R G V N K Q T A M K H L L S F K K E L G T L T S A I N R R S S K Q .
TGGAGAGGTG TGAACAAACA AACAGCGATG AACACCTTC TGAGTTTCAA GAAGGAATA GGGACCTTGA CCAGTGTCTAT CAATCGGCGG AGCTCAAAGC
ACCTCTCCAC ACTTGTTTGT TTGTCGCTAC TTTGTGGAAG ACTCAAAGTT CTTCCTTGAT CCTGGAACT GGTACAGATA GTTAGCCGCC TCGAGTTTCG
F signal
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NS3 cleavage

F1

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401 . K K R G G E L L I L K A N A I T T I L T A V T F C F A S G Q N I T .
AAAAGAAGCG AGGGGGCGAG TTGCTAATCC TCAAAGCAAA TGCAATTACC ACAATCCTCA CTGCAGTCAC ATTTGTGTTT GCTTCGGTC AAAACATCAC
TTTTCTTCGC TCCCGGCTC AACGATTAGG AGTTTCGTTT ACGTTAATGG TGTTAGGAGT GACGTAGTG TAAACAAAA CGAAGACCAG TTTGTAGTG
F1
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501 . E E F Y Q S T C S A V S K G Y L S A L R T G W Y T S V I T I E L S .
TGAAGAATTT TATCAATCAA CATGCAGTGC AGTTAGCAAA GGCTATCTTA GTGCTCTGAG AACTGGTTGG TATACCACTG TTATACTAT AGAATTAAGT
ACTTCTTAAA ATAGTTAGTT GTACGTACG TCAATCGTTT CCGATAGAAT CACGAGACTC TTGACCAACC ATATGGTCAC AATATTGATA TCTTAATTCA
F1
-----

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601 N I K E N K C N G T D A K V K L I K Q E L D K Y K N A V T E L Q L L .
AATATCAAGG AAAATAAGTG TAATGGAACA GATGCTAAGG TAAATTTGAT AAAACAAGAA TTAGATAAAT ATAAAAATGC TGTAACAGAA TTGCAGTTGC
TTATAGTTCC TTTTATTCAC ATTACCTTGT CTACGATTC ATTTAATA TTTTGTCTT AATCTATTTA TATTTTACG ACATTGTCTT AACGTCAACG
F1
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701 . M Q S T P P T N N R A R R E L P R F M N Y T L N N A K K T N V T L .
TCATGCAAAG CACACCACCA ACAACAATC GAGCCAGAAG AGAACTACCA AGGTTTATGA ATTATACACT CAACAATGCC AAAAAACCA ATGTAACATT
AGTACGTTTC GTGTGGTGGT TGTGTTAG CTGGGCTTC TCTGATGGT TCCAATACT TAATATGTA GTGTGTACG TTTTGTGGT TACATTGTA
F1
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801 . S K K R K R R F L G F L L G V G S A I A S G V A V S K V L H L E G  
 AAGCAAGAAA AGGAAAAGAA GATTTCTTGG TTTTITGTTA GGTGTGGAT CTGCAATCCG CAGTGGCGTT GCTGTATCTA AGGTCTGCA CCTAGAAGGG  
 TTCGTTCTTT TCCTTTTCTT CTAAGAACC AAAAACAAT CCACACCTA GACGTTAGCG GTCACCGCAA CGACATAGAT TCCAGGACGT GGATCTTCCC  
 F2

901 E V N K I K S A L L S T N K A V V S L S N G V S V L T S K V L D L K  
 GAAGTGAACA AGATCAAAG TGCTCTACTA TCCACAAACA AGGCTGTAGT CAGCTTATCA AATGGAGTTA GTGTCTTAAC CAGCAAAGTG TTAGACCTCA  
 CTTCACTTGT TCTAGTTTTC ACGAGATGAT AGGTGTTTGT TCCGACATCA GTCGAATAGT TTACCTCAAT CACAGAATTG GTCGTTTAC AATCTGGAGT  
 F2

1001 . N Y I D K Q L L P I V N K Q S C S I S N I E T V I E F Q Q K N N R  
 AAACTATAT AGATAACAA TTGTTACCTA TTGTGAACAA GCAAAGCTGC AGCATATCAA ATATAGAAAC TGTGATAGAG TTCCAACAAA AGAACAACAG  
 TTTTGATATA TCTATTGTTT AACAAATGGAT AACACTTGT CTTTTGACG TCGTATAGTT TATATCTTTG AACTATCTC AAGGTTGTTT TCTTGTGTGTC  
 F2

1101 . L L E I T R E F S V N A G V T T P V S T Y M L T N S E L L S L I N  
 ACTACTAGAG ATTACCAGGG AATTAGTGT TAATGCAGGT GTAACACAC CTGTAAGCAC TTACATGTTA ACTAATAGTG AATTATTGTC ATTAATCAAT  
 TGATGATCTC TAATGGTCCC TTAAATCACA ATTACGTCCA CATTGATGTG GACATTCGTG AATGTACAAT TGATTATCAC TTAATAACAG TAATTAGTTA  
 F2

1201 D M P I T N D Q K K L M S N N V Q I V R Q Q S Y S I M S I I K E E V  
 GATATGCCCTA TAACAATGA TCAGAAAAG TTAATGTCCA ACAATGTTCA AATAGTTAGA CAGCAAAGTT ACTCTATCAT GTCCATAATA AAAGAGGAAG  
 CTATACGGAT ATTGTTTACT AGTCTTTTTC AATTACAGGT TGTTACAAGT TTATCAATCT GTCGTTTCAA TGAGATAGTA CAGGTATTAT TTTCTCCTTC  
 F2

1301 . L A Y V V Q L P L Y G V I D T P C W K L H T S P L C T T N T K E G  
 TCTTAGCATA TGTAAGTACAA TTACCCTAT ATGGTGTAT AGATACACCC TGTGGGAAC TACACACATC CCGTCTATGT ACAACCAACA CAAAGAAGG  
 AGAATCGTAT ACATCATGTT AATGGTGATA TACCACAATA TCTATGTGGG ACAACCTTG ATGTGTGTAG GGGAGATACA TGTTGGTTGT GTTTTCTTCC  
 F2

1401 . S N I C L T R T D R G W Y C D N A G S V S F F P Q A E T C K V Q S  
 GTCCAACATC TGTTTAACAA GAACTGACAG AGGATGGTAC TGTGACAATG CAGGATCAGT ATCTTTCTTC CCACAAGCTG AAACATGTAA AGTTCAATCA  
 CAGGTTGTAG ACAAAATGTT CTGACTGTC TCCTACCATG ACACGTGTAC GTCCTAGTCA TAGAAAGAAG GGTGTTGAC TTTGTACATT TCAAGTTAGT  
 F2

1501 N R V F C D T M N S L T L P S E I N L C N V D I F N P K Y D C K I M  
 AATCGAGTAT TTTGTGACAC AATGAACAGT TTAACATTAC CAAGTGAAAT AAATCTCTGC AATGTTGACA TATCAACCC CAAATATGAT TGTAATAATTA  
 TTAGCTCATA AAACACTGTG TTAAGTTGICA AATTGTAATG GTTCACTTTA TTAGAGAGCG TTCAACTGT ATTAAGTTGGG GTTTATACTA ACATTTTAA  
 F2

1601 . T S K T D V S S S V I T S L G A I V S C Y G K T K C T A S N K N R  
 TGACTTCAAA AACAGATGTA AGCAGCTCCG TTATCACATC TCTAGGAGCC ATTGTGTCTAT GCTATGGCAA AACTAAATGT ACAGCATCCA ATAAATATCG  
 ACTGAAGTTT TTGTCTACAT TCGTCGAGGC AATAGTGTAG AGATCCTCGG TAACACAGTA CGATACCGTT TTGATTTACA TGTCGTAGGT TATTTTTAGC  
 F2

1701 . G I I K T F S N G C D Y V S N K G M D T V S V G N T L Y Y V N K Q  
 TGGAATCATA AAGACATTTT CTAACGGGTG CGATTATGTA TCAAAATAAG GGATGGACAC TGTGTCTGTA GGTAACACAT TATATTATGT AAATAAGCAA  
 ACCTTAGTAT TTCTGTAAAA GATTGCCAC GCTAATACAT AGTTTATTTT CCTACCTGTG ACACAGACAT CCATTGTGTA ATATAATACA TTTATTCGTT  
 F2

1801 E G K S L Y V K G E P I I N F Y D P L V F P S D E F D A S I S Q V N  
 GAAGGTAAAA GTCTCTATGT AAAAGGTGAA CCAATAATAA ATTTCTATGA CCCATTAGTA TTCCCTCTG ATGAATTTGA TGCAATCAATA TCTCAAGTCA

CTTCCATTTT CAGAGATACA TTTTCCACTT GGTATTATT TAAAGATACT GGGTAATCAT AAGGGGAGAC TACTTAACT ACGTAGTTAT AGAGTTCAGT  
F2

TM Domain

1901 . E K I N Q S L A F I R K S D E L L H N V N A G K S T T N I M I T T .  
ACGAGAAGAT TAACCAGAGC CTAGCATTTA TTCGTAAATC CGATGAATTA TTACATAATG TAAATGCTGG TAAATCCACC ACAATATCA TGATAACTAC  
TGCTCTTCTA ATTGGTCICG GATCGTAAAT AAGCATTTAG GCTACTTAAT AATGTATTAC ATTTACGACC ATTTAGGTGG TGTITATAGT ACTATTGATG  
TM Domain

Cytoplasmic Tail

2001 . I I I V I I V I L L S L I A V G L L L Y C K A R S T P V T L S K D  
TATAATTATA GTGATTATAG TAATATTGTT ATCATTAAAT GCTGTGGAC TGCTCTTATA CTGTAAGGCC AGAAGCACAC CAGTCACACT AAGCAAAGAT  
ATATTAAATAT CACTAATATC ATTATAACAA TAGTAATTAA CGACAACCTG ACGAGAATAT GACATTCGG TCCTCGTGTG GTCAGTGTGA TTCGTTTCTA  
FMDV 2A

Transmembrane

domain of WNV E (split)

Cytoplasmic Tail

pre E/NS1 signal

2101 Q L S G I N N I A F S N N F D L L K L A G D V E S N F G P A R D R S .  
CAACTGAGTG GTATAAATAA TATTGCATTI AGTAACAATT TTGATCTGCT CAAACTTGCA GGCGATGTAG AATCAAATCC TGGACCCGCC CGGGACAGGT  
GTTGACTCAC CATATTTATT ATAACGTAAA TCATTGTTAA AACTAGACGA GTTTGAACGT CCGCTACATC TTAGTTIAGG ACCTGGGGCGG GGCCTGTCCA  
NS1

Transmembrane domain of WNV E (split)

2201 . I A L T F L A V G G V L L F L S V N V H A D T G C A I D I S R Q  
CCATAGCTCT CACGTTTCTC GCAGTTGGAG GAGTTCTGCT CTTCCTCTCC GTGAACGTGC ACGCTGACAC TGGGTGTGCC ATAGACATCA GCCGGCAA  
GGTATCGAGA GTGCRAAGAG CGTCACCTC CTCAAGACGA GAAGGRGAGG CACTTGACAG TCGACTGTG ACCCACACGG TATCTGTAGT CGGCCGTT

## PIV-WNV( $\Delta$ CprME)/RSV-F

deleted C protein

5' UTR

1 AGTAGTTGCG CTGTGTGAGC TGACAAACTT AGTAGTGTTF GTGAGGATTA ACAACAATTA ACACAGTGCG AGCTGTTTCT TAGCACGAAG ATCTCGATGT  
TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCACAAA CACTCCTAAT TGTGTGTAAT TGTGTACCGC TCGACAAAGA ATCGTGCTTC TAGAGCTACA  
deleted C protein

NS3 cleavage

. K K F G G P G K S R A V N M L K R G M P R V L S L I G L K Q K K R .

101 CTAAGAAACC AGGAGGGCCC GGCAAGAGCC GGGCTGTCAA TATGCTAAAA CGCGGAATGC CCCGCGTGTG GTCCTTGATT GGACTTAAAGC AAAAGAAGCG  
GATTCTTTGG TCCTCCCGGG CCGTTCCTCGG CCCGACAGTT ATACGATTTT GCGCCTTACG GGGCGCACAA CAGGAACATA CCTGAATTGG TTTTCTTCGC  
F signal

NS3 cleavage

F1

201 . G G E L L I L K A N A I T T I L T A V T F C F A S G Q N I T E E F  
AGGGGGCGAG TTGCTAATCC TCAAAGCAAA TGCAATTACC ACAATCCTCA CTGCAGTCAC ATTTTGTGTT GCTTCTGGTC AAAACATCAC TGAAGAATTT  
TCCCCGCTC AACGATTAGG AGTTTCGTTT ACGTTAATGG TGTTAGGAGT GACGTCAGTG TAAACAAAA CGAAGACCAG TTTGTAGTG ACTTCTTAAA  
F1

301 Y Q S T C S A V S K G Y L S A L R T G W Y T S V I T I E L S N I K E  
TATCAATCAA CATGCAGTGC AGTTAGCAAA GGCTATCTTA GTGCTCTGAG AACTGGTGG TATACCAGTG TTATACTAT AGAATTAAGT AATATCAAGG  
ATAGTTAGTT GTACGTCACG TCAATCGTTT CCGATAGAAT CACGAGACTC TTSACCAACC ATATGGTCAAC AATATTGATA TCTTAATCA TTATAGTTCC  
F1

401 . N K C N G T D A K V K L I K Q E L D K Y K N A V T E L Q L L M Q S  
AAAATAAGTG TAATGGAACA GATGCTAAGG TAAATTTGAT AAAACAAGAA TTAGATAAAT ATAAAAATGC TGTAACAGAA TTGCAGTTGC TCATGCAAGG  
TTTTATTAC ATTACCTTGT CTACGATTC ATTTAACTA TTTTGTTCIT AATCTATTIA TATTTTACG ACATTGTCTT AACGTCAACG AGTACGTTTC  
F1

501 . T P P T N N R A R R E L P R F M N Y T L N N A K K T N V T L S K K  
CACACCACCA ACAAACAATC GAGCCAGAAG AGAACTACCA AGGTITATGA ATTATACACT CAACAATGCC AAAAAACCA ATGTAACATT AAGCAAGAAA  
GTGTGGTGGT TGTGTTGTTAG CTGGTCTTC TCTTGATGGT TCCAAATACT TAATATGTGA GTTGTACGG TTTTGTGGT TACATTGTAA TTCGTTCTTT  
F1

F2

601 R K R R F L G F L L G V G S A I A S G V A V S K V L H L E G E V N K  
AGGAAAAGAA GATTTCTTGG TTTTGTGTTA GGTGTTGGAT CTGCAATCGC CAGTGGCGTT GCTGTATCTA AGGTCTCTGCA CCTAGAAGGG GAAGTGAACA  
TCCTTTTCTT CTAAAGAAC AAAAACAAT CCACAACCTA GACGTTAGCG GTCACCGCAA CGACATAGAT TCCAGGACGT GGATCTTCCC CTTCCTTGT  
F2

701 . I K S A L L S T N K A V V S L S N G V S V L T S K V L D L K N Y I  
AGATCAAAAG TGCTCTACTA TCCACAAACA AGGCTGTAGT CAGCTTATCA AATGGAGTTA GTGTCTTAAC CAGCAAGAGT TTAGACCTCA AAACTATAT  
TCTAGTTTTC ACGAGATGAT AGGTGTTTGT TCCGACATCA GTCGAATAGT TTACCTCAAT CACAGAATTG GTGGTTTCAC AATCTGGAGT TTTTGATATA  
F2

801 . D K Q L L P I V N K Q S C S I S N I E T V I E F Q Q K N N R L L E  
AGATAAACAA TTGTTACCTA TTGTGAACAA GCAAGCTGC AGCATATCAA ATATAGAAAC TGTGATAGAG TTCCAACAAA AGAACAACAG ACTACTAGAG  
TCTATTGTT AACCAATGGAT AACACTTGTT CGTTTCGACG TCGTATAGTT TATATCTTTG AACTATCTC AAGGTGTTT TCTGTGTCG TGATGATCTC  
F2

901 I T R E F S V N A G V T T P V S T Y M L T N S E L L S L I N D M P I  
ATTACCAGGG AATTTAGTGT TAATGCAGGT GTAATACAC CTGTAAGCAC TTACATGTTA ACTAATAGTG AATTAATGTC ATTAATCAAT GATATGCCTA  
TAATGGTCCC TTAATCACA ATTACGTCCA CATGATGTG GACATTCGTG AATGTACAAT TGATTATCAC TTAATAACAG TAATTAGTTA CTATACGGAT  
F2

1001 . T N D Q K K L M S N N V Q I V R Q Q S Y S I M S I I K E E V L A Y  
TAACAAATGA TCAGAAAAG TTAATGTCCA ACAATGTCA AATAGTTAGA CAGCAAGTT ACTCTATCAT GTCCATAATA AAAGAGGAAG TCTTAGCATA  
ATTGTTTACT AGTCITTTTC AATTACAGGT TGTACAAST TTATCAATCT GTCGTTTCAA TGAGATAGTA CAGGTATTAT TTTCTCCTTC AGAATCGTAT  
F2

. V V Q L P L Y G V I D T P C W K L H T S P L C T T N T K E G S N I

1101 TGTAGTACAA TTACCACTAT ATGGTGTAT AGATACACCC TGTGGGAAAC TACACACATC CCCTCTATGT ACAACCAACA CAAAAGAAGG GTCCAACATC  
ACATCATGTT AATGGTGATA TACCACAATA TCTATGTGGG ACAACCTTTG ATGTGTGTAG GGGAGATACA TGTGGTGTGT GTTTTCTTCC CAGGTTGTAG

F2

1201 C L T R T D R G W Y C D N A G S V S F F P Q A E T C K V Q S N R V F  
TGTITACAA GAACGACAG AGGATGGTAC TGTGACAATG CAGGATCAGT ATCTTTCTTC CCACAAGCTG AAACATGTAA AGTTCAATCA AATCGAGTAT  
ACAAATTGTT CTGACTGTC TCCTACCATG ACACTGTTAC GTCTAGTCA TAGAAGAAG GGTGTTCCGAC TTTGTACATT TCAAGTTAGT TTAGCTCATA

F2

1301 C D T M N S L T L P S E I N L C N V D I F N P K Y D C K I M T S K  
TTTCTGACAC AATGAACAGT TTAACATTAC CAAGTGAAT AATCTCTGC AATGTTGACA TATTCACCC CAAATATGAT TGTAATAATTA TGACTTCAAA  
AAACACTGTG TTAATTGTCA AATTGTAATG GTTCACTTTA TTTAGAGACG TTACAACGT ATAAGTTGGG GTTTTATACTA ACATTTTAAT ACTGAAGTTT

F2

1401 T D V S S S V I T S L G A I V S C Y G K T K C T A S N K N R G I I  
AACAGATGTA AGCAGCTCCG TTATCACATC TCTAGGAGCC ATTGTGTCAT GCTATGGCAA AACTAAATGT ACAGCATCCA ATAAAAATCG TGGAAATCATA  
TTGTCTACAT TCGTCGAGGC AATAGTGTAG AGATCCTCGS TAACACAGTA CGATACCGTT TTAGTTTACA TGTCGTAGGT TATTTTATAGC ACCTTAGTAT

F2

1501 K T F S N G C D Y V S N K G M D T V S V G N T L Y Y V N K Q E G K S  
AAGACATTTT CTAACGGGTG CGATTATGTA TCAAATAAAG GGATGGACAC TGTGTCGTGA GGTAACACAT TATATTATGT AATAAGCAA GAAGGTAAAA  
TTCTGTAAAA GATTGCCAC GCTAATACAT AGTTTATTTT CCTACCTGTG ACACAGACAT CCATGTGTGA ATATAATACA TTTATTCGTT CTTCATTTT

F2

1601 L Y V K G E P I I N F Y D P L V P P S D E F D A S I S Q V N E K I  
GTCTCTATGT AAAAGGTGAA CCAATAATAA ATTTCTATGA CCATTAGTA TTCCCTCTG ATGAATTTGA TGCATCAATA TCTCAAGTCA ACGAGAAGAT  
CAGAGATACA TTTTCCACTT GCTTATTATT TAAAGATACT GGGTAAATCAT AAGGGGAGAC TACTTAACT ACCTAGTTAT AGAGTTCAGT TGCTCTCTA

F2

TM Domain

1701 N Q S L A F I R K S D E L L H N V N A G K S T T N I M I T T I I I  
TAACCAGAGC CTAGCATTTA TTCGTAAATC CGATGAATTA TTACATAATG TAAATGCTGG TAAATCCACC ACAAATATCA TGATAACTAC TATAATTATA  
ATTGCTCTCG GATCGTAAAT AAGCATTTAG GCTACTTAAT AATGTATTAC ATTTACGACC ATTTAGGTGG TGTTTATAGT ACTATTGATG ATATTAAATAT

TM Domain

Cytoplasmic Tail

1801 V I I V I L L S L I A V G L L L Y C K A R S T P V T L S K D Q L S G  
GTGATTATAG TAATATGTT ATCATTAAAT GCTGTTGGAC TGCTCTTATA CTGTAAGGCC AGAAGCACAC CAGTCACACT AAGCAAAGAT CAAGTGAATG  
CACTAATATC ATTATAACAA TAGTAATTAA CGACAACCTG ACGAGAATAT GACATTCCGG TCITCGTGTG GTCAGTGTGA TTCGTTTCTA GTTGACTCAG

FMDV 2A

membrane domain of WNV E (split)

Cytoplasmic Tail

pre E/NS1 signal

1901 I N N I A F S N N F D L L K L A G D V E S N P G P A R D R S I A L  
GTATAAATAA TATTGCATTT AGTAACAATT TTGATCTGCT CAACTTGCA GCGGATGTAG AATCAAATCC TGGACCCGCC CGGGACAGGT CCATAGCTCT  
CATATTTATT ATAACGTAAA TCATTGTTAA AACTAGACGA GTTTGAACGT CCGCTACATC TTAGTTTAGG ACCTGGGCGG GCCCTGTCCA GGTATCGAGA

Transmembrane domain of WNV E (split)

NS1

2001 T F L A V G G V L L F L S V N V H A D T G C A I D I S R Q E L R  
CACGTTTCTC GCAGTTGGAG GAGTTCTGCT CTTCCTCTCC GTGACGTGC ACGTGACAC TGGGTGTGCC ATAGACATCA GCCGCAAGA GCTGAGA  
GTGCAAGAG CGTCAACCTC CTCAGACGA GAAGGAGAGG CACTTGCACG TCGACTGTG ACCACACGG TATCTGTAGT CGGCCCTTCT CGACTCT

# PIV-WNV( $\Delta$ C)/RSV-F

1 GATCCTAATA CGACTCACTA TAGASTAGTT CGCCTGTGTG AGCTGACAAA CTTAGTAGTG TTTGTGAGGA TTAACAACAA TTAACACAGT GCGAGCTGTT  
CTAGGATTAT GCTGAGTGAT ATCTCATCAA GCGGACACAC TCGACTGTTT GAATCATCAC AAACACTCCT AATGTGTGTT AATTGTGTCA CGCTCGACAA  
N-terminus of C

101 M S K K P G G P G K S R A V N M L K R G M P R V L S L  
TCTTAGCAGC AAGATCTCGA TGTCTAAGAA ACCAGGAGGG CCCGGCAAGA GCCGGGCTGT CAATATGCTA AAACGCGGAA TGCCCCGCGT GTTGTCTTGT  
AGAATCGTGC TTCTAGAGCT ACAGATTCTT TGGTCTCTCC GGGCCGTTCT CGGCCCGACA GTTATACGAT TTTGCGCCTT ACGGGGCGCA CAACAGGAAC  
N-terminus of C F signal

NS3 cleavage

F1

201 I G L K Q K K R G G E L L I L K A N A I T T I L T A V T F C F A S G  
ATTGGACTTA AGCAAAAGAA GCGAGGGGGC GAGTTGCTAA TCCTCAARGC AATGCAAIT ACCACAATCC TCACTGCAGT CACATTTTGT TTTGCTTCTG  
TAACCTGAAT TCGTTTTCTT CGCTCCCCCG CTCACAGATT AGGAGTTTCG TTTACGTTAA TGGTGTTAGG AGTGACGTCA GTGTAAACA AAACGAAGAC  
F1

301 Q N I T E E F Y Q S T C S A V S K G Y L S A L R T G W Y T S V I T  
GTCAAAACAT CACTGAAGAA TTTTATCAAT CAACATGCAG TGCAGTTAGC AAAGGCTATC TTAGTGCTCT GAGAACTGGT TGGTATACCA GTGTTATAAC  
CAGTTTGTGA GTGACTTCTT AAAATAGTTA GTTGTACGTC ACGTCAATCG TTTCCGATAG AATCAGSAGA CTCITGACCA ACCATATGGT CACAATATTG  
F1

401 I E L S N I K E N K C N G T D A K V K L I K Q E L D K Y K N A V T  
TATAGAATTA AGTAATATCA AGGAAAATAA GTGTAATGGA ACAGATGCTA AGGTAAATTT GATAAAACAA GAATTAGATA AATATAAAAA TGCTGTAACA  
ATATCTTAAT TCATTATAGT TCCTTTTATT CACATTACCT TGTCTACGAT TCCATTTTAA CTATTTTGTG CTTAATCTAT TTATATTTTT ACGACATTGT  
F1

501 E L Q L L M Q S T P P T N N R A R R E L P R F M N Y T L N N A K K T  
GAATTGCAGT TGCTCATGCA AAGCACACCA CCAACAACA ATCGAGCCAG AAGAGAATA CCAAGGTTTA TGAATTATAC ACTCAACAAT GCCAAAAAAA  
CTTAACGTCA ACGAGTACGT TTCGTGTGGT GGTGTTTGT TAGCTCGGTC TTCTCTTGAT GGTTCCAAAT ACTTAATATG TGAGTTGITA CGGTTTTTTT  
F2

F1

601 N V T L S K K R K R R F L G F L L G V G S A I A S G V A V S K V L  
CCAATGTAAC ATTAAGCAAG AAAAGGAAAA GAAGATTCTT TGGTTTTTGT TTAGGTGTG GATCTGCAAT CGCCAGTGGC GTTGCTGTAT CTAAGGTCCT  
GGTTACATTG TAAATCGTTC TTTTCTTTT CTCTAAAGA ACCAAAAAC AATCCACAAC CTAGACGTTA GCGGTCACCG CAACGACATA GATTCCAGGA  
F2

701 H L E G E V N K I K S A L L S T N K A V V S L S N G V S V L T S K  
GCACCTAGAA GGGGAAGTGA ACRAGATCAA AAGTGCCTA CTATCCACAA ACAAGGCTGT AGTCAGCTTA TCNAATGGAG TTAGTGTCTT AACCAGCAAA  
CGTGATCTT CCCCTTCACT TGTCTAGTT TTCACGAGAT GATAGGTTT TGTCCGACA TCAGTCGAAT AGTTTACCTC AATCACAGAA TTGGTCTT  
F2

V L D L K N Y I D K Q L L P I V N K Q S C S I S N I E T V I E F Q Q

801 GTGTTAGACC TCAAAAAC TAATAGATAA CAATTGTTAC CTATTGTGAA CAAGCAAAGC TGCAGCATAT CAAATATAGA AACGTGATA GAGTTCCAAC  
 CACAATCTGG AGTTTTTGAT ATATCTATTT GTTAACAATG GATAACACTT GTTCGTTTCG ACGTCGTATA GTTATATCTT TTGACACTAT CTCAGGGTTG  
 F2

901 · K N N R L L E I T R E F S V N A G V T T P V S T Y M L T N S E L L ·  
 AAAAGAACA CAGACTACTA GAGATTACCA GGAATTTAG TGTTAATGCA GGTGTAAC TAACCTGTAAG CACTTACATG TTAAC TAATA GTGAATTATT  
 TTTTCTTGT GTCTGATGAT CTCTAATGGT CCCTTAAATC ACAATTACGT CCACATTGAT GTSGACATTC GTGAATGTAC AATTGATTAT CACTTAATAA  
 F2

1001 · S L I N D M P I T N D Q K K L M S N N V Q I V R Q Q S Y S I M S I ·  
 GTCATTAATC AATGATATGC CTATAACAAA TGATCAGAAA AAGTTAATGT CCAACAATGT TCAAAATAGT AGACAGCAAA GTTACTCTAT CATGTCCATA  
 CAGTAATTAG TTACTATACG GATATTGTTT ACTAGTCTTT TTCAATTACA GGTTGTACA AGTTTATCAA TCTGTCTTT CAATGAGATA GTACAGGTAT  
 F2

1101 · I K E E V L A Y V V Q L P L Y G V I D T P C W K L H T S P L C T T N ·  
 ATAAAGAGG AAGTCTTAGC ATATGTAGTA CAATTACCAC TATATGGTGT TATAGATACA CCCTGTTGGA AACTACACAC ATCCCTCTA TGTACAACCA  
 TATTTCTCC TTCAGAATCG TATACATCAT GTTAATGGTG ATATACCACA ATATCTATGT GGGACAACCT TTGATGTGTG TAGGGGAGAT ACATGTTGGT  
 F2

1201 · T K E G S N I C L T R T D R G W Y C D N A G S V S F F P Q A E T C ·  
 ACACAAAAGA AGGGTCCAAC ATCTGTTTAA CAAGAATGTA CAGAGGATGG TACTGTGACA ATGCAGGATC AGTATCTTTC TTCCACAAAG CTGAAACATG  
 TGTGTTTTCT TCCCAGGTTG TAGACAAATT GTTCTTGACT GTCTCCTACC ATGACACTGT TACGTCCTAG TCATAGAAAG AAGGGTGTTC GACTTTGTAC  
 F2

1301 · K V Q S N R V F C D T M N S L T L P S E I N L C N V D I F N P K Y ·  
 TAAAGTTCAA TCAAATCGAG TATTTTGTGA CACAATGAAC AGTTTAACAT TACCAAGTGA AATAAATCTC TGCAATGTG ACATAATTCAA CCCCAAATAT  
 ATTTCAAGTT AGTTTAGCTC ATAAACACT GTGTTACTTG TCAAATTGTA ATGGTTCAT TTATTTAGAG ACGTTACAAC TGTATAAGTT GGGGTTTATA  
 F2

1401 · D C K I M T S K T D V S S S V I T S L G A I V S C Y G K T K C T A S ·  
 GATTGTAAAA TTATGACTTC AAAACAGAT GTAAGCAGCT CCGTTATCAC ATCTCTAGGA GCCATTGTGT CATGCTATGG CAAAACATAA TGTACAGCAT  
 CTAACATTTT AATACTGAAG TTTTGTCTA CATTCTGCGA GGCATATGTA TAGAGATCCT CGGTAACACA GTACGATACC GTTTGATTT ACATGTCGTA  
 F2

1501 · N K N R G I I K T F S N G C D Y V S N K G M D T V S V G N T L Y Y ·  
 CCAATAAAAA TCGTGGAAATC ATAAAGACAT TTTCTAACGG GTGCGATTAT GTATCAATAA AAGGGATGGA CACTGTGTCT GTAGGTAACA CATTATTTA  
 GGTATTTTT AGCACCTTAG TATTCTGTA AAAGATTGCC CACGCTAATA CATAGTTTAT TTCCCTACCT GTGACACAGA CATCCATTGT GTATATAAT  
 F2

1601 · V N K Q E G K S L Y V K G E P I I N F Y D P L V F P S D E F D A S ·  
 TGTAAATAAG CAAGAAGGTA AAGTCTCTA TGTAAAGGT GAACCAATAA TAAATTTCTA TGACCCATA GTATTCCCCT CTGATGAATT TGATGCATCA  
 ACATTTATTC GTTCTCCAT TTTCAGAT ACATTTTCCA CTGGTTATT ATTTAAAGAT ACTGGGTAAT CATAAGGGGA GACTACTTAA ACTACGTAGT  
 F2

1701 · I S Q V N E K I N Q S L A F I R K S D E L L H N V N A G K S T T N I ·  
 ATATCTCAAG TCAACGAGAA GATTACACAG AGCCTAGCAT TTATTCGTAA ATCCGATGAA TTATTACATA ATGTAAATGC TGGTAAATCC ACCACAAATA  
 TATAGAGTTC AGTTGCTCTT CTAATTGGTC TCGGATCGTA AATAAGCATT TAGGCTACTT AATAATGTAT TACATTTACG ACCATTTAGG TGGTGTATTAT  
 TM Domain

Cytoplasmic Tail

· M I T T I I I V I I V I L L S L I A V G L L L Y C K A R S T P V T ·

1801 TCATGATAAC TACTATAATT ATAGTGATTA TAGTAATATT GTTATCAITTA ATTGCTGTTG GACTGCTCTT ATACTGTAAG GCCAGAAGCA CACCAGTCAC  
AGTACTATTG ATGATATTAA TATCACTAAT ATCATTATAA CAATAGTAAT TAACGACAAC CTGACGAGAA TATGACATTG CCGTCTTCGT GTGGTCAGTG  
FMDV 2A

Cytoplasmic Tail

· L S K D Q L S G I N N I A F S N N F D L L K L A G D V E S N P G P  
1901 ACTAAGCAAA GATCAACTGA GTGGTATAAA TAATATTGCA TTTAGTAACA ATTTTGATCT GCTCAAACTT GCAGGCGATG TAGAATCAAA TCCTGGACCC  
TGATTGCTT CTAGTTGACT CACCATATT ATTATAACGT AAATCATTGT TAAAACTAGA CGAGTTTGAA CGTCCGCTAC ATCTTAGTTT AGGACCTGGG  
prM

C/prM signal

· G G K T G I A V M I G L I A C V G A V T L S N F Q G K V M M T V N A  
2001 GGAGGAAAGA CCGGTATTGC AGTCATGATT GGCCTGATCG CCTGCGTAGG AGCAGTTACC CTCCTCTAAT TCCAAGGGAA GGTGATGATG ACGGTAAATG  
CCTCCTTICT GGCCATAACG TCAGTACTAA CCGGACTAGC GGACGCTACC TCGTCAATGG GAGAGATTGA AGGTTCCCTT CCACTACTAC TGCCATTAC  
prM

· T D V T D V I T I P T A A Q K N L C I V R A M D V G Y M C D D T I  
2101 CTACTGACGT CACAGATGTC ATCAGGATTC CAACAGCTGC TGGAAAGAAC CTATGCTATG TCAGAGCAAT GGATGTGGGA TACATGTGCG ATGATACTAT  
GATGACTGCA GTGTCTACAG TAGTGCTAAG GTTGTCGACG ACCTTTCTTG GATACGTAAC AGTCTCGTTA CCTACACCCT ATGTACACGC TACTATGATA  
prM

· T Y E C P V L S A G N D P E D I D C W C T K S A V Y V R Y G R C T  
2201 CACTTATGAA TGCCGAGTGC TGTGCGCTGG TAATGATCCA GAAGACATCG ACTGTTGGTG CACAAAGTCA GCAGTCTACG TCAGGTATGG AAGATGCACC  
GTGAATACTT ACGGGTCACG ACAGCCGACC ATTACTAGGT CTCTGTAGC TGACAACCAC GTGTTTCAGT CGTCAGATGC AGTCCATACC TTCTACGTGG  
prM

· K T R H S R R S R R S L T V Q T H G E S T L A N K K G A W M D S T K  
2301 AAGACACGCC ACTCAAGACG CAGTCGGAGG TCACTGACAG TGCAGACACA CGGAGAAAGC ACTCTAGCGA ACAAGAAGGG GCCTTGGATG GACAGCACCA  
TTCTGTGCGG TGAGTTCTGC GTCAGCCTCC AGTGAAGTGC ACCTCTGTGT GCCTCTTTTC TGAGATCGCT TGTCTTTCCC CCGAACCTAC CTGTCTGTGGT  
prM

· A T R Y L V K T E S W I L R N P G Y A L V A A V I G W M L G S N T  
2401 AGGCCACAAG GTATTTGGTA AAAACAGAAT CATGGATCTT GAGGAACCCCT GGATATGCCC TGGTGGCAGC CGTCATTGGT TGGATGCTTG GGAGCAACAC  
TCCGGTGTTC CATAAACCAT TTTGTCTTA GTACCTAGAA CTCTTGCGA CCTATACGGG ACCACCGTCG GCAGTAACCA ACCTACGAAC CCTCGTTGTG  
prM

· M Q R V V F V V L L L L V A P A Y S F N C L G M S N R D F L E G V  
2501 CATGCAGAGA GTTGTGTTTG TGTGCTATT GCTTTTGGTG GCGCCAGCTT ACAGCTTTAA CTGCCTTGGG ATGAGCAACA GAGACTTCTT GGAAGGAGTG  
GTACGTCTCT CAACACAAAC AGCAGGATAA CGAAAACCA CCGGGTCGAA TGTGGAATTT GACGGAACCT TACTCGTTGT CTCTGAAGAA CCTTCCTCAC  
prM

E

· S G A T W V D L V L E G D S C V T I M S K D K P T I D V K M M N M E  
2601 FCTGGAGCAA CATGGGTGGA TTTGCTTCTC GAAGGCGACA GCTCGGTGAC TATCATGTCT AAGGACAAGC CTACCATCGA TGTGAAGATG ATGAATATGG  
AGACCTCGTT GTACCCACCT AAACCAAGAG CTTCGCTGT CGACGCACTG ATAGTACAGA TTCTGTTCG GATGGTAGCT ACACCTCTAC TACTTTATAC  
E

· A A N L A E V R S Y C Y L A T V S D L S T K A A C P A M G E A H N  
2701 AGGCGGCCAA CCTGGCAGAG GTCCGCAATT ATTGCTATTT GGCTACCGTC AGCGATCTCT CCACCAAAGC TGCGTGCCCG GCCATGGGAG AAGCTCACAA  
TCCGCCGGTT GGACCGTCTC CAGGCGTCAA TAACGATAAA CCGATGGCAG TCGTAGAGA GGTGGTTTCG ACGCACGGGC CGGTACCCCTC TTGAGTGT  
E

2801 · D K R A D P A F V C R Q G V V D R G W G N G C G L F G K G S I D T  
 TGACAAACGT GCTGACCCAG CTTTGTGTG CAGACAAGGA GTGGTGGACA GGGGCTGGG CAACGGCTGC GGACTATTTG GCAAAGGAAG CATTGACACA  
 ACTGTTTGCA CGACTGGGTC GAAACACAC GTCTGTTCCT CACCACCTGT CCCCACCCC GTTGCCGACG CCTGATAAAC CGTTTCCTTC GTAACGTGT  
 E  
 2901 C A K F A C S T K A I G R T I L K E N I K Y E V A I F V H G P T T V  
 TGGCCCAAT TTGCCTGCTC TACCAAGGCA ATAGGAAGAA CCATTITGAA AGAGAATATC AAGTACGAAG TGGCCATTTT TGTCCATGGA CCAACTACTG  
 ACGCGGTTTA AACGGACGAG ATGGTICCGT TATCCTTCTT GGTAAACTT TCTCTTATAG TTCATGCTTC ACCGGTAAAA ACAGGTACCT GGTGATGAC  
 E  
 3001 · E S H G N Y S T Q V G A T Q A G R F S I T P A A P S Y T L K L G E  
 TGGAGTCGA CGGAACCTAC TCCACACAGG TTGGAGCCAC TCAGGCAGGG AGATTGACGA TCACTCCTGC GCGCCCTTCA TACACACTAA AGCTTGGAGA  
 ACCTCAGCGT GCCTTTGATG AGGTGTGTCC AACCTCGGTG AGTCCCTCCC TCTAAGTCGT AGTGAGGACG CCGCGGAAGT ATGTGTGATT TCGAACCTCT  
 E  
 3101 · Y G E V T V D C E P R S G I D T N A Y V V M T V G T K T F L V H R  
 ATATGGGAG GTGACAGTGG ACTGTGAACC ACGGTGAGG ATTGACACCA ATGCATACTA CGTGATGACT GTTGGAAACA AGACGTTCTT GGTCCATCGT  
 TATACCTCTC CACTGTCAAC TGACACTTGG TGCCAGTCCC TAACTGTGGT TACGTATGAT GCACTACTGA CAACCTTGT TCTGCAAGAA CCAGGTAGCA  
 E  
 3201 E W F M D L N L P W S S A G S T V W R N R E T L M E F E E P H A T K  
 GAGTGGTICA TGGACCTCAA CCTCCCTTGG AGCAGTGTCT GAAGTACTGT GTGGAGGAAC AGAGAGACGT TAATGGAGTT TGAGGAACCA CACGCCACGA  
 CTCACCAAGT ACCTGGAGTT GGAGGGAACC TCGTCACGAC CTTCATGACA CACCTCCTTG TCTCTGTGA ATTACCTCAA ACTCCTTGGT GTGCGGTGCT  
 E  
 3301 · Q S V I A L G S Q E G A L H Q A L A G A I P V E F S S N T V K L T  
 AGCAGTCTGT GATAGCATTG GGCTCACAG AGGGAGCTCT GCATCAAGCT TTGGCTGGAG CCATTCTGT GGAATTTTCA AGCAACACTG TCAAGTTGAC  
 TCGTCAGACA CTATCGTAAC CCGAGTGTTC TCCCTCGAGA CGTAGTTTCA AACCGACCTC GGTAAAGACA CCTTAAAGT TCGTTGTGAC AGTTCAACTG  
 E  
 3401 · S G H L K C R V K M E K L Q L K G T T Y G V C S K A F K F L G T P  
 GTGCGGTCTT TTGAAGTGA GAGTGAAGAT GGAAAAATG CAGTTGAAGG GAACAACCTA TGGCGTCTGT TCAAAGGCTT TCAAGTTTCT TGGGACTCCC  
 CAGCCCACTA AACTTCACAT CTCCTTCTA CCTTTTAACT GTCAACTTCC CTGTGTGGAT ACCGCAGACA AGTTTCCGAA AGTTCAAAGA ACCCTGAGGG  
 E  
 3501 A D T G H G T V V L E L Q Y T G T D G P C K V P I S S V A S L N D L  
 GCAGACACAG GTCACGGCAC TGTGGTGTG GAATTGCAGT AACTTGGCAC GGATGGACCT TGCAAGTTC CTATCTCGTC AGTGGCTTCA TTGAACGACC  
 CGTCTGTGTC CAGTGCCGTG ACACCACAAC CTTAACGTCA TGTGACCCGT CCTACCTGGA ACGTTTCAAG GATAGAGCAG TCACCAAGT AACTTGCTGG  
 E  
 3601 · T P V G R L V T V N P F V S V A T A N A K V L I E L E P P F G D S  
 TAACGCCAGT GGCAGATTG GTCAGTGTCA ACCCTTTTGT TTCAGTGGCC ACGGCCAACG CTAAGGTCTT GATTGAATTG GAACCAACCT TTGGAGACTC  
 ATTGCGGTCA CCGCTCTAAC CAGTGACAGT TGGGAAAACA AAGTCACCGG TGCCGTTTGC GATTCCAGGA CTAACCTAAC CTGTGTGGGA AACCTCTGAG  
 E  
 3701 · Y I V V G R G E Q Q I N H H W H K S G S S I G K A F T T T L K G A  
 ATACATAGTG GTGGGCAGAG GAGAACAACA GATCAATCAC CACTGGCACA AGTCTGGAAG CAGCATTGGC AAAGCCTTTA CAACCAACCT CAAGGAGCG  
 TATGTATCAC CACCCGTCTC CTCTGTGTGT CTAGTTAGTG GTGACCGTGT TCAGACCTTC GTGTRAACG TTTGGAATAT GTTGTGGGA GTTTCCTCGC  
 E  
 3801 Q R L A A L G D T A W D F G S V G G V F T S V G K A V H Q V F G G A  
 CAGAGACTAG CCGCTCTAGG AGACACAGCT TGGGACTTGT GATCAGTTGG AGGGGTGTTT ACCTCAGTTG GGAAGGCTGT CCATCAAGTG TTCGGAGGAG  
 GTCTCTGATC GGCAGATCC TCTGTGTGA ACCCTGAAC CTAGTCAACC TCCCACAAG TGGAGTCAAC CCTTCCGACA GGTAGTTTAC AAGCCTCTCT

E

-----  
F R S L F G G M S W I T Q G L L G A L L L W M G I N A R D R S I A  
3901 CATTCGGCTC ACTGTTCCGA GGCATGTCCT GGATAACGCA AGGATIGCTG GGGGCTCTCC TGITGTGGAT GGGCATCAAT GCTCGTGACA GGTCCATAGC  
GTAAGGCGAG TGACAAGCCT CCGTACAGGA CCTATTGCGT TCCTAACGAC CCCCAGAGAG ACAACACCTA CCCGTAGTTA CGAGCACTGT CCAGGTATCG  
NSI  
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E

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L T F L A V G G V L L F L S V N V H A D T G C A I D I S R Q E L R  
4001 TCTCACGTTT CTCGCAGITG GAGGAGTTCT GCTCTTCCTC TCCGTGAACG TGCACGCTGA CACTGGGTGT GCCATAGACA TCAGCCGGCA AGAGCTGAGA  
AGAGTGCAAA GAGCGTCAAC CTCTCAAGA CGAGAAGGAG AGGCACTTGC ACSTGCGACT GTGACCCACA CGGTATCTGT AGTCGGCCGT TCTCGACTCT  
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### Other Embodiments

All publications, patent applications, and patents mentioned in this specification are incorporated herein by reference in their entirety as if each individual publication, patent application, or patent were specifically and individually indicated to be  
5 incorporated by reference.

Various modifications and variations of the described viruses, vectors, compositions, and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the  
10 invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the fields of medicine, pharmacology, or related fields are intended to be within the scope of the invention. Use of singular forms herein, such as “a” and “the,” does not exclude indication of the corresponding plural form, unless the  
15 context indicates to the contrary. Similarly, use of plural terms does not exclude indication of a corresponding singular form. Other embodiments are within the scope of the following claims.

What is claimed is:

1. A replication-deficient pseudoinfectious flavivirus comprising a yellow fever or West Nile virus genome comprising (i) a deletion in the nucleotide sequence encoding amino acids 26-93 or 31-100 or 31-93 or 26-100 of capsid (C) protein, and one or more deletions within nucleotide sequences encoding a pre-membrane (prM) and/or envelope (E) protein, and (ii) a sequence encoding one or more heterologous pathogen immunogens inserted in the place of or in combination with the one or more deletions of the pre-membrane (prM) and/or envelope (E) protein,

wherein said pathogen is selected from the group consisting of a rabies virus, *Borrelia burgdorferi*, an influenza virus, a human immunodeficiency virus, a simian immunodeficiency virus, a human papilloma virus, a respiratory syncytial virus, a malaria parasite, and *Mycobacterium tuberculosis*.

2. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises a rabies virus G protein immunogen or an immunogenic fragment thereof.

3. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises *Borrelia burgdorferi* OspA immunogen or an immunogenic fragment thereof.

4. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises an influenza virus hemagglutinin (HA) or an immunogenic fragment thereof.

5. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises a codon-optimized HIV gag protein or an immunogenic fragment thereof.

6. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises an HPV16 capsid protein or an immunogenic fragment thereof.

7. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises a respiratory syncytial virus F glycoprotein.

8. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said flavivirus genome comprises sequences encoding a pre-membrane (prM) and/or envelope (E) protein.

9. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein the flavivirus genome is

a chimera comprising pre-membrane (prM) and envelope (E) sequences of a first flavivirus, and capsid (C) and non-structural sequences of a second, different flavivirus.

10. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said yellow fever or West Nile virus genome is packaged in a particle comprising capsid (C), pre-membrane (prM) and/or envelope (E) amino acid sequences from a flavivirus that is the same or different from that of the genome.

11. A pharmaceutical composition comprising the replication-deficient pseudoinfectious flavivirus of claim 1 and a pharmaceutically acceptable carrier or diluent.

12. The pharmaceutical composition of claim 11 for use in inducing an immune response against the heterologous pathogen immunogen in a subject.

13. The pharmaceutical composition of claim 11 for use in inducing an immune response against the heterologous pathogen immunogen in a subject wherein the subject is at risk of but does not have an infection by said pathogen.

14. The pharmaceutical composition of claim 11 for use in inducing an immune response against the heterologous pathogen immunogen in a subject wherein the subject has an infection by said pathogen.

15. The pharmaceutical composition of claim 11, further comprising an adjuvant.

16. A nucleic acid molecule corresponding to the genome of a pseudoinfectious flavivirus of claim 1.

17. A method of making a replication-deficient pseudoinfectious flavivirus of claim 1, the method comprising introducing a nucleic acid molecule of claim 16 into a cell that expresses the protein corresponding to any sequences deleted from the flavivirus genome of the replication-deficient pseudoinfectious flavivirus.

18. The method of claim 17, wherein the protein is expressed in the cell from the genome of a second, different, replication-deficient pseudoinfectious flavivirus.

19. The method of claim 17, wherein the protein is expressed from a replicon.

20. The method of claim 19, wherein the replicon is an alphavirus replicon.

21. The method of claim 20, wherein the alphavirus is a Venezuelan Equine Encephalitis virus.
22. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises an influenza virus M2 epitope or an immunogenic fragment thereof.
23. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises an influenza virus neuraminidase (NA) epitope or an immunogenic fragment thereof.
24. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises a codon-optimized HIV gp120 protein or an immunogenic fragment thereof.
25. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises a codon-optimized HIV tat/nef protein or an immunogenic fragment thereof.
26. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises an HPV18 capsid protein or an immunogenic fragment thereof.
27. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said heterologous pathogen immunogen comprises a respiratory syncytial virus G glycoprotein.