

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
5 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
10 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.
15 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
20 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
25 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 Δ 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.

Summary of the Invention

The invention 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

(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 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.

The invention also includes compositions including a first replication-deficient pseudoinfectious flavivirus, as described above, and a second (or further), different 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 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.

The invention further includes methods of inducing immune responses to an immunogen in a subject, which involves administering to the subject one or more 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 invention 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

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 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.

The invention 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 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).

Further, the invention 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 Langat virus capsid/pre-membrane signal sequence, or a variant thereof.

The invention 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 invention 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 Further, the invention 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.

The invention also provides methods of making replication-deficient pseudoinfectious flaviviruses as described herein, involving introducing one or more
10 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 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
15 (e.g., an alphavirus replicon, such as a Venezuelan Equine Encephalitis virus replicon; see below).

The invention 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-
20 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
25 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
30 sequences and an inserted sequence encoding a tick saliva protein immunogen, or a replication-deficient pseudoinfectious flavivirus including a genome containing

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 invention. Further, the invention 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 invention 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 invention 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 invention 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
20 a vector to deliver a heterologous immunogen, as the deletion can permit insertion of 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 (Δ C-Rabies G, Δ PrM-E-Rabies G, and Δ 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 (Δ C-Rabies G, Δ PrM-E-Rabies G, and Δ 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 Δ 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 Δ 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 Δ 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, ΔC , $\Delta prM-E$, $\Delta C-prM-E$, $\Delta NS1$, $\Delta NS3$, and $\Delta NS5$. Thus, in one example, in the case of s-PIVs and the ΔC 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 Δ C deletion), 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 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 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 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, 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). 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* OspA₁₆₅₋₁₇₃ (YVLEGLTA) 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:

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1  mkkylllgigl ilaliackqn vssldeknsv svdlpgemkv lvskeknkdg
kydliatvdk
61  lelkgtsdkn ngsgvlegvk adkskvklti sddlgqttle vfkedgkltv
25  skkvtskdks
121  steekfnek g evsekiitra dgtrleytgi ksdgsgkake vlkgyvlegt
ltaekttlvv
181  kegvtltskn isksgevsve lndtdssaat kktaawns g stltitvnsk
ktdlvftke
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/ TWN/DS/AG/N/KT; SN/AGTK/NLEGS/N/K/TAVEIT/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 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, 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 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)

MKAFFVL SLL STAALTNAAR AGRLGSDLD T FGRVHGNLYA GIERAGPRGY PGLTASIGGE
VGARLGGRAG VGVSSYGYGY PSWGYPYGGY GGYGGYGGYG GYDQGFSGSAY GGYPGYYGY
YPSGYGGYG GSYGGSYGGY YTYPNVRASA GAAA

Isac (AF270496)

MRTAFTCALL AISFLGSPCS SSEDGLEQDT IVETTTONLY ERHYRNHSG L CGAQYRNSSH
AEAVYNCTLN HLPVNVNATW EGIRHRINKT IPQFVKLICN FTVAMPQEFY LVYMGSDGNS
DFEEDKESTG TDEDSNTGSS AAAKVTEALI IEAEENCTAH ITGWTETPT TLEPTTESQF EAIP

Salp20 (EU008559)

MRTALTCALL AISFLGSPCS SSEGGLEKDS RVETTTONLY ERYRKHPLG CGAQYRNSSH
AEAVYNCTLS LLPLSVNTTW EGIRHRINKT IPEFVNLCN FTVAMPDQFY LVYMGSGNS
YSEDEDGKT GSSAAVQVTE QLIIQAEENC TAHITGWTTE APTTLEPTE TQFEAIS

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 ΔC 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), NNATFNVTNVNPIHIRGS (NBe), MSLLTEVETPIRNEWGCRCNDSSD (M2e), MSLLTEVETPTRNEWECRCSDSSD (M2e), MSLLTEVETLTRNGWGCRCSDSSD (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 (MSLLTEVETLTRNGWGCRCSDSSD).

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⁻, 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), 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), 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), 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 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., Cnidosporidae spp., Clonorchis sinensis, Dicrocoelium dendriticum, Dicrocoelium hospes, Diphylobothrium latum, Dracunculus spp., Echinococcus spp. (e.g., E. granulosus, E. multilocularis), Entamoeba histolytica, Enterobius vermicularis, 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
5 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
10 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.
15 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
20 example, gp100 (Cox et al., Science 264:716-719, 1994), MART-1/Melan A (Kawakami 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
25 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
30 (Guilloux et al., J. Exp. Med. 183:1173-1183, 1996), p15 (Robbins et al., J. Immunol.

154:5944-5950, 1995), β -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 β -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* (18th 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 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.

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 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).

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 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 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 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 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 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 initiation codon of C can be ablated (in constructs where C protein sequence is unnecessary, e.g., Δ 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.

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 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 (Δ 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 log₁₀ 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 log₁₀ 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 log₁₀ FFU/dose) or chimeric LAV and YF 17D LAV controls (4 log₁₀ PFU). Select PIV-WN, -JE and -YF groups were boosted on day 21 with 5 log₁₀ FFU of corresponding constructs (Table 3).

20 Neutralizing antibody responses were determined in animal sera by standard PRNT₅₀ 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 PRNT₅₀ 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 LD₅₀), 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 LD₅₀) (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., PRNT₅₀ 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 PRNT₅₀ 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) log₁₀ FFU doses, respectively. This was somewhat lower compared to YF/WN LAV 4 log₁₀ 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 1st and 2nd doses. For instance, for PIV-WN after 1st 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 2nd dose, titers were similar). A more pronounced effect was observed for other constructs after both the 1st and 2nd doses. Interestingly, PIV-YF/WN was very highly immunogenic by IP route (titer 1:320 after 1st IP dose vs. 1:20 by SC, and 1:1,280 after 2nd 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 poses). 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 PRNT₅₀ in animals immunized with 5 log₁₀ 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 log₁₀ 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.

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 PRNT₅₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ PFU/ml, which were 0.5 log₁₀ 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 log₁₀ 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 α -Helix I; WO 2006/116182). As expected, the p59 virus grew to lower titers (5.6 and 6.5 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ PFU) showed dose-dependent mortality, while all animals inoculated IP (5 log₁₀
25 PFU) survived, in accord with the knowledge that YF 17D virus is not neuroinvasive. All animals that received graded IC doses (2 – 4 log₁₀ 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 log₁₀ 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 log₁₀ 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 (LD₅₀ ~ 0.1 PFU) and IP (LD₅₀ ≤ 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 PRNT₅₀ 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 (≥ 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 ΔC , $\Delta prM-E$ 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 ΔC deletion (ΔC 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 ΔC construct, or signal for NS1 and NS1-5 genes in $\Delta prM-E$ and $\Delta C-prM-E$ constructs.

Packaged WN(ΔC)-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 log₁₀ 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(Δ 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(Δ 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 Δ C deletion (~ 200 nt), significantly increasing the overall size of the recombinant RNA genome. In contrast, in WN(Δ prME)-rabiesG, and WN(Δ 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 $8 \log_{10}$ 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(Δ 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(Δ prME)-rabiesG and WN(Δ CprME)-rabiesG PIVs will induce humoral immune response only against rabies because they do not encode the WN prM-E genes. WN(Δ C)-rabiesG s-PIV construct can be also co-inoculated with WN(Δ 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(Δ prME)-rabiesG, and a d-PIV sample, WN(Δ prME)-rabiesG + WN(Δ 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(Δ 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- Δ NS1 genome (sequence provided in Sequence Appendix 4) and obtained evidence of co-infection with WN(Δ prME)-rabiesG or WN(Δ 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- Δ 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, Δ NS1 deletions can be replaced with or used in combination with Δ NS3 and/or Δ 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, 5th 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(Δ C)-RSV F, WN(Δ prME)- RSV F, and WN(Δ 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(Δ prME)- RSV F

construct in Fig. 19. The presence of packaged PIVs in the supernatants from transfected cells (titer as high as 7 log₁₀ 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
5 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 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 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)

10

Table 2. Safety: Suckling mouse neurovirulence¹

Construct	Doses (log ₁₀)	Mortality (%)	AST (days) ²
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

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

²AST for mice that died; na, not applicable.

5

10 Table 3. PIV highly immunogenic and efficacious in mice¹

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

¹IP immunization (d0 prime, and d21 boost in select groups); challenge on d35: wt WN NY99, 3 log₁₀ PFU IP, 270 LD50; wt YF Asibi, 3 log₁₀ PFU IC, 500 LD50; N/D, not determined.

15

Table 4. PIV are immunogenic in hamsters and protect against challenge¹

Group	Dose(s)	PRNT Day 38	POST-CHALLENGE		
			Mortality	Morbidity	Peak viremia (log)
PIV-WN	10 ⁵	320	0/5 (0%)	0/5 (0%)	2.3
	10 ⁶	640	0/5 (0%)	0/5 (0%)	1.8
	10 ⁶ +10 ⁵	1280	0/5 (0%)	0/5 (0%)	<1.3
YF/WN control	10 ⁴	≥2560	0/5 (0%)	0/5 (0%)	<1.3
PIV-WN/JE	10 ⁴	20	2/5 (40%)	2/5 (40%)	2.2
	10 ⁵ +10 ⁵	40	0/5 (0%)	0/5 (0%)	<1.3
YF/JE control	10 ⁴	2560	0/5 (0%)	0/5 (0%)	1.3
PIV-YF	10 ⁴	<10	1/3 (33%)	3/3 (100%)	8.3
	10 ⁵	<10	1/5 (20%)	4/5 (80%)	8.3
	10 ⁵ +10 ⁵	20	0/4 (0%)	0/4 (0%)	2.5
YF17D control	10 ⁴	≥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

¹Syrian hamsters, SC inoculation (d0, and d21 in select groups); challenge (d39): wt WN NY385/99 6 log₁₀

- 5 PFU IP, wt JE Nakayama 5.8 log₁₀ PFU IC, or hamster-adapted YF Asibi 7 log₁₀ 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 (log ₁₀)	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)¹

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

¹ 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) (log ₁₀)	Mortality (%)	AST, days ¹	Dose(s) (log ₁₀)	Mortality (%)	AST, days ¹
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

¹AST for mice that died.

5

Table 8. Neutralizing antibody titers (PRNT₅₀) in mice immunized IP (determined against wt TBE virus Hypr), and protection from challenge (postchallenge observation, day 9)

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

¹Numbers in parenthesis correspond to number of mice in each pooled serum sample tested.

²Mortalities 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, WN	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	Mandl, 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
K336D	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

C protein YF17D

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 GGAACGCCG TTCCATGAT GTTCTGACTG TGCATTCCT AATTTGGC ARGCTGGCA TGACATCGC AGCTACGGTT CGC
T CCTTGGCGC AAGGTACTA CAAGACTGAC ACGTTAGGA TTAAACCGG TAGGACCGT ACTGTTAGG TCGATGCCAA GCG

TBEV partial signal

Hypr or LGT E5 prM protein

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

C protein YF17D

YF 17D partial signal

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 GGAACGCCG TTCCATGAT GTTCTGACTG TGCATTCCT AATTTGGC ARGCTGGC GTGTCGGAGC AGCTACCGTG CGA
T CCTTGGCGC AAGGTACTA CAAGACTGAC ACGTTAGGA TTAAACCGG TAGGACCGA CACAGCCTCG TCGATGGCAC GCT

WNV partial signal

Hypr prM protein

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

TBEV signal

WNV C protein

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
CAAAAGAAA CGGGGGGAA CAGACTGGAT GAGCTGGCTG CTCGTAATCG GCATGCTGGG CATGACAATC GCAGCTAGCG TTCGC
GTTTCTTT GCCCCCCCTT GTCTGACCTA CTCGACCGAC GAGCATTAGC CGTACGACCC GTACTGTTAG CGTCGATGCC AAGCG

Hypr prM protein

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

WNV signal

WNV C protein

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 CGGGGGGAA AGACAGGCAT AGCTGTGANG ATAGGATGC TGGCTTGTGT CGGAGCAGCT ACCGTGCGA
GTTTCTTT GCCCCCCCTT TCTGTCCGTA TCGACACTAC TATCCGTAGC ACCGAACACA GCCTCGTCCA TGGCACGCT

Hypr prM protein

Sequence Appendix 2

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

5' UTR

1 AGTAAATCCT GTGTGCTAAT TGAGTGCAT TGGTCTGCA AATCAGTTGC TAGGCAATAA ACACATTTGG ATTAAATTTA ATCGTTCTGT GAGCGATTAG
TCAITTTAGGA CACAGATTA ACTCCACGTA ACCAGAGGTT TAGCTCAAG ATCCGTTATT TGTGTAAACC TAATTAATAAT TAGCAAGCAA CTCGCTAATC

5' UTR

101 CAGAGAACTG ACCAGAACAT GTCTGGTCTT AAGCTCAGG GAAACACCTT GGGCGTCAAT ATGTACGAC GAGGAGTTGC CTCCTTGTCA ACAAATAA
GTCTCTTGAC TGGTCTTGTA CAGACCAGCA TTTCGAGTCC CTTTGTGGA CCGCGAGTTA TACCATGCTG CTCCTCAAGC GAGGAACAGT TTGTTTAT

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 AAAACAATT GGAACAGAC CTGACCTTC AAGAGGTGT CAAGGATTTA TCITTTTCTT TTGTGTCAC ATTTGACTG GAAAAAGAT
TTGTTTTTG TTTTGTTAA CCTTGTCTG GACCTGGAAG TTTCTCAAA GTTCTTAAT AGAANAAGA AACAAGTTG TAAACTGNC 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 CTAAGAGGT TGTGAAAT GTTGACCCA AGACAAGCT TGGCTGTCT AAGGAAGTC AAGAGAGTG TGGCCAGTTT GATGAGGGA
GTGTGGGTG GATTCTCCA ACACCTTTTA CGACTGGGT TGTGTCGA ACCGACAAGA TTCTCTCAG TTCTCTCACC ACCGTCAAA CTACTCTCT

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 TTCCTAAT GTTCTGCTG TGCATCTCT AATTGTGGC ATGTGGCA TGACATCGC AGCTACGTT CGCAAGGAA
AACAGAGTT CCTTGGGC AAGGTACTA CAAGACTGAC AGTTAAGCA TTAACCCG TAGACCCGT ACTGTAGCG TCGATGCCAA GCGTCTCTT

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
GAGAGGGCAG TAGGTGATA CGCGCGGAG GTAAGTNGC CGCTACCAA GTGAGAGTG AATATGTAC CTGGTCAAT CTGGCCACCG ACATGGGCTC
CTCTGCCGTC ATGCCAGTAT GCGCGCCTTC CATTCCTAG CGCATGGGT CACTCTCACC TTITACCATG GACGAGTAA GACCGGTGC 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 GATAGCCTT CTTAAGAGTG CGTAACCAT GATCAAGTG AGGAACCTGT TGCTGTGCT GAACTGTGA TGGGTGTAT
AACCACACTA CTATCGGAA GAATACTCAC GCATTGGTAT CTATTGCCA CTCTTGACA ACTGCACTA ACGAAGACCG CTTTGCACCT ACCCCACATA

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
CTCGAATAG GACGGTGTG TAAACAAGAA GGAACAGAA CCAAGAGCTC AGTGTCTATA CCTCCACG CTCAGGAGA GCTGACCGA CGGGACATA
GAGCTATAC CTGCCACACC ATTTGTCTT CCTTGTCTT GGTCTGCGAG TCACAATAT GGAAGGTGC GAGTCTCTCT CGACTGSCCT GCCCTGTAT

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
AATGGTTGA GGGGACTCA CTCGGAACAC ATTGACCGG CGTCAGGCG TGCGTCTGA AAAATCGGT GTTGGCCTC GCTATGGTGA CAGTCGTTG
TTACCAACCT CCGCTGAGT GAGGCTTGT TAACTGGC GCAGTCCC ACCAGACCT TTTAGCCGA CACCGGAG GATACCACT GTACGMAAC
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 GAGTCTGTG TACTCGCTG GCGAGTCTG TGCTGCTCC TGTGCTG CCGTCTAC GGTCCAGGT GTACTCATTT GGAARACAGA
CGAGTGGAC CTCAGACACC AATGACGCA CCGTACGAC CACACAGAG AGACAGACG GGGACAGATG CGCAGGTCCA CATGAGTAAA CCTTTGTCT
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 CCGCACCCA GGGGAGACT CGGTAAACC TGGTCTGA ACTGGTGGT TGCGTTACTA TTACCGCTGA GGGCAAAACC TCTATGGATG
CTAAACAGT GGCCTGGGT CCGTCTGA GCCATTGG ACCAGAACT TGACCCACCA ACAGCAATGAT NATGGGACT CCGTGTGGG 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
TGTGGTGA TGCAATCTAT CAGGAGATC CCGCACAAAC CAGGAAATAT TGCTTCACG CAAAGCTGTC CGATACAAAG GTCGGGGCTA GGTGCCAAC
ACACCGACT ACCTTAGATA GTCTCTTAG GCGTGTGTG GTCCCTTATA ACAGAGTGC GTTTCGACAG GCTATGTTT 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
AATGGACCG GCCACCTGG CCGAGGAACA TCAGGAGGT ACAGTGTGA AAGCGGACCA GAGTATAGA GCGTGGGTA ATCACTGGG CCGTGTGGC
TTACCTGGC CCGTGGGACC GCCTCTTGT AGTCCCTCCA TGTCACAGT TTGCTCTGT CTCATATCT CCGACCCCT TGTGACGCC GGCACAGCCG
Hypr E protein

1301 K G S I V A C V K A C E A K K A T G H V Y D A N K I V Y T V K V
AAAGGAATG TTGCGCTG CCGTAGGA CCGTGGAG CCAAAAGAA GGTACTGGG CAGCTTATG ACGCCAACAA GATCGTTTAT ACAGTGAAG
TTTCTTTCAT AACAGCGAAC GCAGTCCGT CCGACACTCC GGTITTTCT CCGATGACCC GTGCAGATAC TCGGTTGTT CTACCAATA TGTCACTTTC
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
TGGACACA CACAGGGGAT TACGTGGCG CCAACAGAC TCATTCGGT CCGAAACGG CCGCTTAC CCGTGTATCC GAAAAGACA TCCTCACTAT
ACCTTGTGT GTGTCCCTTA ATGCACCGC GGTGCTCTG AGTAGGCCA GCGTTTGGC GGTGAGTGC GCACAGTAGG CTTTCTGCT 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 CTCTCTCTG CCGGTGGCT AGCGAGTGC ACCTGGCCA GACATCATC CTGGAAGTGG ATAAACAGT TGACATCTG
CCCCCTCAT CCGCTGCAA GAGACGAGAC GGGCCACCGA TCGCTCAGC TGGACCGGT CTGTCACTAG GACCTTGACC TATTTGTCA ACTGTAGAC
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 A E R L V E
CCTACCGCTT GCGAGTGA CAGGATGG TTAAACACC TTGCGCTGCC ATGGAACAT ATGAGAGCA GAAATGGA TAAGCAGAG CCACTCGTAG
GGATGGCGAA CCGTCCACT GTCCCTAAC AAATGTCTG AACGGACCG TACCTTGTGA CTTCTCTGCT CTGTGACCT ATTACGCTC GCTGACATC
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
AATCGGTGC CCTCATGCC GTNAGATGG AGCTTACAA TCTGGTGTAT CAGACGGCG TTCTCTTAA AGCTCTGCT GGGTACCAAG TTGCCACAT
TTAGCCACG GGGAGTACG CACTTCTACC TCGATGTT AGACCACTA GTCTGCGCC AGAGGAAT TCGAGAGCA CCGCATGGTC AACGGTGT
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 CCATGTRACT TCCGAGTGG GCTTGAGAA GTTGAATG AAAGTCTTA CGTACAAAT GTGTGACRAG
GCTTCCTTGC TTCAAGTGG ACTTCAGTCC GGTACATGA ACGTCCACC CGGACCTCTT CAACTTTTAC TTCCAGAT 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 CATGAGAGG GGCCTCCACA GATAGCGCC AGATACTGT GGTATGGAG GTGACCTTTT CTGACACAAA ACCCTGCHGA ATACCGTGC
TGGTTCAAGT GTACCTTCTC CCGGGGTGT CTATCGCGG TGTATGACA CCACTACCTC CACTGGAANA GACCTTGTTT TGGACGCTT TATGGSCACG
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
GGCTGTAGC TCACGATCT CCGGATCTA ATGTGCTAT GCTATGCTA CCAACCTTA CCACTGAGAA TAACGTGGT GGTTTATG AGATGACGCT
CCGACATCG AGTGCCTAGA GGGCTACAGT TACAACGATA CGACTAATGT GGTATGGAT GGTAGCTCTT ATTGCCACCA CCAAAATPAC 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
TCCGCCAGG GATAACATCA TCTAGTGG CGAATCTCT TACCAAGGT TTCAGAGG GAGTCAAT GGGCGGTCT TCCAAAAC GAGAGAGGA
AGCGGTCCG CTATGTAGT AGATGACCC GCTTGAGAG ATGTACCA AAGTCTTCC CTCAGTTAA CCGGCCAGA AGGTTTITG CTCTTCCCT
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
ATCGAAGAT TGACGGTAT CCGGAGCAC GCATGGAT TTGTTCCG AGGGGATC CTGCTTCTA TTGTTAAGC ACTGCATAC GTGTGGGG
TAGCTTGCTA ACTGCCAATA CCGCTCGTG CGTACCCTA AACCAAGCG TCCCTTAAG GACAGAGAT AACCATTCG TGACGTATG CACGACCCC
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
GGCATTCAA TTCTATTTT CCGGCGTGG GGTCTCTGCC TAACTCTG CTGGAGTAG CCGTGGCTG GTTGGACTG AATATGCGA ATCCGACGAT
CGGTAGIT AGATAAAG CCGCGCAC CCGAGAGCG ATTGAGGAC GACCTCATC GGGACCGAC CAACCTGAC TTATACGCT TAGGCTGCTA
Hypr E protein

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
GTCCATGCA TTCCTCTTG CCGCGTGTCT TGTACTGCC ATGACACTGG GGTGGCGC CGATCAGGA TGGCCATCA ACTTGGCAA GAGAGAGTC
CAGGTACAGT AAGGAGAAC GCGCGCACG ACATGACCG TACTGTGACC CGCAACCGG GCTAGTCTCT ACGCGTAGT TGAACCGTT CTCTCTGAG

NS1 gene of YF17D

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

5' UTR

1 ACTAATCTT GTGTGCTAAT TGAGTGCAAT TGGTCTGCAA ATCAGTTGC TAGGCAATAA ACATTTGG ATTAATTTTA ATGTTCTGTT GAGGATTAG
TCATTTAGGA CACACGATTA ACTCCAGTA ACCAGAGTGT TAGCTCAACG ATCCGTTAT TGTGTAAC TAATTAAT TAGCAAGCAA CTGCTAATC
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
CAGAGACTG ACCAGAACAT GTCTGGTGT AAAGTCAGG GAAACCTCT GGGCTCAAT ATGTAGAC GAGGAGTTCG CTCCTTGTCA ACAAATAA
GTCTCTTGAC TGGTCTGTGA CAGACCAGCA TTCTGAGTCC CTTTTGGGA CCGCAGTTA TACCATGCTG CTCCTCAAGC GAGGACAGT TTGTTTAT
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 F N I L T G K K I

201 AACAAACAAAC ABAACAAATT GGAACACAGC CTGGACCTTC AAGAGGTGTT CAAGGATTTA TCTTTTCTT TTTGTTCAAC ATTTTGACTG GAARAAGAT
TTGTTTTTG TTTTGTTTAA CTTTGTCTG GACCTGGAAG TTCTCCACAA GTTCTCTAAT AGAARAAGAA ABAACAGTTG TAAACTGAC CTTTTTCTA
C protein YF17D

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
301 CACACCCAC CTAAGAGGT TGTGGAAT GTTGACCCCA AGACAGGTG TGTGTTCTT AAGCAAGTC AAGAGGTG TGTGAGTTG TGTGAGTTG TGTGAGTTG
GTGTCGGTG GATTTCTCCA ACACCTTTTA CGACCTGGT TGTGTTCCA ACGACAGA TTCTTTTCTG TGTGTTTCTG TGTGTTTCTG TGTGTTTCTG
C protein YF17D

YF 17D partial signal

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
401 TTGCTCTCA GGAACGCG TTCCATGAT GTTCTGCTG TGAATCTCT AATTTGGG ATGCTGGCT GTGTCGGAG AGTACCGTG CCAAAAGAAC
AACAGAGTT CTTTGGCG AAGGTACTA CAAGACTGAC AGTTTAGA TTAACCCG TACGACCGA CACAGCCTCG TCGATGGCAG CTTTCTCTG
Hypr prM protein

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
501 GCGACGGAAG CACGTGATA AGGCTGAG GTAAGATGC GGTAAGCAG GTGAGTAG AGATGGAC TTGCTTAATA CTGCGACTG ATATGGGATC
CGTGCCTTC GTGCACTAT TCCGACTCC CATTCTACG CCGATGCTC CACTCTAT TCTTACCTG AACGATTTAT GAGCGCTGAC TATACCTTAG
Hypr prM protein

W C 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
601 CTGCTGTGAC GATAGCTTCA GTTATGATG CATAACATA GACGAGGCG AAGAACCTGT GAGCTGTGAC TGTCTGTGA GAAATGTGA TGGCTTTAT
GACCACTG CTATCGGAGT CAATCTTAC GCATTGTTAT CTGTCCCG TCTTGGACA CTTGCACTG ACAAGACAT CTTTACACT ACCGCAATA
Hypr prM protein

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
701 CTGAGTAG GCGCTGGS AAACAGGAG GGTCTAGAA CTGAGATC TGTGCTGAT CCAAGTACG CCAAGGAGA GTTGACCGGT AGAGCCACA
GACCTCAGC CGCGACACC TTTTGTCTC CCGAGTCTT GAGCTCTAG ACACACTAA GGTTCAGTC GGTCTCTCT CAACTGGCCA TCTCGGTGT
Hypr prM protein

W L E G D S L R T H L T R V E G W V W K N R L A L A M V T V V W
801 AGTGTCTGA AGGGACTCA TTGAGGACC ACTGACTAG GTGAGGAGT TGGGTTTGA AGATGCTG GCTCGGCTC GTATGTGCA CCGTGTGTG
TCACCGAAT TCCCTGAGT AACTCTGG TGAAGTATC CCACTCCA ACCCAACCT TCTTAGCCA CAGGCGCAG CATAACCAT GCGACACAC
Hypr prM protein

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
901 GCTGACTG GAGGTGTG TACTCGGT TGTGTTCTG GTTCTCTCC TGTGTTGCG CCAAGTAC CCAAGTAC GTACTCATTT GGAACACAGA
GACTGTGAC CTCTACAGC ACTGAGCCA ACACACAC ACACAGGAG AGACAAACG GGTTCACATG CCGAGTCCA CATGAGTAA CTTTGTCT
Hypr E protein

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
1001 GATTTGTCA CCGGACCA CCGGACGACT CCGGTAACC TGGTCTTGA ACTGCTGTG TGGTCTTGA TTACCTGTA GGCACACCC TCTATGGATG
CTAAACAGT GCGGTGGGT CCCCTGCTGA GGCCTGTTG ACCACGAACT TGACCCACA ACACAGAT AATGGGACT CCCGTTGGG AGATACCTAC
Hypr E protein

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
1101 TGTGCTGGA TGAATCTAT CAGGAGATC CCGACAAAC CAGGATAT TGCCTTACG CAAAGTGTG CAAAGTGTG GTGCGGCTA GGTGCCCCA
ACACGACT ACCTAGATA GTCTCTTAG GCGGTGTTG GTCTCTTATA ACGGAGTGC GTTTCAGAG CTAATGTTT CAGCGCGAT CCACGGGTTG
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 GCCACCTGCG CGGAGGAACA TCAGGAGGTG ACAGTGTGCA AACGGGACCA GAGTGATAGA GCGTGGGTA ATCACTGCGG CCGTGTGCGC
TTACCTTGGC CGGTGGGACC GCGTCTTGT AGTCCCTCCA TGTACACGT TTGCCCTGGT CTCACTAICT CGGACCCCAT TAGTGACGCC GGACNAGCCG
Hypr E protein

1301 K G S I V A C V K A A C E A K K A T G H V Y D A N K I V Y T V K V
AAAGGAAGTA TTGTGCTTG CGTCAGGCA GCGTGTGGG CCAAAAGAA GGTACTGCG CACGTCTATG AGCCCAACA GATCGTTTAT AGAGTGAAG
TTTCCCTTAT AACAGCGAAC GCAGTTCCGT CGGACACTCC GGTATTTCTT CCGATGACCC GTGCAGATAC TGGCGTTGTT CTAGCAATA TGTCACTTTC
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
TGAACACA CACAGGGAT TACGTGGCG CCAACAGAC TCAITCCGT CGCAACGG CCAGCTTAC CCGTCTATCC GAAAGACCA TCCTCACTAT
ACCTTGGTGT GTGTCCCTTA ATGCACGCC GGTGTCTCTG AGTAAGCCA GCGTTTGGC GGTGAGTG GCACAGTAGG CTTTCTGCT AGAGTGATA
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 GCGGACGTTT CTCTGCTCTG CCGGTGGT AGCGAGTGC ACCTGGCCA GACATCATC CTGGAACCTG ATAAACAGT TGAGCATCTG
CCCCCTCATA CCGCTGCATA GAGACGAGAC GGCCACCGA TCGCTCAGC TGGACCGGT CTSTCAGTAG GACCTTGACC TATTTTGICA 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 GCGAGTGCA CAGGATGCG TTAAACGACC TTGCTGCGT ATGGAACAT GAAGAGCGA GAACTGGAA TAATGACGAG CGACTCGTAG
GGATGGCAA CCGTCCAGT GTCCCTAACC AATGTGCTG AACGGACCG TACCTTGTA CTTCCTGCT 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
AATCGGTGC CCCTCATGCC GTGAAGATGG ACCTTACAA TCTGGGTGAT CAGACGGCG TTCTCTTAA AGCTCTGCT GCGTACCGAG TTGCCACAT
TTAAGCCAG GGGATACCG CACTTAC TGCAGTGT AGACCACTA GTCTGCCCG AAGAGGAAT TCGAGACGA CCGCATGCTC AACGGGTGA
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 TGAAGTCAG CCACTGTAAT TCGAGGTGG GCGTGGAGA GTTGAAGATG AAAGTCTTA CGTACACAT GTGTGACAG
GCTTCTTGC TTCAATGTTG ACTTCAGTCC GGTACATGCA AGCTTCCAC CCGACCTCTT CACTTTTAC TTCCAGAT GCATGTGTTA CACACTGTT
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
ACCACTTCA CATEAGACG GCGCCCCACA GATAGCGCC AGTACTGT GGTATGGG GTGATGGG GTGACCTTTT CTGGAACAA ACCCTGCAGA ATACCGTGC
TGGTTCAAGT GTACCTTCTC CCGGGGTGT CTATCGCGGT TGCTATGACA CCACTACCTC CACTGAAA GACCTTGT TTGGACGCTT TATGGGACG
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
GGCTGTAGC TCAGGATCT CCCGATGCA ATGTGCTAT CTTGATTA CCAACCTTA CCACTGAGA TAACGTGGT GGTTTATG AGATGACGT
CCCGACATG AGTGCCTAGA GGGTACAGT TACACGATA CCACTAAT GTATGGAT GGTAGTCTT ATTGCCACA CCAAAATAAC TCTACGTGA
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
TCCGCCAGC GATAACATCA TCTAGTGGG CGNACTCTT TACCGTGT TCGAAGAG GAGTCAAT GGGCGGTCT TCCAAACAA GAAGAAGGA
AGGCGTCCG CTATTGTAGT AGATGCACC GCTTGAGA ATGTACCA AGTCTTCC CTCAAGTTAA CCGGCCAGA AGGTTTTTG CTCTTCCCT
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
ATCGAACAT TGACGGTAT CCGGAGCAC GCAATGGAT TTGTTCCG AGGGGATTC CTGTCTTA TTGTAAGG ACTGCATACC GTGCTGGGG
TAGCTTGCTA ACTGCCAATA CCGCTGCTG CGTACCTTA AACCAAGGG TCCCCCTAAG GACAGAGAT AACCATCCG TGAGGTATGG CACGACCCC
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
 GCGCAATCAA TTCTATTTTC GGGGCGCTGG GGTTCCTGCC TAACTCTCTG CTGGAGTAG CCTGGCCTG GTTGGACTG ATATGCGGA ATCCGACGAT
 CCGTAAGTT AAGATAAAG CCCGCGACC CCAAGGACG ATTGAGGAC GACCTCTATC GCGACCGGAC 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
 GTCCATGTA TTCTCTTGG CCGGCTGCT TGTACTGACC ATGACACTGG GGGTGGCGC CGATCAAGGA TCGGCCATCA ACTTTGGCA GAGAGAGCTC
 CAGGTACAGT AAGGAGAACC GCGCCACGA ACATGACCG TACTGTGACC CGCAACCGG GCTAGTTCTT ACGCGTAGT TGAACCGTT CTCTCTCGAG

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

5' UTR

1 AGTAAATCCT GTGTGCTAAT TGAGTGCAT TGGTCTGCA ATCGAGTTGC TAGGCAATAA ACACATTTGG ATTAATTAA ATCGTTCTGT GAGCGATTAG
 TCATTAGGA CACACGATTA ACTCCAGTA ACCGAGGTT TAGCTCAAG ATCCGTTATT TGTGTAAACC TAATTAAAT TAGCAACGA 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
 CAGAGACTG ACCAGACAT GTCTGGTCTT AAAGCTCAG GAAACACCT GGGCTGCAAT ATGCTAGAC GAGGATTCG CTCCTTGTC AACAAATAA
 GTCTCTTGAC TGTCTTGTA CAGACCAGCA TTTCGAGTCC CTTTTGGGA CCGCAGTTA TACCATGCTG CTCCTCAAGC GAGGAACAGT TTGTTTATT
 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 L F N I L T G K K I
 AACAAAAAC AAACAATT GGAACAGAC CTGGACTTC AGAGGTCTT CAAGGATTTA TCTTTTCTT TTGTGTCAC ATTTGACTG GAAAAAGAT
 TTGTTTTTG TTTGTTTAA CCTTGTCTG GACCTGGAG TTCTCCCAA TTCTTAAT AGAATAAGAA AACAAGTTG 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 TGTGAANT GCTGACCCA AGACAAGCT TGGCTTTCT AGGAAGTC AAGAGTGS TGGCCAGTTT GATGAGAGA
 GTTCGGTG GATTTCTCA ACACCTTTA CGACCTGGT TCTGTCGA ACCGACGA TTCTTTTCAG TTCTCTACC ACCGTCAA CTACTCTCT
 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 F L I L G M L G M T I A A T V R R E R
 TTGCTCTCA GGAACGCG TTCCATGAT GTTCTAGT TGAATTCCT AATTGGC ATCTGGGA TGACATGCG AGTACTGTG CGAAGGAGA
 AACAGAGTT CCTTTGGGC AAGGTACTA CAAGCTGAC AGTTAAGA TAAACCG TAGACCCCT ACTGCTAGC TCGATGAC GCTTCCCTCT
 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
 GAGCGGCTC TATGGTGATC AGAGCGAAG GTAGGACGC TGGACCCAG GTGAGGTG AAATGGCAC CTGTCTTAT CTGGCGACCG ACATGGGCTC
 CTCTGCCGAG ATACCACTAG TCTGGCTTC CATCCCTGG ACCTGGGTC CACTCCAGC TTTCACCGT GACACAATA GACCGTGGC TGTACCCGAG
 prM protein Langat E5

601 . W C D D S L A Y E C V T I D Q G E E P V D V D C F C R G V E K V T
 CTGGTGTGAT GATTCCTGG CTTATGAATG TGTACTATT GATCAGGTG AAGAGCTGT GAGCGTGC TGTTCTGTA GAGCGTGA GAAAGTGACC
 GACCACTA CTAGAGACC GAATACITAC AATGATAA CTAGTCCAC TTCTCGCA CTGACCTG ACAAAGACAT CTCGCGAGCT CTTTCACG

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
CTGGATATG GACGATGCG CCGGAGAGAA GGTCTCAGGA GTCCGAGATC CGTGTGATC CTTTCACATG CGCAGCGGA TCTGACAGG AGGGGTACCC
GACCTTATAC CTGCTACACC GCGCGCTCTT CCGAGGTCTT CAGCTCTAG GCACACTAG GGAAGTGTAC GCGTCCGCT 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
AGTGGCTGA AGCGAGACA GTCCAGGCC ATCTGACTCG CGTGTAGCG TGGGTGGA AAAACAACT CTTTACCTT AGCTGTGGA TGGTCCGCTG
TCACCGAGCT TCCGCTTCTG CAGTTCGGG TAGACTGAGC GCACTTCGG ACCCACCT TTTTGTGGA GAATGGGAA 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
GCTGATGTA GAGGACTCC TTCCCGCAT TCTCATGTT GTGTGGCTC TCGGCTGCG CCCTGCATAC CGTCCAGGT GTACGCACT CGAAATCGA
CGACTACCAT CTGCTGAGG AAGGGCGTA AGAGTAACA CACCACCGAG AGCGGAGCG GGGACGTATG CGCAGTCCA 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
GATTTCGTA CAGGCTCCA AGTACTACC CGGCTACCC TGTGTGGA GCTGGAGGC TGTGTACTG TTACAGCCGA CGGAAACCT AGTCTGGATG
CTAAGCAGT GTCCGCGAGT TCCATGAG GCGGAGTGG AGCAGGACT CGACCTCG ACACAGTGAC AATGTGGCT 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
TGTGGCTGA CTCATCTAT CAGGAGACC CGGCACAGC CAGGAGTAC TGCCTCCAG CTAAGCTGAC TGGACACAAAG GTAGCCCAA GATGTCCAC
ACACGACCT GAGTAGATA GTCTCTCG GCGGTCTG GTCCCTATG ACGAGTGC GATTCAGTG ACCCTGTTT CACGCGGCT CTACAGGCTG

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
AATGGGCTT GCCACCTTG CCGAGGAACA CCAATCCGT ACGTATGCA AGCAGATCA GTCTGATCG GGTGGGGA ATCATTCGG CCTCTCGGT
TTACCCCGA CCGTGGNAG GGTCTCTGT GTTAGGCCA TGCATACGT TCGTCTAGT CAGACTAGCG CCTACCCCT TAGTAAGCC 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
AAAGCAGCA TTGTCACTG CGTGAAGTG ACATGCGAG ACAGAGAGAA GGCCACAGT CAGTATATG ATGTGAACAA AATCAGATAT ACCATTAAAG
TTTCGTCTG ACAGTGAAC GCACTTCCAC TGTACGCTC TGTCTCTT CCGGTGTTCA GTACATATAC TACACTTGT TTAGTGATA 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 F T V S S E K T I L T L
TAGAACACA TACAGGGAA TTGTTGGCAG CAACAGAGC TCATAGCGA CGAAGTCCG CTTCTTCC CGTCTCTCC GAGAAACA TCTTGACCT
ATCTTGCTG ATGTCCCTT AAGCACCTG GTTGTCTG AGTATCGCT GTTTCAGGC GGAGGAGTG GCAGAGGAGG CTCTTTTGT AGGACTGGA

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 GCGACGTAT CTTGTCTG CAGGTGACC AGCGGTG ACCTGTCTCA GACGTGCTG TTGGCCCTG ACAAGACA TGAGACTTG
GCCTCTGAT CCGCTGCATA GAAACGACAC GTCCACCGG TCCCGCAC TGAACAGT CTGTACAC AACCGGACC TGTCTGTGT ACTGCTGAAC

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
CCACAGCTT GCGAGTGA CAGGACTGG TTACAGACC TGGCGTCCC GTGAACAT GACGGCTG AGCNVGA TCGAGGAGG AGACTGTGG
GTTTGTGGA CCGTCCAGT GTCCCTGACC AAATGTCTG ACCGAGGG CACTTTGTA CTGCCGAG TCTGTACCTT ACTCCCTCC TCTGACCACT

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
 AATTGGAC CCACACGCC GTAAAGATGG ACGTTTCAA TCTTGTGAC CAGACGGGG TGCTCTGAA ATCACTGGG GCGTGCCTG TAGCCAGCAT
 TTAACCTTG GGGTGTGGG CATTCTIACC TGCAAAGTT AGRACCACTG GTCTGTCCC ACGAGGACTT TAGTGACCGC CCGACCGAC ATCGGTGTA
 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
 CGAGGCACA AAGTATCACG TGAAGTCTGG GCATGTAACC TGGAGATGG GCCTGMAAA GCTGAAGATG AAGGACTTA CGTACACTGT TTGTGATAAG
 GCTCCCGTGT TTCATAGTGG ACTTCAGACC CGTACATGG ACCTTCACG CCGACCTTTT CGACTTCTAC TTTCCTGAAT GCATGTGACA AACACTATTTC
 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
 ACCRAGTTA CATGGAAGC AGCCCAACG GATTCCGCC ATCATAGCGT CGTATAGAG GTTGTTTCT CCGCACCGG ACCATGTAGA ATACCACTGA
 TGGTTCAAT GPACCTTCG TCGGGGTTC CTAAGCCCG TACTATGCA GCATACCTC CAACCAAGA GGCCTGTGTC TGCTACATCT TAAGTCACT
 E protein Langat E5

2001 . A V A H 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
 GAGTGTGCG CCACGGTGA CCGAGGTAA ACGTGGCCAT GCTGATTACA CCGAATCCA CTATGAGAA CAATGGCGGA GGGTTCATCG AATGTCAGCT
 CTCGACAGC GGTGCCACAT GGGCTCCATT TGCACCGTA GCACTAATGT GGCTTAGGT GATACCTTT GTTACCGCTT CCAAGTAGC TTACGTGCA
 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
 GCGCCTGGA GACAACATCA TTATATGCG GACCTCGAT CATCAATGGT TCCAGAAAGG GTCTTCATC GCGCGCTCC TTCAGAGAC ACGAAAGGC
 CCGCGGACCT CTGTTGTAGT AAATACAGCC GCTGAGGCTA GTAGTTACCA AGGTCTTTC CAGAGGTAG CCGCGCAGG AAGTCTCTG TGCTTTTCG
 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
 AITGAAGAC TTACATCTT GGGGAACAT GCTGGGACT TGGGTGAGT TGGGGGGA ATGCAAGCA TAGCAGAGC TAGTCACACC GTTCTCGGTG
 TAACCTTCTG AATGTCAGGA CCGCTTGTA CGGACCTCA AGCCGCTCA ACCGCCCAT TACTGTCTG ATCGTCTCG ATACGNTGG 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
 GGCATTAA TACTCTGTTG GTTGGCGTGG GTTCTTTC GAAATCTG CTGCGTCTG CAATGGCTG GCTTGGACTG AATATGCGCA ATCTACACT
 CCCGTAATT ATGAGACAAC CCACCGCAC CAAAGAGG CTTTATAGGAC GAGCCACAGC GTTACCGGAC CGAACCTGAC TTATAGCGT 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 TTCTCTCTGT CAGAGGCCT GTCTCTGGA ATGACTCTGG GAGTGGGCG CGATCAAGGA TGGCCCATCA ACTTGGCAA GAGAGAGCTC
 CTCATACCCC AAGAAGACA GTCTCTCGGA CCAGACCGT TACTAGACC CTCACCGCG CTAGTCTCT ACAGGCTAGT TGAACCGGT CTCTCTCGAG

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

5' UTR

1 AGTAATCCT GTGTCTAAT TGAGTGGCAT TGGTCTGAA ATCGAGTGC TAGCAATAA ACACATTGG ATTAATTTA ATCGTCTGT GAGCGATTAG
 TCATTATGGA CACAGATTA ACTCCAGTA ACCAGAGCTT TAGCTCAAG ATCGTTATT TGTGTAAAC TAATTAAT TAGCAAGCA CTCGTATC
 5' UTR

C protein

101 CAGAGAACTG ACCAGAACAT GTCTGTGTG 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
GTCTCTTGAC TGGTCTTGTA CAGACGACA TTTCAGATCC CTTTGTGGA CCGCAGTGA TACCATGTG CTCCTCAAGC GAGAACAGT TTGTTTATT
dc2 deletion (FSR)

201 AACAAAAAC AAACAAATT GGAACAGAC CTGAGGTGT TCAAGATTT ATCTTTTCT TTITTCTCAA CATTTGACT GGAATAAAGA TCACAGCCCA
TTGTTTGTG TTITGTTTAA CCTTTGCTG GACTCTACA AGTCTCTAA TAGAATAAGA AAACAAGTT GTAAACTGA CCTTTTCT AGTGCGGGT

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 TTGTGGAAGA TGCTGACCC AGACAAAGC TTGCTGTTC TAAGAAAGT CAAGAGAGT GTGGCAGTT TGATGAGAGG ATGTCTCTCA
GGATTCTCC AACACCTTT AGCACTGGG TTCTGTCCG AACCCACAG ATCTCTTCA GTTCTCTAC CACCGGTCAA ACTACTCTCC TAACAGAGT
YF17D partial signal

TBEV partial signal

C protein Hypr prM protein

401 R K 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
AGAAAGCC GTTCCATGA TGTCTGACT GTCAATCC TAAITGGG CATCTGGC ATGCAATCG CAGTACGTT TCGCAGGAA AGAGAGGCA
TCCTTTGCG CAAGGTACT ACAAGACTGA CAGTTAAG ATTAAACCC GTACAGCCG TACTGTAGC GTGATGCCA AGCGTTCCT TCTCTGCCG
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
GTACGTCT ACBCCGGA GGTAAAGTG CCGTACCA AGTAGAGTG GAAATGTA CTGCTCAT TCTGGCCACC GACATGGCT CTGTGTGA
CATGCCAGTA TGCAGCCTT CCATTCCTAC GCGATGGT GTCACTCTAC CTATTACAT GACGCGTGG CTGTACCCGA GAACCACT
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
TGATAGCTT TCTTATGAGT GCGTAACTAT AGATCAAGT GAGAACCTG TTGACGTGA TTGCTCTGC GGAACGTGG ATGGGTGTA TCTCGAATAT
ACTATCGGA AGAATACTCA CGCATGTGA TCTAGTTCCA CTCTTGGAC AACTGCACT AACGAGAGG GCTTTCACC TACCCACAT AGAGCTTATA
Hypr prM protein

701 G R C G K Q E G S R T R S V L I P S H A Q G E L T G R G H K W L E
GGACGTGTG GTAACAGA AGAAGCAGA ACCAGAGCT CAGTCTTAT ACCCTCCAC GCTCAGAG AGCTGACCG ACGGGACAT AATGGTTGG
CCTGCCACAC CATTTGTCT TCCTTGTCT TGGTCTGCA GTACAGATA TGGAGGGTG CGAGTCTCTC TCGACTGGCC TGCCCTGTA TTACCAACC
Hypr prM protein

801 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
AGGCGACTC ACTCGAACA CATTTGACC GGTGAGGG CTGGGTCTG AAAATCGC TGTGGCCCT CGTATGGT ACAGTCTGTT GGCTCACGCT
TCCCGCTGAG TGAGCTTGT GTAAACTGG CCGAGCTCC GACCAGACC TTTTAGCCG ACAACCGGA GGAATACC GATCAGCAA CCGAGTGGC
Hypr E 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
GGAGTCTGT GTTACTCGG TGGAGTGT GTGTCTG CTGTCTG CCGCTCTA CGCTCAGG TGTACTCAT TGAATAACAG AGATTGTG
CCTCAGAC CAATGAGGC ACCGTACGA CCACACAG GAGACAGAC GGGACAGT GGCAGGTCC ACATGATGA ACCTTTGTCT 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 ACCGCACCC AGGGACGAC TCGGTAACC CTGTGCTTG AACTGGTGG TTGCTTACT ATTACCGCTG AGGGCAACC CTCTATGAT GTGTGCTGG
TGGCCCTGGG TCCCTGCTG AGCCATTGG GACCACCAAC TTGACCCACC AAGCAATGA TAATGGCGAC TCCCGTTGG 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 TCAGGAGAT CCCGACAAA CAGAGGATA TTGCTTACG GCAAGCTGT CCGATACAA GGTGCGGCT AGGTGCCAA CAATGGGACC
TAGCTTAGT AGTCTCTTA GGGCGTGT GTTCCCTTAT AACGNACTG CTTTGCACA GGCTATGTT CCAGCCCGA TCCACGGGT GTTACCCCTGG

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 GCGAGGAGC ATCAGGAGG TACAGTGGC AAACGGAGC AGAGTAGAG AGGTGGGT ATCACTGCG GCCTGTTGG CAAGGAGT
CGGTGGGAC CGCTCCTTG TAGTCCCTCC AITGACACG TTGGCCTGG TCTCACTATC TCCGACCCA TTATGACGC CGGACAGCC GTTTCCTCA

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
ATTGCGCTT GGTCAAGC AGCCTGTAG GCCAAAAGA AGGCTACTGG GCAGTCTAT GACGCCACA AGATCGTTA TACAGTGAA GTGGAACAC
TAACAGCAA CGCAGTTCCG TCGACACTC CGGTTTTCT TCCGATGACC CGTCAGATA CTGCGTTGT 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
ACAGAGGGA TTACGTGGG GCCAAGCAGA CTCAATCCGG TCGCAAAAGC GCGAGTCTCA CCGTGTATC CGAAAAGACC ATCCTCACTA TGGGGGAGTA
TGTGTCCCT AATGCACCGC CGTTGCTCT GAGTAAGCC AGCGTTTTC GGTGCTAGT GGCACAGTAG GCTTTTCTG TAGSAGTAT 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
TGGCGAGTT TCTGTCTT CCGGCTGC TACGAGTC GACCTGGCC AGACAGTCAT CCTGGAAGT GNTAAACAG TTGAGCATCT GCCTACGCT
ACCGTGCAG AGACAGCAGA CGGCCACCG ATCCCTCAG CTTGACCGG TCTGTACATA GGACCTGAC CTATTTGTC AACTCGTAGA CGGATGCGA

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
TGGCAGTGC ACAGGATG GTTTACGAC CTGCGCTGC CATGAACAA TGAAGAGCG AGAACTGGA ATATGAGA GCGACTGTA GAATCGGTG
ACCGTCAAG TGTCCCTAAC CAAATTGCTG GAACGGAGC GTACCTTGT ACTTCTCGC TCTTTGACCT TATTAGCTCT 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
CCCTCATGC CGTGAAGATG GACGTCTACA ATCTGGTGA TCAGACCGC GTTCTCTTA AGCTCTGC TGGCGTACCA GTTCCCCACA TCGAAGGAAC
GGGAGTACG GCCTTCTAC CTGCAGATGT TAGACCACT AGTCTGGCG CAAGAGGAT TTGAGAGCG ACCGATGCT CAAGGGTGT AGCTTCTTG

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 GCCATGTAC TTGCGAGTG GGCCTGGAG AGTGAATAT GAAGGTCTT AGTACACAA TTGTGACAA GACCAAGTTC
CTTCATGGTG GACTTCACT CCGTACATG AAGCTCCAC CCGGACCTCT TCACTTTTA CTTTCCAGAA TGCATGTT ACACACTGT CTGTTTCNAG

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
ACATGAAGA GGGCCCCAC AGATAGCGC CAGATACTG TGGTATGA GTGACCTTT TCTGAAACA AACCTGSCAG AATACCCGTG CGGGCTGTAG
TGTACCTTCT CCGGGGGTG TCTATGCCG GTCTATGAC ACCATACCT CCACTGAAA AGACTTGT TTGAGAGTC 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
CTCAGGATC TCCGATGTC AATGTGCTA TGTGNTAC ACCTAACCTT ACCATGAGA ATACGGTGG TGTITTTAT GAGATGACG TTCGCCAGG
GAGTGCCTAG AGGCTACAG TTACAAGAT ACAGTAATG TGAATGGA TGGTAGTCT TATTGCCACC ACCAATAA CTCTACGTG AAGCGGTCC

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 ATCTAGCTGG GGAACCTCTC TTACAGTGG TTTCAAGG GAGTTCAT TGGGGGGTTC TTCCAAAAA CGAAGAAGG AATCGAACGA
 GCTATTGTAG TAGATGCACC CGCTTGAGG AATGTCACC AAGTCTTTC CCTCAGTTA ACCGCCCCAG AAGTTTTTTT GCTTCTCC 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 H T V L G G A F N
 TTGACGTTA TCGCGAGCA CGATGGGAT TTGCTTCG CAGGGGAT CTGTCTTCT ATTGTAAG CACTGCATAC CGTCTGGG GCGGCATTC
 AACTGCCAAT AGCCGCTCGT GGTACCTA AACCAAGCG GTCCCTCTAA GACAGAGA TAACCATTC GTACGATG GCACGACCC 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
 AITCTATTT CGGGGGG GGTCTCTGC CTAACTCT GCTGGAGTA GCGCTGCT GGTGGGACT GAATATCGG AATCGACGA TGTCCATGTC
 TAAGATAAA GCGCCGCGAC CCCAAGACG GNTTGAAGA GACCTCTAT CCGGACCGGA CCAACCTGA CTATACGCC TTAGCTGCT ACAGGTACG
 Hypr E protein
 NS1 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
 ATTCCTCTTG GCGGCGTGC TTGTACTGCG CATGACACTG GCGGTGGCG CGATCAAGG ATGCCCATC AACTTTGGCA AGAGAGACT C
 TAGGAGAAC CGGCGCGCAG AACATGACCG GTACTGTGAC CCGCAACCG GGTAGTTCC TAGCGGTAG TTGAACCGT TCTCTCTGA G

Sequence Appendix 3

PIV-WNTBEV Hypr with TBEV signal (p39)

deleted C

5' UTR

1 AGTAGTTCG CTGTGTGAC TGACAACTT AGTAGTGTG GTGAGATTA ACAACATTA ACACAGTGC AGCTGTTTCT TAGCAGGAG ATCTCGATGT
 TCATCAAGCG GACACACTCG ACTGTTTGA TCAACARA CACTCTAAT TGTGTAAAT TGTGTACGC TCGACAAAGA ATCTGTCTTC TAGAGCTACA
 WNV 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
 CTAAGAACC AGAGGGGCC GGCAGAGCC GGTCTGTCTA TTGTGTAAG CCGGTAATG CCGCGTGT GTCTTGAAT GACTTAAGC GGAGCTCCAA
 GATCTTTGG TCCTCCCGG CCGTCTCG CCGACAGAT AAACGATTT GCGCTTACG GCGCGCACAA CAGGAATTA CCTGAATTCG CCTCGAGTT
 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
 ACNAAAGAA CCGGGGGGAA CAGACTGGAT GAGCTGGCTG CTCGTAATCG GCATGCTGG CATGACAATC GCAGCTACGG TTGCAAGGA AAGAGACGGC
 TGTTCCTTTT GCGCCCTCTT GTCTGACCTA CTCGACCGAC GAGCATTAAG CBTACGACCC GTACTGTTAG CBTGATGCC AAGGTTCTCT 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
 AGTAGCGTCA TAGCGCGGGA AGGTAAGGAT GCGCTACCC AAGTAGAGT GGAATAGT ACCTGGCTCA TTCTGCGCAC CGACATGGGC TCTTGGTGTG

TCATGCCAGT ATGGCGCCT TCCATTCTTA CGGCGATGG TTCACTCTCA CCTTTTACCA TGGACGAGT AAGACCGGTG GCTGTACCCG AGRACCACAC
 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 TCTTATGAG TGGTAACCA TAGATCAAGG TGAGAACCT GTTGACGTG ATGCTTCTG CCGAAACGTG GATGGGTGT ATCTCGAATA
 TACTATCGGA AAGNATACIC ACCCATTTGT ATCTAGTTCC ACTCTCTGGA CAATGCAAC TAACGAGAC GGCCTTGCAC 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 GGTAAACAG AAGGACGAG AACGACGCT TCACTCTTA TACCTCCCA CGCTCAGGA GAGTCACCG GACGGGACA TAAATGGTTG
 ACCTGCCACA CCATTGTTC TTCTCTGTC TTGCTCTGCG AGTCAGAT ATGGAGGCT CCGAGTTCTT CCGACCTGC CTGCCCTGT 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
 GAGGGGACT CACTCGAAC ACATTTGACC CGGTGCGAGG GTTGGGTG GAAATCGG CTGTGGGCC TCCTATGGT GACAGTCGT TGGCTCAGCG
 CTCCCGCTGA GTGAGGCTTG TGTAACTGG GCGCAGCTCC CGACCCAGAC CTTTTAGCC GACAACCGG AGGATACCA CTGTACAGCA 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 GGTACTGCG GTGGCAGTGC TGGTGGTGT CCTGTCTT GCGCTGCT ACCTGCTCAG GTGTACTCAT TTGGAACA GAGATTTGT
 ACCTCAGACA CCATGAGCG CACCTCAG ACCACCA GAGACAGAA CGGGACAGA TCGCAGGTC CACATGAGTA AACCTTTGT CTCTAAACA
 E Hypr

801 T G T Q G T R V T L V L E L G C V T I T A E G K P S M D V W L
 CACGGGACC CAGGGACGA CTCGGTAA CCTGGTCTT GACTGGTG GTTGGGTAC TATTACGCT GAGGGCAAC CCTCTATGA TGTGTGCTG
 GTGGCGTGG GTCCCTGCT GAGCCCATG GGACACGAA CTGACCCAC CAAGCAATG ATAATGCGA CTCCGCTTG GAGATACCT 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 ATCGAGGAA TCCCGACAA ACCAGGAAAT ATTGCTCA CGCAAGCTG TCCGATACAA AGTTCGGCG TAGTSCCCA ACAATGGAC
 CTAGCTTGA TAGTCTCTT AGGGGCTGT TGGTCCCTTA TAAGGAGT GCGTTTCGAC AGGCTATGTT TCCAGCGCG ATCCAGGCT TGTACCTG
 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
 CGGCCACCT GCGGAGGAA CATCAGGAG GTACAGTGT CAAAGGAG CAGAGTGATA GAGGTGGG TAATCACTGC GGCCTGTG GCAAGGAAG
 GCGGTGGA CCGCTCTCTT GTAGTCCCTC CATGTACAC GTTTCCTG GTCTCATAT CTCCACCCC ATTAGTACG CCGACAAAG CBTTCCTTC
 E Hypr

1101 I V A C V K A A C E A K K A T G H V Y D A N K I V Y T V K V E P
 TATTGTGCT TGGTCAAG CAGCTGTGA GGCACAAAG AAGGTACTG GGCAGCTTA TGACCCAA AGATCTGTT ATACAGTAA AGTGAACCA
 ATAACAGGA ACGCAGTTC GTGACACT CCGTTTTC TTCCATGAC CCGTGCAAT ACTGCGTGT TTCTAGCAA TATGTCACT TCACCTTGT
 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
 CACACAGGG ATTAGTGGC GGCACAGAG ACTCATTCG GTCCAAAC GGCAGCTTC ACCGTGCTAT CCGAAAGAC CATCTCACT ATGGGGAGT
 GTGTGTCCC TAATGACCG CCGTTGCTC TGATTAAGC CAGCTTTTG CCGTTCAG TGGCACAGTA GCTTTTCTG GTAGAGTGA 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
 ATGGCGAGT TTCTGTGTC TGGCGGTGG CTAGCGTGT GAGCTTGC CAGACAGT TCTGTAACT TCGTAACA CATTGACATC TGCCTACCG
 TACCGCTGA AAGAGACAG ACGGCCACC GATGCTCTCA GCTGACCG GTCTGTAGT AGSACCTGA CCTATTTGT CAATCTGTAG ACGGATGGC
 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 P G
TTGGCAGTG CACAGGATTT GGTTAACGA CTTTGCCTG CCATGGAAC AGAAGAGC GAGAACTGG AATATGACG AGAATCGGT AGAATCGGT
AACCGTCCAC GTGTCCCTAA CCRAATTGCT GGAACGGAC GTTACTCTG TACTTCTCG CTTTGTACC TTATTACGTC TCGCTGACCA TCTTAAGCCA
E HypE
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
1501 GCGCTCATG CCGTGAGAT GGAGCTTAC AATCGGTG ATCAGACCG CTTTCTCTT AAAGCTCTCG CTGGGTACC AGTGGCCAC ATCGAAGAA
CGGGAGTAC GGCATCTTA CCGTCAATG TTAGACCCAC TAGTCTGCC GCAAGAGAA TTTCGAGAGC GACCCGATGG TCAACGGGTG TAGCTTCCYT
E HypE
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
1601 CGAAGTACCA CCGTCAATG GGCATGTAA CTTGCGAGT GGGCTGAGG AAGTTGAAA TGAAGGTCT TACGTACACA ATGTGTGACA AGACCAAGTT
GCTTCATGTT GGAATTCAGT CCGGTACAT GACGCTCCA CCGGACCTC TTCAACTTTT ACTTCCAGA ATGCAATGT TACACACTGT TCTGGTTCAA
E HypE
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
1701 CACATGGAAG AGGCCCCCA CAGATAGCG CCACGTACT GTGTGATGG AGGTACCTT TTCTGGAACA AACCTTGA ATATACCGT CGGGCTGTA
GTGTACCTTC TCCCGGGGT GTCTATGCC GGTGTATGA CACCATACC TCCACTGAA AGACCTTGT TTGGGACGT TTATGGSCA CGCCGACAT
E HypE
A H G S P D V N V A M L I T P N P T I E N N G G F I E M Q L P P G
1801 GCTCAGGAT CTCCGAGT CAATGTTCT ATGCTGATTA CACTTAACC TACCATCGAG AATAACGTT GTGTTTAT TGAGATGACG CTTCGCCAG
CGAGTGCTTA GAGGGTACA GTTACACGA TACGACTAT GTGATTTGG ATGTAGCTC TTATTGCCAC CACCAATA ACTTACGTC GAAGCGGTC
E HypE
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
1901 GCGATACAT CATCTACGT GCGCACTCT CTACCATG GTTTCAGAA GGAATTCAA TTGGGCGGT CTTCACAAA ACGAAGAGG GAATCGAAG
CGCTATTGTA GTAGATGAC CCGCTTGAGA GAATGTGAC CAAGTCTTT CCTCAAGT AACCCGCCA GAAGTTTTT TGTCTCTCC CTAGCTTGC
E HypE
L T V I G E H A W D F G S A G G F L S I G K A L H T V L G G A P
2001 ATTGACGTT ATCGGAGC ACGATGGA TTGTGTTCC GCAGGGGAT TCTGTCTTC TATGTGTAAG GCACTGCAAT CCGTGTGGG GGGGCGATC
TAATGCCAA TAGCCGCTG TCGTACCTT AAACCAAG CGTCCCTTA AGGACAGAG ATACCATTC CGTCAAGTAT GSCACGACCC CCGCGTAA
E HypE
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
2101 AATTCATTT TCGGGGCGT GGGTTCCTG CTTAACTCC TGTGAGT AGCCTGGC TGGTTGGAC TGAATATGG GAATCGAGC ATGTCCATGT
TTAGATATA AGCCCCGCA CCGAAGAC GATTTGAGG ACGACCTCA TCGGACCGG ACCAACCTG ACTTATACG CTAGGCTGC TACAGGTACA
E HypE
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
2201 CATTCCTTT GCGGGCGT GTGTACTGG CCATGACAT GGGGTTGGC GCGGACTG GTGTGCCAT AGACATCAG CGGCA
GTAGAGAA CCGGCCGCAC GAACATGACC GGTACTGTA CCGCAGCG CGGTGTGAC CCACAGGTA TCTGTAGTC GCGGTT

MNV NS1 protein

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

deleted C

5' UTR

1 AGTAGTTCCG CTGTGTGAGC TGACAAACTT AGTAGTGTGT GTGAGATTG AACAAATTA ACACAGTGG AGCTGTTTCT TAGCACGAAG ATCTCGATGT
TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCACAAA CACTCTTAAT TGTGTTTAAT TGTGTACCG TCACACAAGA ATCGTCTTC TAGAGCTACA

M S

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 AGGAGGGCC GGCAGAGCC GGGCTGTCTA TTGTCTAATA CCGGATGTC CCGGCTGT GTCTTGATTT GGACTTAAGC GGAGTCCAA
GATTCTTTGG TCTCTCCGGG CCGTCTCTCG CCGACACAGT AAACGATTTT GCGCTTACG GGGGACACAA CAGGAATTA 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
GCAAGAAA CCGGGGGNA AGACAGCAT AGCTGTATG ATAGCATGC TGCTTGTGT CGGAGCAGCT ACCGTGGAA AAGAACGCA CGAAGCACC
CGTTTCTTT GCGCCCTTT TCTGTCCGTA TCGACACTAC TATCGTACG ACCGAACACA GCCTGTGCGA TGGCAGCTT TTCTTGCGCT GCCTTGCTGG

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
GTGATAGGG CTGAGGGTAA GGATGGGCT ACGAGGTGA GAGTAGAAA TGGCACTGC GTATACTCG CGACTGATAT GGGATCTGCG TGTGACGATA
CACTATTCCC GACTCCCATT CCTACCCGA TGGCTCCACT CTCATCTCTT ACCTGTAACG CATTATGAC GCTGACTATA CCCTAGGACC ACCTGCTAT

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
GCCTCAGTA TGAATGCGTA ACAATAGACC AGGGGGAAGA ACCTGTGGAC GTTGACTGTT TCTGTAGAAA TGTGGATGCG GTTATCTGG AGTACGGCCG
CGGAGTCAAT ACTTACGCAAT TGTATCTGG TCCGCTTCT TGSACACCTG CCACTGACNA AGACTCTTT ACACCTACCG CNAATAGACC TCATGCGCGC

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
CTGTGAAA CAGGAGGCT CAGCACTCG AGATCTGTG CTGATTCCAA GTACGGCA AGGAGGTG ACCGTAGAG GCCACAGTG GCTTGAAGGG
GACACCTTT GTCTCCCGA GTGCTGAGC TTCTAGACAC GACTAAGTT CAGTGGCGT TCCTCTCAAC TGCCCATCTC CGGTGTTTAC CGAATTCTCC

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
GACTCATGA GGACCCACT GACTAGGGTG GAGGGTGGG TTGGAAGAA TCGGTCTC GCCTCGCTA TGGTACCGT CGTGTGCTG ACCTGGAGA
CTGAGTAAT CCTGGGTGA CTGATCCCAC CTCCACCC ACACCTTCTT AGCCACAGG CCGAGCGAT ACCAGTGGCA GCACACCGAC TGTGACTCT

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
GTGTGTGAC TCGGTTGCT GTGTTGGTTG TCTCTCTCTG TTGCCCCA GTGTAGCGT CCAGGTGAC TCATTGGAA ACAGAGATT TTGTACCCG
CACAGCATG AGCCCAACGA CACACCAAC AGGAGGAGAC AAACGGGT CACATGGCA GTTCCACATG AGTAACCTT TTGTCTCTAA AACAGTGGC

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
CACCCAGGG ACCTAGCGG TAACCTGGT GGTGGTGG TACTATTAC CGCTGAGGC AACCTCTA TGGATGTG GCTGGATGA
GTGGGTCCC TGCTGAGCC ATTGGACCA CGACTTGAC CCACCAACG AATGATAAG GCGACTCCG TTGGAGAT ACCTACAC CGACTACGT
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 AGATCCGC ACATACCGG GAATATTGC TTACCGCAA GCTGTCCGAT ACAGGTGCG CGGTAGGTG OCCACATG GGACCGCCA
TAGATAGTCC TCTTAGGCG TGTGTGTCC CTATATACG AGTGTGGT TTGCAGGCTA TGTTCCAGC GCGATCCAC GGTGTGTAC CTTGCCGT
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
CCCTGGCGA GGACATCAG CGAGTACG TGTGACAGG GACAGAGT GATAGGCT GGGTAATCA CTGCGCCTG TTGGGMAAG GAAGTATTGT
GGACCGCT CTTGTATG CTTCCATG ACAGTTG CTTGTCTCA CTATCCGA CCCATTAGT GACGCGGAC AGCGGTTC CTTCATACA
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
CGTTTCGTC AAGCAGCT GTAGGCCAA AAGAAGCT ACTGGCAG TCTATGAGC CAACAGATC GTTTATACAG TGAAGTGA ACCACACA
GCGAAGCAG TTCCGTGGA CACTCGGT TTCTTCGA TGACCGTGC AGATCTGCG GTTGTCTAG CAATATGTC ACTTCACCT TGGTGTGT
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
GGGATTAC TGGCGCAA CGAGTCTAT TCGGTGCA AAGCGCAG CTTACCGTG TCATCCGAA AGACATCCT CACTATGGG GAGTATGGC
CCCTAATGC ACCGCGGT GCTCTAGTA AGCCAGCT TTTCGCTG GAGTGGCAG AGTAGGCTT TCTGTAGGA GTGATACCC CTATACCG
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
AGTTTCTCT GCTTGGCG GTGCTAGCG GAGTGCACCT GGCCAGACA GTCTCTGG AACTGGATA AACAGTGG CATCTGCCTA CCGTTGGCA
TGCAAGAGA CGAGCGCC CACCGATCG CTCAGCTGA CCGGTCTGT CAGTAGACC TTGACCTATT TTGTCACTC GTAGACGAT GCGAACCCT
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
GGTGCACAG GATGTGTTA ACGACTTGC CTTGCCATG AAGCATAG GAGCGAGAA CTGAATAT GCAGAGCAG TCGTAGAAT CCGTCCCT
CCAGTGTCC CTACCAAT TGTGGAAG GAGCGTACC TTGTACTTC CTGCTCTTT GACCTTATTA GCTCTGCTG AGCATCTTAA GCCCGGGGA
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
CATGCGTGA AGATGAGCT CTACATCTG GGTATCAG CCGGTCTCT CTTAAGCT CTGCTGCG TACAGTTGC CCATCTGAA GGAAGAGT
GTACGCACT TCTACCTGCA GATGTAGAC CCACTAGTCT GCGCGCAGA GGAATTCGA GAGCGACGC ATGTCAAG GGTGTAGCT CTTGCTCA
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 GTACGCGCT GATCTGCG AGTGGGCT GAGAGAGT AATATGAAG GTCTTAGTA CACATGTGT GACAAGACCA AGTTCACATG
TGGTGGACT CAGTCCGTA CATTGAACG TCCACCGGA CTTCTTCA CTTTACTTC CAGATGAT GTGTACACA CTGTTCTGT TCAAGTGTAC
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
GAAGAGGCC CCCACATA GCGCCAGA TACTGTGTG ATGAGGGA CTTTCTG AACAACCC TGCAGATAC CCGTGGGCG TGTAGCTCAC
CTTCTCCCG GGTGTCTAT GCGCGTGT ATGACACCAC TACTCTCAT GGAAGACC TTGTTTGGG AGTCTTANG GGCACGCGCG ACATCGAGT
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
GGATCTCCG ATGTCAATGT TGTATGCTG ATTACCTA ACCTACCAT CGAGATAC GGTGTGTGT TTAATGAT GAGCTTCCG CCAGCGATA
CCTAGAGGC TACAGTTACA ACGATACGAC TAATGTGAT TGGATGTA GCTCTATTG CCACACCA AATACTCTA CGTGAGGC GGTCCGCTAT
E Hypr

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1901  . 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
      ACATCATCTA CDTGGGCGAA CTCTCTTACC AGTGGTTTCA GAAGGGAGT TCAATTGGGC GGGTCTTTCCA AAAAACGAG AGGGGATCG AACGATTGAC
      TGTAGTAGAT GCACCCGCTT GAGAGATGG TCACCAAGT CTTTCCCTCA AGTTAACCG CCACAGAGT TTTTGTCTT TCCCTTAGC TTGCTACTG

2001  . 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
      GGTATCGGC GAGCAGCAT GGGATTTGG TTCCGAGGG GGAATCTCTGT CTCTATTGG TAAGGCACTG CATACCGTGC TGGGGGCGC ATTCAATTCT
      CCAATAGCG CTGCTGCGTA CCTAAACC AAGGCTTCC CCTAAGACA GAAGATAACC ATTCCGTGAC GTATGGCAG ACCCCCGCG TAGTTAAGA

2101  . I F G G V G F L P K L L L L G V A L A W L G L N M R N P T M S M S F L
      ATTTTCGGG GGTGGGTTT CCTGCTTAA CTCTCTTGG GAGTAGCCT GGCCTGGTGG GACTGATA TCGGATCC GAGATGTC ATGTCATTCC
      TAAAGCCCC CGCACCCCAA GGAGGATTT GAGGAGACC CTATCGGA CCGGACCAAC CCTGACTTAT AGCCTTAGG CTGCTACAGG TACAGTAGG

2201  . 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
      TCTTGGCCGG CGTGTGTGTA CTGCCATGA CACTGGCGT TGGCGCGAC ACTGGGTGG CCATAGACAT CAGCGGCAA
      AGACCGGCC GCACGACAT GACCGTACT GTGACCCGA ACCGCGCTG TGACCCACAC GGTATCTGTA GTCGCCGTT

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WNV NS1 protein

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

WN (Δ CprME)-Rabies PIV sequence (partial)

N-terminus of C -----

5' UTR

M S

1 AGTAGTTCGC CTGTGTGAGC TGACAAACTT AGTAGTGTTC GTGAGGATTA ACAACAATTA ACACAGTGGC AGCTGTTTCT TAGCACGAAG ATCTCGATGT
TCATCAAGCG GACCACTCG ACTGTTTGAA TCATCAGAAA CACTCTTAAT TGTGTTAAT TGTGTACGC TCGACAAAGA ATGCTGCTTC TAGAGCTACA
N-terminus of C

· 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 Q K K R ·

101 CTAAGAAACC AGGAGGGCCC GGCAAGAGCC GGGCTGTCTA TTGCTAAAA CGCGGAATGC CCCTGCTGTT GTCCTTGATT GGACTTAAGC AAAAGAAGCG
GATTCTTTGG TCCTCCCGGG CCGTTCTCGG CCGACAGAT AAACGATTTT GCGCTTACG GGGCGCACAA CAGGAACATA CCTGAATCG TTTTCTTCGC
N-terminus of C Rabies-G signal

partial C signal Rabies-G protein

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

201 AGGGGGCAAG ACTGGTATAG CTGTGATCGT TCCTCAGGCT CTTTGTGTTG TACCTTGTCT GGTATTTCCT CTTTGTGTTG GTAAATTTCC TATCTATACC
TCCCCCGTTC TGACCATATC GACACTAGCA AGGAGTCCGA GAAACAAAC ATGGGAACGA CCATAAAGGG GAAACGAAAC CATTAAAGG 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 TTGGAGTCCC ATTGATATTC ACCATTGAG CTGCCCAAC AACCTCGTCG TTGAGGATGA AGGGTGCCT AATCTTCTG
TAGGGACTAT TCGAGCCCGG AACCTCAGGG TAACATAAG TGTAAGTCTC GACGGGTTTG TTGGAGCAGC AACTCCTACT TCCACGTGA TTAGAAAGAC
Rabies-G protein

· 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 Y T N ·

401 GATTTTCTTA CATGGAGTTG AAAGTGGGCT ATATTTGAGC CATTAAGATG AACGGCTTTA CTGTACAGG AGTGTGACC GAAGCCGAGA CATATACAAA
CTAAAAGGAT GTACCTCAAC TTTCACCGCA TATAAGTTCG GTAATCTTAC TTGCGGAAT GAACATGTCC TCAGCACTGG CTTCGGCTCT GTATATGTTT
Rabies-G protein

· F V G Y V T T T F 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 TTTCGTGGGA TACGTACCA CCACCTTCAA GAGAAACAC TTCCGCCCAA CGCTGACGC TTGTGCGGCC GCTTACAAC GGAAGATGGC AGGAGATCCT
AAAGCACCTC ATGCAGTGGT GGTGGAAGTT CTCTTTTGTG AAGGCGGGT GCGGACTGCG AACAGCCCGG CGAATGTGA CCTTCTACCG TCCTCTAGGA
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 CAACCGTAT CCTGATTACC ATTGGCTGCG GACAGTCAAG ACTACCAAGG AGAGTCTGCT CATTATATCA CCAAGCGTGG
GCTATACTTC TTAGAGACGT GTTGGGCATA GGACTAATGG TAACCGACGC CTGTCAGTTC TGATGGTTCC TCTCAGACCA GTAATATAGT GGTTCGCACC
Rabies-G protein

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

701 CCGATCTTGA TCCTTATGAT AGATCCCTGC ACAGTAGGGT TTTTCTGCG GGAATTTGTA GCGGTGTTGC AGTATCAAGT ACCTACTGCT CCACTAACCA
GGCTAGAACT AGGAATACTA TCTAGGGACG TGTCATCCCA AAAAGGACCG CCCTTAACAT CGCCACAACG 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 GAACCTACCG GGCAAGCGGG CATCTAAGGG GTCTGAAACA
GCTGATGTGA TATACCTACG GACTCTTGGG AGCTGAGCCA TACTCAACGC TGTAATAAGT CTGAGTGGC CCGTTCGCCC 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 AAATCTCTTA AAGGCGCCTG TAAGCTGAAA CTCTGTGGCG TACTGGGGCT GCGCCTGATG GACGGCACAT
AGCCCCAAC AACTACTGCG CCGCAACATA TTTAGAGAAT TTCCGCGGAC ATTGACTTT GAGACACCGC ATGACCCCGA CGGGGACTAC CTGCGGTGTA
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 F R S D E I E H L V V ·

1001 GGGTGGCTAT GCAGACAAGC AATGAAACAA AGTGGTGTCC CCTGCTCAG CTGGTTAATC TGCACGACTT TAGGTCTGAC GAAATCGAGC ACCTGTGGT
CCCACCGATA CGTCTGTTG TTAATTTGTT TCACCAACAG GGGACCAAGT GACCAATTAG ACGTGTCTGA ATCCAGACTG CTTTAGCTCG TGGAACACCA
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 TGGAAGAAC GCGAAGAGTG CCTGGACGCA CTTGAGAGTA TTATGACCAC CAAATCCGTT TCCTTCAGAA GACTGAGCCA CTGCGAAAG
      CCTCCTTGAC CACTTCTTTG CGCTTCTCAC GGACCTGCGT GAAGTCTCAT AATACGGTG GTTAGGCAA AGGAAGTCTT CTGACTCGGT GGACGCTTTC
      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 GGTTCCGGAA GGCTTATACT ATTTTCAACA AGACTCTTAT GGAGGCGGAT GCCCATTATA AGTCAGTTAG GACTTGGAAT GAGATAATTC
      GACCACGGTC CCAAGCCCTT CGGAATATGA TAAAAGTTGT TCTGAGAATA CCTCCGCGTA 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 ATGTCTGAGA GTGCGTGGGA GATGCCACCC CCATGTCAAT GGGGTGTTCT TTAACGGAAT CATCCTGGGA CTGACGGGA ACGTCTGTAT
      GGAGGTTTCC TACAGACTCT CAGCCACCCT CTACGGTGGG GGTACAGTTA CCCACAGA AATTGCCITTA GTAGGACCCT 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  TCCCGAGATG CAATCTTCCC TTCTGCAGCA ACACATGGAA CTCCTGGTGT CTTAGTGATG ACCCCTGATG CACCCACTGG CCGACCCAG CACTGTGTTC
      AGGGCTCTAC GTTAGAAGGG AAGACGTGCT TGTGTACCTT GAGGACCACA GAAGTCACTA 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  AAAAATGGCG ATGAGGCCGA AGACTTTGTG GAAGTTCACC TGCCCGATGT ACACGAAAGG ATATCTGGAG TAGACCTGGG CCTTCTAAT TGGGTAAAGT
      TTTTACCGC TACTCCGGCT TCTGAACAC CTTCAAGTGG ACGGGCTACA TGTGCTTCC TATAGACCTC ATCTGGACCC GGAAGGATTA ACCCCATTCA
      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  ACGTGCTCCT GAGTGCGGGT GCCTTGACCG CTTTGATGCT GATCATTTTT CTGATGACCT GCTGGGGAG GGTGAATGCG TCCGAGCCGA CACAGCACAA
      TGCACGAGGA CTCACGCCCA CGGAAGTGGC GAAACTACGA CTAGTAAAAA GACTACTGGA GACCCGCTC CCACTTAGCG AGGCTCGGCT GTGTCTGTGT
      Rabies-G protein
      FMDV 2A
      . 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  TCTCAGAGGG ACAGGCCGGG AAGTAAGTGT GACTCCGCAA TCTGGCAAGA TTATTAGTAG TTGGGAGAGT TACAAGTCTG GAGGAGAGAC TGGGTGAAT
      AGAGTCTCCC TGTCCGSCCC TTCATTACA CTGAGGCGTT AGACCGTTCT AATAATCATC AACCCCTCTCA ATGTTCAAGC CTCTCTCTG ACCCAACTTA
      preNS1 signal
      FMDV 2A
      . 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  TTTGATCTGC TCAAACCTGC AGGCGATGTA GAATCAAATC CTGGACCCGC CCGGACAGG TCCATAGCTC TCAGTTTCT CGCAGTTGGA GGAGTTCTGC
      AAAC TAGACG AGTTTGAACG TCCGCTACAT CTTAGTTTAG GACCTGGGCG GGCCTGTCC AGGTATCGAG AGTGCAAGA GCGTCAACCT CCTCAAGACG
      NS1 signal
      NS1
      . 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  TCTTCTCTC CGTGAACGTG CACGCTGACA CTGGGTGTGC CATAGACATC AGCCGGCAAG AGCTGAGATG TGAAGTGA GTGTTTATAC ACAATGATGT
      AGAAGGAGAG GCACTTGACG GTGCGACTGT GACCCACACG GTATCTGTAG TCGCCGTTTC TCGACTCTAC ACCTTCACCT CACAAGTATG TGTTACTACA

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WN (Δ C)-Rabies G PIV sequence (partial).

5'UTR	
terminus of C	N-
	M S
1	AGTAGTTCGC CTGTGTGAGC TGACAACTT AGTAGTGTTC GTGAGGATTA ACAACAATTA ACACAGTGC AGCTGTTTCT TAGCACGAAG ATCTCGATGT TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCACAAA CACTCCTAAT TGTGTGTAAT TGTGTCACGC TCGACAAAGA ATCGTGCTTC 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 AGGAGGGCCC GGCAAGAGCC GGGCTGTCAA TATGCTAAAA CGCGGAATGC CCGCGGTGTT GTCCTTGATT GGACTTAAGC AAAAGAAGCG GATTCTTTGG TCCTCCGGG CGTTCTCGG CCGACAGATT ATACGATTTT GCGCTTACG GGGCGCACAA CAGGAACATA CCTGAATTCC TTTTCTTCG 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 P P L C F G K F P I Y T
201	AGGGGCAAG ACTGGTATAG CTGTGATCGT TCCTCAGGCT CTTTGTGTTG TACCTTGTCT GGTATTTCCT CTTTGTCTTG GTAAATTTCC TATCTATACC TCCCCGTTTC TGACCATATC GACACTAGCA AGGAGTCGCA GAAACAAC ATGGGAACGA CCATAAAGG GAAACGAAC CATTTAAGG 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 TTGGAGTCCC ATTGATATTC ACCATTGTAG CTGCCCAAAC AACCTCGTCG TTGAGGATGA AGGGTGCCT AATCTTCTG TAGGGATAT TCGAGCCCGG AACCTCAGG TAACTATAAG TGGTAACTC GACGGGTTTG TTGGAGCAGC AACTCCTACT TCCCACGTGA TTGAAAGAC 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 AACGGCTTTA CTGTACAGG AGTCGTGACC GAAGCGAGA CATATACAA CTAAAGGAT GTACCTCAAC TTTCACCGA TATAAGTCG GTAATTTCTAC TTGCGAAAT GAACATGTCC TCAGCACTGG CTTGCGCTCT GTATATGTT Rabies-G protein
	F V G Y V T T T F 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 CCACCTTCAA GAGAAACAC TTCCGCCCAA CGCTGACGC TTGTGCGGCC GCTTACAAC GGAAGATGGC AGGAGATCCT AAAGCACCT ATGCAGTGGT GGTGGAAGTT CTCTTTGTG AAGGGGGTT GCGGACTGCG AACAGCCCG CGAATGTGA CCTTCTACCG TCCTCTAGGA 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 CCGATTACC ATTGGCTGCG GACAGTCAAG ACTACCAAG AGAGTCTGGT CATTATATCA CCAAGCGTGG GCTATACTTC TTAGAGACGT GTTGGGCATA GGACTAATGG TAACGAGCG CTGTCACTTC TGATGGTTCC TCTCAGACCA GTAATATAGT GGTTCGACCC 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 TCCTATGAT AGATCCCTGC ACAGTAGGCT TTTTCTGGC GGGAAATGTA GCGGTGTTC AGTATCAAGT ACCTACTGCT CCATAACCA GGCTAGAAT AGGAATACTA TCTAGGACG TGTCATCCCA AAAAGGACCG CCCTTAACAT CGCCACAACG 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	CGACTACCT ATATGGATGC CTGAGAACC TCGACTCGGT ATGAGTTGCG ACATTTTAC GAACTCACGG GGCAAGCGG CATCTAAGGG GTCTGAAACA GCTGATGTA TATACCTACG GACTCTTGG AGCTGAGCCA TACTCAACGC TGTAATAATG CTGAGTGCC CCGTTCGCC 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	TGCGGTTTG TTGATGAGCG GGGGTTGTAT AAATCTCTTA AAGGGGCTG TAAGCTGAAA CTCTGTGGCG TACTGGGGCT GCGCCTGATG GACGGCACAT ACGCCAAAC AACTACTCGC CCCCACATA TTAGAGAAT TTCCGCGGAC ATTGACTTT GAGACACCG ATGACCCGA CGCGGACTAC CTGCGGTGTA 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 F R S D E I E H L V V
1001	GGTGGCTAT GCAGACAAGC AATGAAACAA AGTGGTGTCC CCTGGTTCAG CTGGTTAATC TGCACGACTT TAGGTCTGAC GAAATCGAGC ACCTGTGGT CCCACGATA CGTCTGTCG TTACTTTGTT TCACCACAG GGGACCACT GACCAATTAG ACGTGCTGAA ATCCAGACTG CTTAGTCTCG TGAACACCA Rabies-G protein
	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	GGAGGAACG GTGAAGAAC GCGAAGAGT CCTGGACGA CTGAGAGTA TTATGACCAC CAAATCCGTT TCCTTCAGAA GACTGAGCCA CCTGCGAAAG CCTCTTAC CACTTCTTTG CGTTCTCAC GGACCTGCT GAACCTCAT AATACCTGGT GTTAGGCAA AGGAAGTCT CTGACTCGGT GGACGCTTC 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 GGTTCGGGAA GGCTTATACT ATTTTCAACA AGACTCTTAT GGAGGCGGAT GCCCATTATA AGTCAGTTAG GACTTGGAAAT GAGATAATTC
 GACCAAGGTC CCAAGCCCTT CCGAATATGA TAAAAGTTGT TCTGAGAATA CCTCCGCCTA CGGTAATAT 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 I .
 1301 CCTCCAAAGG ATGTCTGAGA GTGGGTGGGA GATGCCACCC CCATGTCAAT GGGGTGTCTT TTAACGGAAT CATCTGGGA CTGACGGGA ACGTCTGTAT
 GGAGGTTTCC TACAGACTCT CAGCCACCCCT CTACGGTGGG GGTACAGTTA CCCCACAAGA AATTGCCTTA GTAGGACCCCT 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 P .
 1401 TCCCGAGATG CAATCTTCCC TTCTGCAGCA ACACATGGAA CTCCTGGTGT CTTCAGTGAT ACCCTGATG CACCCACTGG CGACCCCGAG CACTGTGTTC
 AGGGCTCTAC GTTAGAAGGG AAGAGCTCGT TGTGTACCTT GAGGACCACA GAAGTCACTA 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 AAAATGCGG ATGAGGCGGA AGACTTTGTG GAAGTTCACC TGCCCGATGT ACACGAAAGG ATATCTGGAG TAGACCTGGG CCTTCTAAT TGGGGTAAGT
 TTTTACCGC TACTCCGGCT TCTGAAACAC CTTCAAGTGG ACGGCTACA TGTGCTTTC TATAGACCTC ATCTGGACCC GGAAGGATTA ACCCAATTCA
 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 ACGTGTCTCT GAGTGGGGT GCCTTGACCG CTTTGATGCT GATCATTTT CTGATGACCT GCTGGCGGAG GGTGAATCGC TCCGAGCCGA CACAGCACAA
 TGCACGAGGA CTCACGCCCA CGGAAGTGGC GAAACTACGA CTAGTAAAAA GACTACTGGA CGACCGCCTC CCACTTAGCG AGGCTCGGCT GTGTCTGTGT
 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 TCTCAGAGGG ACAGGCGGGG AAGTAAGTGT GACTCCGCAA TCTGGCAAGA TTATTAGTAG TTGGGAGAGT TACAAGTCTG GAGGAGAGAC TGGGTGAAT
 AGAGTCTCCC TGTCGGGCC TTAATTACCA CTGAGGGGTT AGACGGTCTT AATAATCATC AACCTCTCA ATGTTACAGC CTCCTCTCTG 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 TTTGATCTGC TCAAACCTGC AGGCGATGTA GAATCAATC CTGGACCCGG AGGAAAGACC GGTATTGCAG TCATGATTGG CCTGATCGCC TGCSTAGGAG
 AAACATAGAC AGTTTGAAAG TCCGCTACAT CTTAGTTAG GACCTGGGCC TCCTTCTCG CCATACAGTC AGTACTAACC GGACTAGCGG ACCCATCTC
 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 CTCTAACTTC CAAGGGAAGG TGATGATGAC GGTAATGCT ACTGACGTCA CAGATGTCAT CAGGATTCCA ACAGCTGCTG GAAAGAACCT
 GTCAATGGGA GAGATTGAAG GTTCCCTTCC ACTACTACTG CCATTACGA TGAATGACGT GTCTACAGTA GTGCTAAGGT TGTGACGAC CTTTCTTGGA
 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 ATGCATGTCT AGAGCAATGG ATGTGGGATA CATGTGCGAT GATACTATCA CTTATGAATG CCCAGTCTGT 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 H G .
 2101 TGTGGTGCA CAAAGTCAGC AGTCTACGTC AGGTATGGAA GATGCACCAA GACACGCCAC TCAAGACGCA GTCGGAGGTC ACTGACAGTG CAGACACAG
 ACAACCACT GTTTCAGTGC TCAGATGCAG TCCATACCTT CTAAGTGGTT CTGTGCGGTG AGTTCTGOST CAGCTCCAG TGACTGTAC GTCTGTGTGC
 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 CTTGGATGGA CAGCACCAAG GCCACAAGGT ATTTGGTAAA AACAGARTCA TGAATCTTGA GGAACCCCTG
 CTCCTTCTG AGATCGCTTG TTCTTCCCC GAACCTACCT GTGCTGGTTC CGGTGTTCCA TAAACCATTT TTGTCTTAGT ACCTAGAACT 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 GTGGCAGCCG TCATTGGTGT GATGCTTGGG AGCAACACCA TGCAGAGAGT TGTGTTTGTG GTGCTATTGC TTTTGGTGGC CCCAGCTTAC
 TATACGGGAC CACCGTCGGC AGTAACCAAC CTACGAACCC TCGTTGTGTT ACGTCTCTCA ACACAAACAG CAGGATAACG AAAACCAACG 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 AGCTTTAACT GCCTTGGAAAT GAGCAACAGA GACTTCTTGG AAGGAGTGTG TGGAGCAACA TGGGTGGATT TGGTCTCGA AGGCGACAGC TGGGTGACTA
 TCGAAATTGA CGGAACCTTA CTCGTTGTCT CTGAAGAACC TTCTTCACAG ACCTCGTTGT ACCCACTTAA ACCAAGAGCT TCGCTGTGCG 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 GGGGCCAACC TGGCAGAGGT CCGCAGTTAT TGCTATTGG CTACCGTCAG
 AGTACAGATT CCTGTTCCGA TGGTAGCTAC ACTTCTACTA CTTATACCTC CGCCGGTTGG ACCGTCTCCA GGCCTCAATA ACGATAAACC 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 G V V D R .
 2601 CGATCTCTCC ACCAAAGCTG CGTGCCCGGC CATGGGAGAA GCTCACAATG ACAAACGTGC TGACCCAGCT TTGTGTGCA GACAAGGAGT GGTGGACAGG
 GCTAGAGAGG TGGTTTCGAC GCACGGGCGG GTACCCCTCT CGAGTGTTC TGTTCGCAGC ACTGGGTGCA AAACACACGT CTGTTCTCA CCACCTGTCC
 E
 G W G N G C G L F 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 GGTGGGGCA ACGGCTGCGG ACTATTGGC AAAGGAAGCA TTGACACATG CGCCAAATTT CCTGTCTCTA CCAAGGCAAT AGGAAGAACC ATTTTGAAAG
 CCGACCCCGT TGCCGACGCC TGATAAACCG TTCTCTCGT AACTGTGTAC GGGTTTAAA CGGACGAGAT GGTTCGGTGA TCCTTCTGG TAAAACTTC
 E
 . N I K Y E V A I F 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 GCCATTTTG TCCATGGACC AACTACTGTG GAGTGCACAG GAAACTACTC CACACAGGTT GGAGCCACTC AGGCAGGGAG
 TCTTATAGTT CATGCTTCAC CGGTAAAAAC AGGTACCTGG TTGATGACAC CTCAGCGTGC CTTTGATGAG GTGTGTCCAA CCTCGGTGAG 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 CACACTAAG CTGGAGAAAT ATGGAGAGGT GACAGTGGAC TGTGAACAC GGTGAGGAT TGACACCAAT
 TAAGTCGTAG TGAGGACGCC GCGGAAGTAT GTGTGATTTC GAACCTCTTA TACCTCTCCA CTGTCACTG ACACITGGTG CAGTCCCTA ACTGTGGTTA
 E
 A Y Y V M T V G T K T F L V H R E W F M D L N L P W S S A G S T V W .
 3001 GCATACTACG TGATGACTGT TGAACAAAG ACGTCTTGG TCCATCGTGA GGGTTTCATG GACCTCAACC TCCCTGGAG CAGTGTCTGA AGTACTGTGT
 CGTATGATGC ACTACTGACA ACCTGTGTTT TGCAAGAAC AGGTAGCACT CACCAAGTAC CTGGAGTTGG AGGGAACCTC GTCACGACCT TCATGACACA
 E
 . R N R E T L M E F 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 ATGGAGTTG AGGAACCACA CGCCACGAAG CAGTCTGTGA TAGCATTGGG CTCACAAGAG GGAGCTCTGC ATCAAGCTTT
 CCTCTGTGC TCTGTCAAT TACCTCAAC TCCTGGTGT GCGGTGCTTC GTGAGACACT ATCGTAACCC GAGTGTCTC CCTCGAGACG 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 ATTCTGTGG AATTTTCAAG CAACACTGTC AAGTTGACGT CGGGTCATTT GAAGTGTAGA GTGAAGATGG AAAAATTGCA GTTGAAGGGA
 CCGACCTCGS TAAGGACACC TTAAGAGTTC GTTGTGACAG TTCAACTGCA GCCCAGTAAA CTTACATCT CACTTCTACC TTTTAAAGT CAACTTCCT
 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 ACAACCTATG GGTCTGTTC AAAGGCTTTC AAGTTTCTTG GACTCCCGC AGACACAGGT CACGGCACTG TGGTGTGGA ATTGAGTAC ACTGGCAGG
 TGTGTGATAC CGCAGACAAG TTTCCGAAAG TTCAAGAAC CCTGAGGGCG TCTGTGTCCA GTGCGGTGAC ACCACAACCT TAAGTCTATG TGACCGTGCC
 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 CAAAGTTCT ATCTCGTCAG TGGCTTCATT GAACGACCTA AGCCAGTGG GCAGATTGGT CACTGTCAAC CCTTTTGTTC CAGTGGCCAC
 TACCTGGAAC GTTTCAAGGA TAGAGCAGTC ACCGAAGTAA CTGCTCGAT TGGGTGACCC CGTCTAACCA GTGACAGTTG GGAACACAA GTACCCGTG
 E
 . A N A K V L I E L E 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 AAGGTCTGA TTGAATTGGA ACCACCTTT GGAGACTCAT ACATAGTGGT GGCAGAGGA GAACAACAGA TCAATCACA CTGGCACAAG
 CCGGTTGCGA TTCCAGGACT AACTTAACCT TGGTGGGAAA CCTCTGAGTA TGTATCACA CCGTCTCCT CTGTGTGCT AGTTAGTGGT GACCGTGTTC
 E
 S G S S I G K A F 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 GCATTGGCA AGCCTTTACA ACCACCTCA AAGGAGGCGA GAGACTAGCC GCTTAGGAG ACACAGCTTG GGACTTTGGA TCAGTTGGAG
 AGACCTTCGT CGTAACGTT TCGGAATGT TGGTGGAGT TTCTCGCGT CTCTGATGG CGAGATCCTC TGTGTGCAAC CCTGAAACCT 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 ATCAAGTGTG CGGAGGAGCA TTCCGCTCAC TGTTCGGAGG CATGTCTGG ATAACGCAAG GATTGCTGG
 CCCACAAGTG GAGTCAACCC TTCCGACAGG TAGTTCACAA GCCTCTCGT AAGGCGAGTG ACAAGCCTCC GTACAGGACC TATTGCTTC CTAACGACCC
 E
 . A L L L W M G I N A R D R S I A L T F L A V G G V L L P L S V N V .
 3801 GGCTCTCTTG TTGTGGATGG GCATCAATGC TCGTGACAGG TCCATAGCTC TCACTTTCT CCGAGTTGGA GGAGTTCTGC TCTCTCTCTC CGTGAACGTG
 CCGAGAGGAC AACACCTACC CGTAGTTAG AGCACTGTCC AGGTATCGAG AGTGCAAGA GCGTCAACCT CCTCAAGAG AGAAGGAGAG GCACTTGAC
 E

NS1

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 AGCTGAGATG TGGAACTGGA GTGTTTCATAC ACAATGATGT GGAGGCTTGG ATGGACCGGT
 GTGGCACTGT GACCCACACG GTATCTGTAG TCGGCCGTTT TCGACTCTAC ACCTTCACCT CACAAGTATG TGTACTACA CCTCCGAACC TACCTGGCCA

WN (Δ prME)-Rabies G PIV sequence (partial)

C protein

5' UTR

M S

1 AGTAGTTCGC CTGTGTGAGC TGACAAACTT AGTAGTGTTC GTGAGGATTA ACAACAATTA ACACAGTGGC AGCTGTTTCT TAGCAGCAAG ATCTCGATGT
 TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCACAAA 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 AGGAGGGGCC GGCAAGAGCC GGGCTGTCTA TTTGCTAAAA CGCGGAATGC CCGCGTGTTC GTCTTGTATT GGACTTAAGA GGGCTATGTT
 GATTCTTTGG TCCTCCCGGG CCGTCTCTCG CCCGACAGAT AAACGATTTT GCGCCTTACG GGGCGCACAA CAGGAAGTAA CCTGAATTCT CCGATACAA
 C protein
 S L I D G K G P I R F V L A L L A P F R F T A I A P T R A V L D R
 201 GAGCCTGATC GACGGCAAGG GGCCAATACG ATTTGTGTTC GCTCTCTTGG CGTCTCTCAG GTTCACAGCA ATTGCTCCGA CCGAGCAGT GCTGGATCGA
 CTCGGACTAG CTGCCGTTCC CCGTTATGCT TAAACACAAC CGAGAGAACC GCAAGAAGTC CAAGTGTGCT 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 TGGAGAGGTS TGAACAAACA AACAGCGATG AAACACCTTC TGAGTTTCAA GAAGGAACCTA GGGACCTTGA CCAGTGCTAT CAATCGGCGG AGCTCAAAGC
 ACCTCTCCAC ACTGTTTGT TGTGCTCTAC TTTGTGGAAG ACTCAAAGTT CTTCCTTGAT CCCTGGAACT GGTCACGATA GTTAGCCGCC TCGAGTTTCG
 Rabies-G signal
 C protein partial C signal Rabies-G
 protein
 K K R 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
 401 AAAAGAGGCG AGGGGGCAAG ACTGGTATAG CTGTGATCGT TCCTCAGGCT CTTTGTGTTG TACCTTGGCT GGTATTTCCC CTTTGCTTTG GTAAATTTCC
 TTTCTTCGCG TCCCGGTTTC TGACCATATC GACACTAGCA AGGAGTCCGA GAAACACAAC ATGGGAACGA CCATAAAGG GAAACGAAAC 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 AGCTCGGGCC TTGGAGTCCC ATTGATATTC ACCATTTGAG CTGCCCAAAC AACCTCGTCG TTGAGGATGA AGGGTGCACCT
 ATAGATATGG TAGGGACTAT TCGAGCCCGG AACCTCAGGG TAACATAAG TGGTAAACTC GACGGGTTTG TTGGAGCAGC AACTCCTACT 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 AATCTTTCTG GATTTTCTTA CATGGAGTTG AAAGTGGGCT ATATTTACGC CATTAAAGATG AACGGCTTTA CTGTACAGG AGTCGTGACC GAAGCCGAGA
 TTAGAAGAC CTAAGAGGAT GTACCTCAAC TTTCACCGCA TATAAGTGC GTAATTCTAC TTGCGAAAT GAACATGTCC TCAGCACTGG CTTGCGCTCT
 Rabies-G protein
 Y T N F V G Y V T T T F K R K H F R P T P D A C R A A Y N W K M A
 701 CATATACAAA TTTCGTGGGA TAGCTACCA CCACCTTCAA GAGAAACAC TTCCGCCCAA CGCCTGAGC TTGTGGGGCC GCTTACAACT GGAAGATGGC
 GTATATGTTT AAAGCACCTC ATGCACTGGT GGTGGAAGTT CTCTTTTGTG AAGGGGGGTT GCGGACTGCG AACAGCCCGG CGAATGTTGA 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 AGGAGATCCT CGATATGAAG AATCTCTGCA CAACCCGTAT CCTGATTACC ATTGGCTGCG GACAGTCAAG ACTACCAAGG AGAGTCTGGT CATTATATCA
 TCCTCTAGGA GCTATCTTC TTAGAGACCT GTTGGGCATA GGACTAATGG TAACCGACGC CTGTCACTTC TGATGGTTCC TCTCAGACCA GTAATATAGT
 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 TCCTTATGAT AGATCCCTGC ACAGTAGGGT TTTCTCTGGC GGGAAATGTA GCGGTGTTGC AGTATCAAGT ACCTACTGCT
 GGTTCGACCC GGCTAGAACT AGGAATACTA TCTAGGGACG TGTCAATCCA AAAAGGACCG CCCTTAACAT CGCCACAACG 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 GGCAAGCGGG CATCTAAGGG
GGTGATTGGT GCTGATGTGA TATACCTACG GACTCTTGGG AGCTGAGCCA TACTCAACGC TGTAAAAATG CTGAGTGCC CCGTTCGCC GTAGATTCCC
Rabies-G protein

· S E T C G F 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 ·

1101 GTCTGAACA TCGGGTTTG TTGATGAGCG GGGGTTGTAT AAATCTCTTA AAGGCGCTG TAAGCTGAAA CTCTGTGGCG TACTGGGGCT GGGCTGATG
CAGACTTTGT ACGCCCAAC AACTACTCGC CCCCAACATA TTTAGAGAAT TTCGCGCGAC ATTGCACTTT GAGACACCGC ATGACCCCGA CGCGGACTAC
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 AATGAAACAA AGTGGTGTCC CCCTGGTCAG CTGGTTAATC TGCACGACTT TAGGTCTGAC GAAATCGAGC
CTGCCGTGTA CCCACCGATA CGTCTGTTCC TACTTTGTT TCACCACAGG GGGACCAAGT GACCAATTAG ACGTGCTGAA ATCCAGACTG CTTTAGCTCG
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 ACCTTGTTGGT GGAGGAACCT GTGAAGAAC GCGAAGAGTG CCTGGACGCA CTTGAGAGTA TTATGACCAC CAAATCCGTT TCCTTCAGAA GACTGAGCCA
TGGAACACCA CTCCTTGAC CACTTCTTG CGCTTCTCAC GGACCTGCGT GAATCTCAT AATACTGGT GTTTAGGCAA AGGAAGTCTT CTGACTCGGT
Rabies-G protein

· L R K L V P G F 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 ATTTTCAACA AGACTCTTAT GGAGGCGGAT GCCCATTATA AGTCAGTTAG GACTTGAAT
GGACGCTTTC GACCAACGTC CCAAGCCCTT CCGAATATGA TAAAGTTGT TCTGAGAATA CCTCCGCTTA CGGGTAATAT TCAGTCAATC CTGACCTTA
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 F P N G I I L G P D G N ·

1501 GAGATAATTC CCTCAAAGG ATGTCTGAGA GTCGGTGGGA GATGCCACCC CAATGTCAAT GGGGTGTTCT TTAACGGAAT CATCCTGGGA CTGACGGGA
CTCTATTAG GGAGTTTCC TACAGACTCT CAGCCACCT CTACGGTGGG GGTACAGTTA CCCCACAAGA AATTGCCTTA GTAGGACCT GACTGCCT
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 ACGTGCTGAT TCCGAGATG CAATCTCCC TTCTGCAGCA ACACATGGAA CTCCTGGTGT CTTCACTGAT ACCCCTGATG CACCCACTGG CCGACCCAG
TGACAGACTA AGGGCTCTAC GTTAGAAGG AAGAAGTGT TGTGTACCT GAGGACCACA GAAGTCACTA TGGGGACTAC GTGGTGACC GGTGGGTC
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 CCTTCTAAT
GTGACACAAG TTTTACCAG TACTCCGCT TCTGAAACAC CTTCAAGTGG ACGGCTTACA 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 ACGTGCTCCT GAGTGCGGT GCCTTGACCG CTTGATGCT GATCATTTT CTGATGACCT GCTGGCGGAG GGTGAATCGC TCCGAGCCGA
ACCCCATTA TGCACGAGGA CTCACGCCA CGGAAGTGGC GAACTACGA CTAGTAAAAA GACTACTGGA CGACCGCTC CCACTTAGCG AGGCTCGCT
Rabies-G protein

· Q H N 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 ·

1901 CACAGCAAA TCTCAGAGG ACAGGCGGG AAGTAAGTGT GACTCCGCAA TCTGGCAAGA TTATTAGTAG TTGGGAGAGT TACAAGTCTG GAGGAGAGC
GTGTCGTGTT AGAGTCTCCC TGTCCGGCCC TTCATTACA CTGAGGCGTT AGACGTTCT AATAATCATC AACCTCTCA ATGTTGAGC CTCCTCTCTG
PMDV 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 TTTGATCTG TCAACTTGC AGGCGATGTA GAATCAATC CTGACCCGC CCGGACAGG TCCATAGCTC TCAGTTTCT CGCAGTTGA
ACCCAACTTA AAAGTAGAG AGTTTGAAG TCCGCTACAT CTAGTTTAG GACCTGGCG GGCCTGTCC 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 CTGGGTGTC CATAGACATC AGCCGCAAG AGCTGAGATG TGAAGTGA GTGTTCTATC
CTCAAGAGC AGAAGGAGAG GCACTTGAC GTGCGACTGT GACCCACAG GTATCTGTAG TCGGCGTTC TCGACTCTAC ACCTTCACT CACAAGTATG

Sequence Appendix 4 (continued)

PIV-WNV helper ΔNS1

5' UTR
C

1 AGTAGTTGCG CTGTGTGAGC TGACAAACTT AGTAGTGTTT GTGAGGATTA ACAACAATTA ACACAGTGG AGCTGTTTCT TAGCACGAAG ATCTGATGT
TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCAAAA CACTCCTAAT TGTGTGAAT TGTGTCAAGC TCGACAAAGA ATGCTGCTTC TAGAGCTACA
C

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 AGGAGGCCC GGCAGAGCC GGGCTGTCAA TATGCTAATA CCGGAATGC CCGCGTGT GTCTTGAAT GGACTTAAGA GGGCTATGTT
GATTCCTTGG TCCTCCCGGG CCGTCTCGG CCGGACAGTT ATAGCATTTT GCGCTTACG GGGCGACAA CAGGAATCT CCCGATACAA
C

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
GAGCTGATC GACGGCAAG GGCNAATAG ATTGTGTTG GTCTCTTGG CTTCTTTCAG GTTCACAGCA ATTGCTCCGA CCCGAGCAGT GCTGATCGA
CTCGGACTAG CTGCGGTCC CCGGTTATGC TAAACACAAC CGAGAGAAC GCAAGAGTC CAAGTGTGT TAACGAGGCT GGGCTCGTCA CGACCTAGCT
C

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 GAAGAACTA GGGACCTGA CCAGTGTCTAT CAATCGGCG AGCTCAAAAC
ACCTCTCCAC ACTGTTTGT TTGTGCGTAC TTGTGGAAG ACTCAAGTT CTCTCTTGT CCCTGGAAT GTTCAGGATA GTTAGCCGCC TCGAGTTTTS
Signal peptide

C

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
AAAGAAAG AGGAGGAAG ACCGAATG CAGTCATGAT TGGCTGATC GCGAGGTAG GAGCAGTAC CCTCTTAAC TTCCAAGGA AGGTGATGAT
TTTTCTTTC TCCTCCTTC TGGCCTTAA GTCACTACTA ACCGACTAG CCGTCCATC CTGCTCAATG GAGAGATTG AAGTTCCCT 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
GACGGTAAT GCTACTGACG TCACAGATGT CATCAGATT CCAACAGTG CTGAAGAA CCTATGCTT GTACAGCAA TGGATGTGG ATACATGTC
CTGCCATTTA CGATGACTG AGTGCTACA GTAGTCTAA GGTGTGAC GACCTTCTT GGATACGTA CAGTCTCGT ACCTACACCC TATGTACAG.
prM

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
GATGATCTA TCACTTATGA ATGCCAGTG CTGTGCGTG GTATGATCC AGAAGATC GACTGTGTT GTACAAAGTC AGCAGTCTAC GTCAAGTATG
CTACTATGAT AGTGAATACT TACGGGTAC GACAGCCGAC CATTAATAGG TCTTCTGTAG CTGACACCA CGTGTTCAG TCGTCAATG CAGTCCATAC
prM

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 CAGACACGC CACTCAAGAC CGAGTCGAG GTCACTGACA GTGACACAC ACCGAGAAG CACTCTAGCG AACAAGAGG GGGCTTGGAT
CTTCTACG GTTCTGTGCG GTGAGTTCTG CGTACGCTC CAGTCACTGT CAGCTCTTC TGCCTCTTC GTGAGATCGG 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 AAGGCGACAA GGTATTGGT AAAACAGAA TATGATCT TGAGAACCC TGGATATGCC CTGGTGGGAG CCGTCAATGG TTGGATGCTT
CCTGCTGGG TTCCGGTGT CCATAACCA TTTTGTCTT AGTACCTAGA ACTCTTGGG ACCTATACGG GACCACCGTC GACAGTAACC 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
GGAGACAA CAATGAGAG AGTTGTGTT CTGCTGCTAT TGTGTGGT GGCCTCAGT TACAGCTTA ACTGCTTGG AATGACAAAC AGAGACTTCT
CCCTCGTTGT GTTACGCTC TCACACAA CAGCAGATA ACAGAACCA CCGGGTGA ATGTGAAAT TGACGGAACT TTACTGTTG TCTCGAGA
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
TGAAGGAGT GTCTGAGCA ACATGGGTGG ATTGTGTTCT CGAGGCGAC AGTGGCTGA CTATCATGTC TAAGACAAAG CCTACCATCG ATGTGAAGAT
ACCTTCTCA CAGACTCGT TGTACCCACC TAAACNAGA GCTTCGCTG TCGAGCGACT GATGTACAG ATTCTGTGTC GATGTGAGC 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
GATGAATAG GAGGCGCCA ACCTGGCAGA GGTCCGCGAT TATGTAT TGGTACCGT CAGCATCTC TCACACCAAG CTGCTGSCC GGCCATGGGA
CTACTTATAC CTCCGCGGT TGGACCGTCT CCAGGCTCA ATACGATAA ACCGATGCA GTGCTAGAG AGTGTGTTT GACGACGGG CCGGTACCT
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
GAAGCTACA ATGACAAAG TGTGACCCA GCTTTGTGT GCAGACAGG AGTGTGAC AGGGCTGGG GCAACGGCTG CGGACTATT GGCAGAGAA
CTTCGAGTGT TACTGTGTC ACAGTGGGT CGAAACACA CTTCTGTCTC TCACCACCTG TCCCGACCC CGTGTCCGAC GCCTGATAA CCGTTTCTT
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
GATTCACAT ATGCGCAA TTGCTGCTCT CTACAGGC ATAGGAGA ACCATTGA AAGAAATAT GTGGCAATTT TTGTCCATGG
CGTAACTGT TACGGGTTT AAACGGACGA GATGTTCCG TTATCTCTCT TGTAATAAT TTCTCTATA GTTCAUGCTT CACCGGTAA 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
ACCACTACT GTGGAGTCG ACGGAACCTA CTCACACAG GTTGGAGCCA CTCAGGCGG GAGATTGAC ATCACTCTG CGGCGCTTC ATACACACTA
TGGTTGATGA CACCTCAGG TGCTTTGAT GAGGTGTGTC CAACTCGGT GAGTCCGTC CTCTAAGTC TAGTGAGAC CCGCGGAG TAGTGTGAT
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
AAGCTTGAG AATATGGAG GGTGACAGT GACTGTGAAC CAGGTCAGG GATTGACACC AATGCATCT ACCTGATGAC TGTGGAACA AAGACGTTCT
TTGGAACCTC TTATACCTT CCACTGTAC CTGACACTGT GTGCCAGTCC CTAAGTGTG TTACGTATGA TGCACTACTG ACACCTTGT 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 P
TGGTCCATCG TGAGTGGTTC ATGGACCTCA ACCTCCCTTG CAGCTGCTG TGTGGAGAA CAGAGACG TTAATGAGT TTGAGGAACC
ACGAGGTAGC ACTCACCAG TACCTGGAGT TGGAGGGAAC CTTGACGGA CTTTATGAC ACACCTCTT GTCTCTGCA 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
ACAGGCCAG AAGCAGTCTG TGATAGCAT GGGTCAACA GAGGAGCTC TGCATCAAGC TTGTGCTGA GCCATTCTG TGGATTTC AAGCAACAT
TGTGCGGTGC TTCGTCAGAC ACTATCGTAA CCGAGTGT TCCCTCGAG ACSTAGTTCG AAACGACCT CGSTAGGAC ACCTTAAAG TTGTTGTGA
E

1801 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
 GTCAAGTTGA CGTGGGTCA TTGAAGTGT AGAGTGAAGA TGAANAAT GCAGTTGAAG GGAACAACCT ATGGGTCTG TTCAARGCT TTCAGTTTC
 CAGTTCAACT GCAGCCAGT AAATTCACA TCTCACTTCT ACCTTTTAA CGTCAACTTC CCTTGTGGA TACCAGAC AGTTTCCGA AGTTCAAG
 E

1901 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
 TTGGACTCC CGCAGACACA GTTCACGCA CTGTGTGT GTGAATGAG TACACTGGCA CGGATGACC TTGCAAGTT CCTATCTGCT CAGTGGCTTC
 AACCTGAGG GCCTCTGT CTAGTCCGT GACACACAA CCTTACGTC AGTGAACCT GCTTACCTGG AACGTTCAA GGATAGACA GTACCCGAG
 E

2001 L N D L 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
 ATTGAAGAC CTAAGCCAG TGGCAGAT GTCACTGTC AACCTTTTG TTCACTGGC CAGGCCAC GCTAAGTCC TGATTAAT GGAACACCC
 TAACCTGCTG GATTCGCTC ACCGCTTAA CGATGACAG TTGGAAAC AAAGTCAAC GTCCGCTG CGATTCCAG ACTAATTA CTTTGTGG
 E

2101 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 F T T L
 TTGGAGACT CATACTAGT GGTGGGCGA GGAGACAC AGATCAATCA CCACTGGCAC AGTCTGGAA GCAGATGG CAAAGCCTT ACAACACCC
 AAACCTCTGA GTATGTATCA CCACCGTCT CCTCTGTG TCTAGTAGT GTGACCGTG TTCAGACCTT CGTCGTACC GTTTCGAAA TGTGTGGG
 E

2201 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
 TCAAGAGGC GCAGAGACTA GCGCTCTAG GAGACACAGC TTGGGACTTT GGATCAGTG GAGGGTGT CACCTCAGT GGAAGGCTG TCCATCACT
 AGTTCTCTG CGTCTCTGAT CGCGAGATC TCTGTGTG AACCTGAAA CCTAGTCAAC CCCCCACAA GTGGAGTCAA CCTTCCGAC AGTAGTTCA
 E

2301 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
 GTTCGAGGA CATTCCGCT CACTGTGAG AGGCATGTC TGATAGGC AAGATGCT GGGGCTCTC CTGTGTGGA TGGGATCAA TGTCTGTGAC
 CAAGCCTCT CATTAGGGA GTGACAAGC TCCGTACAG ACCTATTGCT TTCTAACA CCCCCGAG GACAACACT ACCGTAGT ACAGCACTG
 deleted NSI

E

2401 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
 AGGTCCATAG CTCTCAGT TCTCAGT GTGAGGATC TGCTCTCT CTCCGTGAC GTGACGCTG ACCTGGAT CCACCGTGA CCTGCCACTC
 TCCAGTATC GAGATGCAA AGAGCTCA CCTCTCAG ACAGAGGA GAGGACTTG CAGTGGAC TGTACCCCTA GTTGGACCT GGAAGGTGAG
 deleted NSI

2501 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
 GCACCACAC AGAGAGGGA AGTTGATTA CAGATTGTTG CTGAGGAGC TGCACCTTAC CACCACTGCT TACCAACT GACAGGGCT GTTGTATGG
 CGTGTGTTG TCTCTGCTT TCAACTAT GTCTAACCA GACCTCTCTG ACGTGGAATG GTGTGAGC GATGTTTGA CTGTGCGCA CAACCATACC
 deleted NSI

NS2A

2601 M E I R P Q R H D E K T L V Q S O V N A Y N A D M I D P F Q L G L
 TATGAGATC ACACACAGA GACTATGA AAGACCTC GTGAGTAC AGTGAATG TTATATGCT GATATGATG ACCCTTTCA GTTGGCCTT
 ATACCTTAG TCTGTGTCT CTGTACTACT TTCTGGGAG CAGCTCAGT TTCACTAGC ATATATACA CTATACTAC TGGGAAGT CAACCCGAA

Sequence Appendix 5

PIV-WNV(Δ prME)/RSV-F

	5' UTR	C protein	M S
1	AGTAGTTGCG CTGTGTGAGC TGACAAACTT AGTAGTTT GTGAGGATTA ACAACAATTA ACACAGTGG AGCTGTTTCT TAGCACAAG ATCTCGATGT TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCAGAAA CACTCTTAAT TGTGTTAAT TGTGTACAGC TCGACAAGA ATCGTCTTC TAGAGCTACA	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 A M L CTAAGAAACC AGGAGGCCCG GGCAGAGCC GGGCTGTCTA TTGTCTAATA CGCGAATGC CCGCGTGT GTCTTGTATT GGACTTAAGA GGGCTATGTT GATTCCTTGG TCCTCCGGG CCGTCTCGG CCGACAGAT AACAGATTTT GCGCCTTAGG GGGCGCACAA CAGGAATAA CTTGAATTTCT CCGATACAA	C protein	
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 GAGCTGATC GACGCAAGG GGCATATAG ATTGTGTTG GCTCTCTGG CGTCTTCAG GTTCACAGCA ATTGCTCCGA CCGAGCAGT GCTGGATCGA CTCGACTAG CTGCGTTC CCGTTATGC TAACACAAC CGAGAGAACC GCAAGATGC CAAGTGTCT TAAGGAGGCT GGGTCTGTC CGACCTAGCT	NS3 cleavage	
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 TGGAGAGTG TGAACAACA ACACGCGATG AACACCTTC TGATCTCAA GAGGAACCTA GGGACCTTGA CCAATGCTAT CAATCGCGG AGCTCAAGC ACCTCTCCAC ACTTGTGTTGT TTGTGCTAC TTGTGGAAG ACTCAAGTT CTCTCTTGAT CCGTGGACT GTTCACGATA GTTAGCCGCC TCGAGTTTCG	F signal	
401	NS3 cleavage 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 AAAAGAGCG AGGGGCGAG TTGCTAATCC TCNAGCAAA TGCATATACC ACAATCTCA CAGCAGTCA ATTTGTTT GCTTCTGGT AAAACATCAC TTTTCTTCGC TCCCGCGTC AACGATTAGG AGTTTCGTT ACGTTAAGG TGTTAGAGT GACGTCAGT TAAACAAAA CGAAGACCAG TTTTGTAGTG	F1	
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 TGAAGATTT TATCAATCAA CATGCACTGC AGTTAGCAAA GGTATCTTA GTGCTCTGAG AACTGTTGG TATACCACTG TATTAATAT AGAATTAAGT ACTTCTTAAA ATAGTTAGTT GTACGTCAGC TCAATCGTT CCGATAGAT CACGAGACTC TTGACCAACC ATATGCTAC ATATGATGATA TCTTAATTC	F1	
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 AATATAGTG TATGGAACA GATGCTAAG TAAATATGAT AAAACAAGAA TTAGATAAAT ATAAAAATGC TGTACAGAA TTGCAAGTGC TTATAGTCC TTTTATTCAC ATTACCTTGT CTACGATTC ATTAACTA TTTTGTCTT AATCTATTA TATTTTACG ACATGTTCT AACGTCACG	F1	
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 TCATGCAAG CACACACCA ACAACAATC GAGCGAAG AGAATACCA AGTTTATGA ATTATACAT CAACAATGCC AAAAACAACA ATGTAACATT AGTACGTTTC GTTGTGTTGT TGTGTTAG CTGCTCTTC TCTTGATGTT TCCAAATACT TAATATGTA GTTGTACGG TTTTGTGTT TACATTGTA	F1	

F2

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
AAGCAAGAA AGAARAGAA GATTTCTGG TTTTGTGTA GGTGTGGAT CTGATCCG CAGTGGGT GCTGATCTA AGTCTCTGA CCTAGAGGG
TTCTGTTCTT TCCTTTCTT CTRAAGAACC AAAAACAAT CCACACCTA GACGTAGG GTCCACGCA CGACATAGT TCCAGGAGGT 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
GARGNAAC AGATCAAG TGCTCTACTA TCCACAACA AGGTGTAGT CAGCTTATCA AATGGAGTTA GTGTCTTAC CAGCAAGTG TTAGACCTCA
CTTCACTTGT TCTAGTTTC ACGAGATGAT AGGTGTGTGT TCCGACATCA GTCGATAGT TTACCTCAAT CACGAATTG GTGTTTAC 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 TTGTACCTA TTGTGAACA GCAAGCTGC AGCATATCA ATATAGAAC TGTATAGAG TTCCAACAA AGAACAACAG
TTTTGATATA TCTATTGTT AACRATGGAT AACACTGTT CGTTTCAGG TCGTATGTT TATATCTTG ACATATCTC AAGTTTGT TCTTGTGTC
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 ATTACGAGG AATTAGTGT TAATGAGT GTACTACAC CTGTAGCAC TTACTGTTA ACTATAGTG AATTATGTC ATTATCAAT
TGATGATCTC TAATGTCCC TTAATCACA ATTACGTCCA CATGATGTG GACATTCGT AATGTACAAT TGAATATCAC TTAATACAG 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 K E E V
GATATGCCTA TAACAATGA TCAGAAAG TTAATGTCCA ACAATGTCA AATAGTTAGA CAGCAAGTT ACTCTATCAT GTCCATAATA AAAGAGGAG
CTATACGGAT ATGTCTTACT AGTCTTTTC AATTACAGT TGTACAGT TTATCAATCT GTCGTTTCAA TGATATAGTA CAGTATAT TTTCTCTTC
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 TGTAGTACAA TTACCACAT ATGTGTAT ATATACACCC TGTGGAAC TACACATC CCCTCTATGT ACAACACACA CAAGAAGG
AGAATCGTAT ACATCATGTT ANTGGTGATA TACCACATA TCTATGTGG ACAACCTTGT ATGTGTAG GGGAGATACA TGTGTGTGT GTTTCCTTC
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 TGTTHAACA GAATGACAG AGGATGGTAC TGTGCAATG CAGATCAGT ATCTTCTTC CCACAGCTG AACATGTAA AGTTCAATCA
CAGGTGTAG ACARATGTT CTGACTGTC TCTACCAG ACATGTATG GTCTTAGTCA TAGAAGAAG GGTGTGAC TTTGTACAT 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
AATCGATAT TTGTGACAC AATGAACAGT TTAACATTAC CAGTGAAT AAATCTGTC AATGTGACA TAITCAACCC CAAATATGAT TGTAAATTA
TTAGTCTATA AACACTGIG TTACTTGICA AATTGTAAT GTTCACTTA TTTAGAGAGG TTACAACGTG ATAGTTGGG GTTTTACTA ACATTTTAT
F2

1601 . T S K T D V 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
TGACTTCAA ACAGATGTA AGCAGTCCG TTATCAATC TCTAGAGCC ATGTGTCTAT GCTATGGCA AACTAATGT ACAGATCCA ATAAATCG
ACTGAAGTTT TTGTCTACAT TCGTGGAGC AATAGTGTAG AGATCTCTCG TAACACAGTA CGATACCGTT TGAATTTACA TGTGTAGGT TAITTTTAGC
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
TGGATCATA AAGACATTTT CTACGGGTG CGAATATGTA TCAATTAAG GGTGGACAC TGTGTCTGTA GGTAAACAT TATATATGT AATATAGCA
ACCTTAGTAT TTCTGTAATA GATTGCCAC GCTAATACAT AGTTATTTT CCTACTGTG ACACAGACAT CCATGTGTA ATATATACA 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
GAAGTAAA GTCTCTATGT AAGGTGAA CCAATAATA AATTCTATGA CCCATTAGTA TTCCCCTCTG ATGAATTTGA TGCATCAATA TCTCAGTCA
F2

CTTCCATTTT CAGAGATACA TTTTCCACIT GGTATTATT TAAGATACT GGGTAATCAT AAGGGGAGAC TACTTAACT ACCTAGTTAT AGAGTTCTAGT
F2

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
ACGAGAGAT TAACGAGAC CTAGCATTTA TTGCTAAATC CGATGAATTA TTACATPAG TAAATGCCRC TAAATCCACCT AAGCAAGAT
TGCTCTICTA ATGGTCTCG GATCGTAAT AAGCATTTAG GCTACTTAAT AATGTATTAC ATTACGACC ATTTAGGTGG TGTATTAGT ACTATTGATG
TM Domain

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 ATCATTAAIT GCITGTGAC TGCTCTTATA CTGTAGGCC AGAAGCACAC CAGTCACACT AAGCAAGAT
ATATTAAAT CACTAATATC ATTATAACAA TAGTAATTA CAGCAACCTG ACAGAAATAT GACATTCGG TCTTCGTGTC GTCAAGTGGA TTGCTTTCTA
FMDV 2A

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 P G P A R D R S
CAACTGAGTG GTATAAATAA TATTGCAITT AGTAACAAT TTGATCTGCT CAACCTGCA GGCATGTAG AATCAATCC TGGACCCGCC CGGGACAGGT
GTTGACTCAC CATATTATT ATACGTAAA TCATTGTTAA AACTAGACGA GTTTGAAGT CCGCTACATC TTAGTTAGG ACCTGGGCGG GCCCTGTCCA
NS1

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 GTGAAGTGC ACGCTGACAC TGGGTGTGCC ATAGACATCA GCCGGCAA
GGTATCGAGA GTGCAAGAG CGTCAACCTC CTCAGACGA GAAGGAGAGG CACTTGCACG TGCAGCTG ACCCACACGG TATCTGTAGT CGGCCGTT

PIV-WNV(Δ CprME)/RSV-F

1 ASGTAGTGC CTGTGTGAGC TGACAACTT AGTAGTGTIT GTAGAGNTTA ACAACATTA ACACAGTGG AGCTGTTTCT TAGCACGAG ATCTCGATGT
TCATCAAGCG GACACACTCG ACTGTTTGAA TCATCAAAA CACTCTTAAT TGTGTGTAAT TGTGTACGC TCGACAAGA ATCGTGTCTC TAGAGCTACA
deleted C protein

NS3 cleavage

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

deleted C protein

5' UTR

deleted C protein

pre E/NS1 signal

domain of WNV E (split)

Cytoplasmic Tail

Transmembrane domain of WNV E (split)

Transmembrane

101 CTAAGAAACC AGGAGGCC GGCAGAGCC GGGCTGTCAA TATGCTAAAA CGCGAATGC CCCGGTGT GTCTTGATT GGACTTAAGC AAAAGAAGCG
 GATTCCTTGG TCCTCCCGG CGTCTTCGG CCGACAGTT ATACGATTTT GCGCTTACG GGGCGACAA CAGGAATCA CCTGAATCG TTCTCTCGC

F signal

NS3 cleavage

F1

201 AGGGGGCAG TTGCTAATCC TCAAAGCAAA TGCAATTACC ACAATCTCA CTGCAGTCAC ATTTGTTTT GCTTCTGTC AAAACATCAC TGAAGAATTT
 TCCCCCGCTC AACGATTAGG AGTTTCGTTT ACGTTAATGG TGTTAGAGT GACGTCAAGT TAAACAAAA CGAAGACCAG TTITGTAGTG ACTTCTTARA

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 CATGAGTGC AGTAGCAAA GGCTATCTTA GTGCTCTGAG AACGTTGG TATACAGTG TTATACTAT AGAATTAAGT AATATCAAGG
 ATAGTTAGTT GTACGTACG TCAATCGTTT CCGATAGAT CAGGAGCTC TTGACCAACC ATATGTCAC ATATTGATA 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
 AAAATAGTG TAATGGAACA GATGCTAAGG TAAATTTGAT AAAACAAGAA TTAGATAAT ATAAAATGC TGTAAACAGAA TTGCAGTTGC TCATGCAAG
 TTTTATCAC ATTACCTTGT CTAGGATCC ATTTAACA TTTTGTCTT AATCTATTA TATTTTACG ACATTTGCTT 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 ACAACAATC GAGCCAGNAG AGAATACCA AGGTTATGA ATTATACAT CAACATGCC AAAAACAACCA ATGTAACTT AGCAAGAAA
 GTGTGTGTTT TGTGTTTGG CTGGTCTTC TCTGTATGT TCCAAATACT TAATATGTA GTTGTACGG TTTTGTGGT TACATTGTA TTGTTCTTT

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
 AGGAAGAA GATTCCTGG TTTTGTGA GTGTGTGAT CTGCAATGC CAGTGGCTT GCTGTATCA AGTCTCTCA CCTAGAAGG GAGTGAACA
 TCCTTTTCT CTAAAGAAC AAAAACAAT CCACACCTA GAGCTTACG GTACCGCA CGACATAGT TCCAGGAGT GGATCTCCC CTTCACCTGT

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
 AGATCAAG TGCTCTACTA TCCACAACA AGGCTGTAGT CAGCTTATCA AATGGAGTTA GTGTCTAAC CAGCAAGTG TTAGACCTCA AAAACTATAT
 TCTAGTTTC ACAGATGAT AGTGTGTTGT TCCGACATCA GTCGATAGT TTACTCAAT CACAGATTS GTGTTTCAC AATCTGGAGT TTTGTATATA

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
 AGATAACAA TTGTTACCTA TTGTGAACA GCAAGCTGC AGCATATCAA ATATAGAAC TGTGATAGG TTCCAACAAA AGAACACAG ACTACTAGAG
 TCTATTGTT AACATGGAT AACACTTGT CGTTTCGAG TCGTATAGT TATATCTTG ACATATCTC AAGTGTGTT TCTTGTGTC 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
 ATTACCAGG AATTAGTGT TAATGAGGT GTAATACAC CTGTAGCAC TTACATGTTA ACTAATAGT AATTAATGTC AATTAATCAAT GATATGCTTA
 TAATGGTCCC TTAATCACA ATTACGTCCA CATTGATGT GACATTCGT AATCTACAT TGATATCAC TTAATAACAG TAATTAGTTA CTATACGAT

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
 TAACAATGA TCAGAAAG TTAATGTCCA ACATGTTCA AATAGTTAGA CAGCAAGTT ACTCTATCAT GTCCATAATA AAGAGGAG TCCTAGCATA
 ATTGTTTACT AGTCTTTTC AATTACAGT TGTTACAAGT TTATCAATCT GTGTTTCAA TGAGATAGTA CAGGTATAT TTCTCTTC 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 TGTGGAAAC TACACACATC CCCTCTATGT ACAACCAACA CAAAAGAAGG GTCCAACATC
ACATCATGTT AATGGTGATA TACCACAATA TCTATGGGG ACACCTTTG ATGTGTGTAG GGGAGATACA TGTGGTGTGT GTTTCTTCC CAGGTGTAG
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
TGTGTAACA GAACTGACAG AGGATGGTAC TGTGACAATG CAGATCAGT ATCTTTCTC CCACAGCTG AAACATGTAA AGTTCNATCA AATCGAGTAT
ACRAATGTT CTTGACTGTC TCCTACCATG ACACGTGTAC GTCCGAGCA TAGAAGAAG GGTGTTCCAG TTTGACNT 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
TTTGTGACG AATGAACAGT TTAACATAC CAAGTGAAT AATCTCTGC AATGTGACA TATCAACCC CAAATAGAT TGTAAATTA TGACTTCAAA
AAACACTGCG TTACTTGTCA AATCTGAATG GTTCACITTA TTAGAGAGG TTACACAGT ATAAAGTTGG GTTTTACTA ACATTTTAAT ACTGAAGTTT
F2

1401 T D V 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
AACAGATGA AGCAGCTCCG TTATCACAATC TCTAGGAGCC ATTGTGTAT GCTATGGCAA AACTAAATGT ACAGATCCA ATAAATATCG TGAATCATA
TTGTCTACAT TCGTCGAGGC AATAGTGTAG AGATCCTCGG TACACAGTA CGATACCGTT TTGATTTTACA TGTCTAGGT TATTTTAGC 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 TCAAAATAAG GGATGGACAC TGTGTCTGTA GGTAAACAT TATATTATGT AATAAGCAA GAAGGTAAAA
TTCTGTAAA GATGCCCCAC GCTAATACAT AGTTTATTC CCTACTCTG ACACAGACAT CCAATGTGTA 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 ARAAGTGAA CCAATATTA ATTCTATGA CCAATATGA TTCCCTCTG ATGAATTTGA TGCATCAATA TCTCAAGTCA ACAGAAAGAT
CAGAGATACA TTTTCCACTT GATTATTAAT TAAAGATACT GGTAATCAT AAGGGAGAC TACTTAACT ACGTAGTTAT AGATTTTCA GTCTCTCTA
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
TAACAGAGC CTAGCAITTA TCGTAATATC CGATGAATTA TTACATATG TAAATGCTGG TAAATCCACC ACAATATCA TGATAACTAC TATATTATA
ATTGGTCTCG GATCGTAAT AAGCATTTAG GCTACTTAAT AATGTATTAAC ATTAGGACC ATTAGGTGG TGTATTATGT ACTATTGATG ATATTAAAT
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 TAATATTGTT ATCATTAAT GTGTGTGGAC TGTCTTATA CTGTAGGCC AGAAGCACAC CAGTACACT AAGCAAGAT CAATCGAGTG
CACTAATATC ATTATACAA TGTATATTA CGACACCTG ACAGAGATAT GACATTCGG TCTTCGTGTG GTCAAGTGA TTGTTTCTA GTTGACTCAC
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
GTATAATTA TATGCTATTT AGTAACATTT TGTATGCT CAACITGCA GGGATGTAG AATCAATCC TGGACCCGCC CGGACAGGT CCATAGCTCT
CATATTATTT ATACAGTAAA TCAATGTTAA AACTAGCAGA GTTTGAAGT CGCTACATC 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
 CACGTTCTC GCAGTGGAG GAGTCTGCT CTTCCTCTCC GTGAACGTGC ACGTGCAC ACCTGATGCGGCAAGA GCTGAGA
 GTGCAAGAG CGTCAACCTC CTCAGACGA GAAGGAGAG CACTTCACG TGCAGTGTG ACCACACGG TATCTGTAGT CGGCCGTTCT CGACTCT

PIV-WNV(Δ C)/RSV-F

1 GATCCTAATA CGACTCACTA TAGAGTAGTT CGCTGTGTG AGCTGACAAA CTTAGTAGTG TTGTGAGGA TTAACAACAA TTAACACAGT GCGAGCTGTT
 CTAGGATTAT GCTGAGTGAT ATCTCATCA GCGACACAC TCGACTGTIT GAATCATCAC AAACACTCTT ANTTGTGTT AATTGTGTC ACGTCGACAA
 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 ACCAGGAGG CCGGCAAGA GCCGGGTGT CAATATGCTA AAACGGGAA TGCCTGCTGT
 AGAATCGTGC TTCTAGAGCT ACAGATTCTT TGGTCTCCC GGGCCGTTCT CGGCCGACA GTTATACGAT TTTCGCCCTT ACGGGCGCA CAACAGGAAC
 N-terminus of C
 P signal

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 GCGAGGGGC GAGTTGTAA TCTCAAGC AAATCAAT ACCCAATCC TCACTGAGT CACATTTGT TTGCTCTGT
 TRACCTGAT TCGTTTCTT CGTCCCGC CTCAAGAT AGAGTTTCTG TTTAGTTAA TGGTGTAGG AGTGCATCA GTGTAAACA AAACGAAGAC
 F1

301 Q N I T 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
 GTCAAACAT CACTGAGAA TTTATCAAT CAACATGAG TCGATGAGC AAGGCTATC TTAGTCTCT GAGACTGTG TGGTATACCA GTGTATAAC
 CAGTTTGTG GTGACTTCTT AAAATAGTTA GTGTAGCTG ACGTCAATCG TTTCGATAG AATCAGAGA CTCTTGACCA ACCATATGTT CACATATTTG
 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 AGGAAATAA GTGTATGGA ACAGATGCTA AGTAAAT GATAAACA GAATAGATA AATATAAAA TGCTGTACA
 ATATCTTAAT TCATTATAGT TCCTTTATT CACATTACCT TGCTACGAT TCCATTTTAA CTATTTTGT CTTAATCTAT TTATATTTT 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
 GAATGAGT TGCTCATGCA AAGCACACCA CCAACAAACA ATCGAGCAG AAGAGAATA CCAAGTTTA TGAATATAC ACTCAACAAT GCCAAATAAA
 CTTAAGCTCA ACGAGTACGT TTGCTGTGTT GGTGTGTTGT TAGCTCGTC TTCTTTGAT GGTTCRAAT ACTTAATATG TGAGTGTGA CGGTTTTTTT
 F2

601 N V T L S K K R 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 ATTAGCAAG AAAGGAAAA GAAGTTTCT TGGTTTTG TTAGTGTG GATCTCAAT CGCAGTGGC GTTGTGTAT CTAAGGTCTT
 GGTACATG TANITCGTTC TTTCTCTTTT CTCTAAGA ACCAAATAAC AATCCACAAC CTAGAGTTA GCGGTCCCG CAACACATA GATTCACGA
 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 GGGAGAGTGA ACAGATCAAA AAGTCTCTA CTATCCAAA ACAAGGTGT AGTACGCTA TCAATGAG TTAGTGTCTT AACGACAAA
 CGTGGATCTT CCCCTTCACT TGTCTAGT TTACAGAT GATAGTGT TTGTCGACA TCAGTCAAT AGTTTACCTC AATCAGAAA TTGTCGTTT
 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 TCAAAAACATA TATAGATAAA CAAATGTTTAC CTATGTTGAA CAAAGCAAGC TGCAGCATAT CAAATATAGA AACTGTGATA GAGTTCACAC
CACAATCTGG AGTTTTGAT ATATCTATTT GTTAACAATG GATACACATT GTTCGGTTCC AGCTCGTATA GTTTATATCT TTGACACTAT CTCAGGTTG
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
AAAAGACAA CAGACTACTA GAGATTACCA GGAATTTAG TGTAAATGA GGTGTAACCA CACTGTAAAG CACTTACATG TTAACATATA GIGRAATTAT
TTTTCTGTT GTCGTATGAT CTCTAATGGT CCCTTAATC ACAATTAGT CCACATTGAT GTGACATTC 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
GTCAATTAATC AATGATAGC CTATACAAA AGTTAAATG CCAACATGT TCAATAGTT AGACAGCAAA GTTACTCTAT CATGTCCATA
CAGTAATTAG TTAATATAGC GATATGTTT ACTAGTCTTT TTAATATGA GGTGTTTACA AGTTATCAA TGTGTGTTT CAAATGAGATA GTACAGGTAT
F2

1101 I K 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 AATGTAGTA CAATTACCAC TATATGGTGT TATAGATACA CCGTGTGGA AACTACACAC ATCCCTCTTA TGTACAACCA
TATTTCTCC TTCAGATCG 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 P Q A E T C
ACACAAAGA AGGTCCCAAC ATCTGTTTAA CAGAATCGA CAGAGGATG TACTGTGACA ATGAGGATC AGTATCTTTC TTCCCAACAAG CTGAACACATG
TGTGTTTCT TCCAGGTG TAGACAAAT GTTCTTGACT GTCTCTACCC ATGACACTGT TACGTCTTAG TCATAGAAAG AAGGTGTTC GACTTGTAC
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
TAAAGTCAA TCAATCAG TATTTGTGA CACAATGAC AGTTAACTT TACCAGTGA AATTAATCTC TGCATGTTC ACATATTCAA CCCCAATAT
ATTCAAGTT AGTTAGTCTC ATAAACACT GTGTTACTTG TCAATTTGA ATGGTTCACT TTATTAGAG AGTTACAAC TGTATAAGTT GGGGTTTATA
F2

1401 D C K I M T S K T D V S S V I T S L G A I V S C Y G K T K C T A S
GATTGTAAA TTATGACTTC AAAACAGAT GTAAAGCAGT CCGTTATCAC ATCTTAGGA GCCATTGTT CATGCTATGG CAAACTAAA TGTACAGCAT
CTAACATTT AATACTGAG TTTTGTCTA CATTCGTGA GGCATAGT TAGAGATCT CCGTAACACA 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
CCAATAAAA TCGTGGATC ATAAAGACAT TTCTACCG GTGCGATTAT GTATCAATA AAGGATGGA CACTGTGTCT GTAGTAACA CATTATATTA
GGTTATTTT AGCACCTTAG TATTTCTGTA AAAGATTGCC CACGCTATA CATAGTTTAT TTCCCTACCT GTGACACAGA CATCCATTGT GTAATATAAT
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
TGTAAATAG CAAAGAGTA AAGTCTCTA TGTAAAGGT GAACCAATAA TAAATTTCTA TGACCCATA GTATTCCTCT CTGATGANT TGTATCATCA
ACATTTATTC GTTCTCCAT TTTCAGAGAT ACATTTTCCA CTGGTTTAT ATTAAAGAT ACTGGTAAAT CATAGGGA 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 TCAACAGNA GATTAACAG AGCTAGCAT TTATTCGTAA ATCCGATGA TTAATACATA ATGTAATGC TGTAAATCC ACCCAATA
TATAGATTC AGTTGCTCTT CTAATGGCTA TCGGATCGTA AATAAGCAT TAGGCTACTT AATAATGAT TACATTACG ACCATTAGG TGGTGTAT
TM Domain
Cytosolic 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 TACTATAAAT ATAGTGATTA TAGTAATAAT GTTATCANTA ATTGCTGTTG GACTGCTCTT ATACTGTAAG GCCAGAAGCA CACCAGTCAC
AGTACTATTG ATCATATTAA TATCACTAAT ATCAATTATAA CAATAGTAAT TAACGACAAC CTGACAGAA TATGACAATC CGGTCTTCGT GTGGTCAGTG
FNDV 2A

1901 . 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
ACTAAGCAAA GATCAACTGA GTGGTATAAA TAATATTGCA TTTAGTAAACA ATTGTGATCT GCTCAAACTT GCAGCGGATG TAGAATCAAA TCCTGGACCC
TGATTGCTTT CTAGTTGACT CACCAATATT ATTAATACGT AAATCAATGT TAAACTAGA CGAGTTTGAA CGTCGCTAC ATCTTAGTTT AGGACCTGGG
prM

2001 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
GGAGGAAGA CGGTATTGC AGTCATGAT GGCCTGATCG CCGCGGATG AGCACTTACC CTCTCTAACT TCCAGGGA GGTGATGATG ACGGTAATG
CCTCCTTTCT GGCCTAAGC TCAGTACTAA CCGGACTAGC GAGCGCATCC TCGTCAATGG GAGAGATTGA AGGTCCCTT CCACTACTAC TGCCATTAC
prM

2101 . 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 D D T I
CTACTGACGT CACAGATGC ATCAGATTC CAACAGCTGC TGGAAAGAAC CTATGCAATG TCAGAGCAAT GGATGTGGA TACATGTGCG ATGATACTAT
GATGACTGCA GTGTCTACAG TAGTGCTAAG GTTGCTGACG ACCTTCTTG GATAGTAAC AGTCTCTGTA CCTACACCT ATGTACACGC TACTATGATA
prM

2201 . 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
CACATTAGAA TGCCAGTGC TGTGCGTGG TAATGATCCA GAAGACATCG ACTGTGTGG CACAAGTCA GCAGTCTAG TCAGGTATGG AAGATGCACC
GTGAATACTT ACGGGTCACG ACAGCCGACC ATTACTAGGT CTCTGTGAGC TGACAACCAC GTGTTTCAGT CGTCAGATGC AGTCCATACC TTCTACGTGG
prM

2301 K T R H S R R S 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
AAGACAGCC ACTCAAGACG CAGTCGGAGG TCAGTACAG TGAGACACA CGGAAGAAGC ACTTAGCGA ACAGAAGGG GGTCTGGATG GACAGCACCA
TTCTGTGCGG TGAGTTCTGC GTACGCCCTC AGTACTGTC ACGTCTGRT GCCTCTTTCG TGAGATCGCT TGTTCTTCCC CGGAACCTAC CTGTGCTGCT
prM

2401 . 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
AGGCACAAG GTATTGGTA AAGACAGAAAT CATGGATCTT GAGGAACCTT GGATATGCC TGGTGGCAGC CGTCAATGTT TGGATGCTTG GGAGCAACAC
TCCGGTGTTC CATRAACCAT TTTGTCTTA GTACCTAGAA CTCTTGGA CCTATACGG ACCACCGTGG GCAGTAACCA ACCTACGAAC CCTCGTTGTG
prM

2501 . M Q R V V F 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
CATCAGAGA GTTGTTGTTG TCGTGTATT GCTTTTGGTG GCCCAGCTT ACAGCTTTAA CTGCTTGA ATGAGCAACA GAGACTTCTT GGAAGGAGTG
GTACGCTCT CAACACAAC AGCAGATAA CGAAACCAAC CCGGGTCGAA TGTGGAATT GAGGGAACCT TACTGTTGT CTCTGAAGA CCTTCCTCAC
prM

2601 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
TCTGGAGCA CATGGTGA TTTGTTCTC GAAGGACA GTCGCTGAC TATCATGCTT AAGGACAGC CTACATCGA TGTGAGATG ATGATATGG
AGACTCGTT GTACCCACCT AAACCAAGC CTTCGCTGT CGAGCAGCT ATAGTACAGA TTCTGTGTTG GATGTAGCT ACACCTTCTAC TACTATACC
E

2701 . 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
AGCGGCCAA CCTGGCAGAG GTCCGAGTT ATTGCTATT GGTACGCTC AGCAGTCTT CCAACCAAGC TGCCTGCCG GCGATGGAG AAGCTCACAA
TCCGCCGTT GACCCGTCTC CAGCGTCA TAACGATAA CCGATGACG TCGCTAGAGA GGTGGTTTGG ACGCAGGCG CGGTACCCCTC TTCGAGTGT
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
TGACAACTG GTGACCCAG CTTTGTGTG CAGACAGGA GTGTGGACA GGGGTGGG CACGGTGC GGACTATTG GCAAAGGAG CATTGACACA
ACTGTTTGA CGACTGGTC GAAACACAC GTCTGTTCCT CACCACCTGT CCCCAGCCCC GTTGCCGAGC CCGTATTAAC CGTTTCCTTC GTACTGTGT
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 TTGCTTGTCT TACCAAGCA ATAGGAAGAA CCAATTTGAA AGAGATATC AGTACGAAG TGGCCATTTT TGTCATGGA CCAACTACTG
ACGGGTTTA AAGGAGCAG ATGGTTCCGT TATCCTTCTT GGTAATACTT TCTCTTAG TTCTATGTTT ACCGTAATAA ACAGGTACCT GGTTGATGAC
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 CGGAACACTAC TCCACACAGG TTGGAGCCAC TCAGGAGGG AGATTGAGA TCACTCTGCG GCGGCTTCA TACACACTAA AGCTTGGAGA
ACCTCAGCGT GCCTTTGATG AGGTGTGTC AACCTCGGIG AGTCGGTCCC TCTAAGTCTG ASTGAGGAGC CCGGGAAGT ATGTGTGATT TCGAACCTCT
E

3101 . 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 V H R
ATATGGAGG GTGACAGTGG ACTGTGAACC ACGGTGAGG ATTGACACCA ATGCATACTA CGTGATGACT GTTGGAAACAA AGACGTTCTT GGTCCATCGT
TATACCTCTC CACTGTCAAC TGACACTTGG TGCCAGTCCC TAAGTGTGT TACGTATGAT GCACTACTGA CAACCTGT TCTGCAAGAA CCAGTAGCA
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
GAGTGGTTCA TGGACCTCAA CCTCCCTTGG AGCAGTGTG GAAGTACTGT GTGGAGGAAC AGAGAGACGT TAATGGAGT TGAGGAACCA CACGCCACGA
CTCACCAAGT ACCTGGAGT GGAGGGAACC TGCTCAGGAC CTTCAAGACA CACCTCCITG TCTCTGCA ATTACTCAA ACTCTTGGT 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 GATAGCATG GGCTCACAG AGGAGCTCT GCATCAGCT TTGGTGGAG CCAATCTGT GGAATTTTCA AGCAACACTG TCAAGTTGAC
TCGTCAGACA CTATCGTAAC CCGAGTGTTC TCCTCGAGA CGTAGTCTGA AACCGACCTC GGTAGGACA CCTTAAGT TCGTTGAGC 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
GTCGGTCAAT TTGAAGTGA GAGTGAAGAT GGAAATG CAGTTGAAG GAACAACCTA TGGCGTCTGT TCAAGGCTT TCAAGTTCT TGGGACTCC
CAGCCCACTA AACTTCAT CTCACTTCTA CCTTTTAACT GTCAACTTCC CTTGTGTGAT ACGGACAGA AGTTCCGAA AGTTCAAGA 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
GCAGACAG GTACCGGCAC TGTGTGTGTA GAATTCAGT ACATGGAC GTATGGACT TGCAAAGTTC CTATCTGTC AGTGGCTTCA TTGACGACC
CGTCTGTGTC CAGTGGCGTG ACACCACAAC CTTAAGCTCA TGTACCGTG CCTACTGGA AGTTTCAAG AGTTTCAAG TCAACCAAGT AACTTGTGCTG
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 F G D S
TAAGCCAGT GGGCAGATTG GTCACTGTCA ACCCTTTTGT TTGAGTGGCC ACGGCCAAGS CTAAGGTCTT GAATGAATG GAACCACTT TGGAGACTC
ATTGCGGTCA CCGTCTAAC CAGTGACAGT TGGGAACA AACTCACCGG TGCCGTTTC GATTCCAGGA CTAACCTAAC CTGTTGGGA AACCTCTGAG
E

3701 . Y I V V G R G E Q Q I N H H W H K S G S I G K A F T T T L K G A
ATACATAGT GTGGCAGAG GAGACACA GATCAATCAC CATGTGACA AGTCTGGAAG CAGCATTTGG AAGCTTTA CAACCACTT CAAGGAGCG
TAATATATCAC CACCCGTCTC CTCTGTGT CTAGTTAGT GTGACCTGT TCAAGCTTTC GTGTAACCG TTTCGGAAT GTTGGTGGGA GTTTCTCTGC
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
CAGAGCTAG CCGCTCTAGG AGACACAGCT TGGGAGTTG GATCAGTTG AGGGGTCTC ACTCAGTT GGAAGGTGT CCATCAGT TCGGAGGAG
GTCTCTGATC GCGAGATCC TCTGTGTGTA ACCCTGAAC CTAGTCAACC TCCCACAG TGGAGTCAAC GGTGATTCAC AAGCTCTCTC

E

3901 . . 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 .
CAITCCGCTC ACTGTTGGA GGCATGTCCT GGATAACGCA AGGATTGCTG GGGCTCTCC TGTGTGGAT GGGCATCAAT GCTCGTAGCA GGTCCATAGC
GTAAGGCGAG TGACAAACCT CCGTACAGCA CCTATTGCGT TCCTAACGAC CCGGAGAGG ACAACACCTA CCGTAGTTA CGAGCACTGT CCAGGTATCG

NSI

E

4001 . . 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
TCTCACGTTT CTCGAGTTG GAGGAGTTCT GCTCTTCTC TCGTGAACG TGCAGCTGA CACTGGTGT GCCATAGACA TCAGCGGCA AGAGCTGAGA
AGAGTGCAA GAGCGTCAC CTCTCAGA CGAGAGGAG AGGCACTGC AGTGGGACT GTGACCCACA CGGTATCTGT AGTGGCGGT TCTCGACTCT

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 flavivirus genome comprising (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 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5), and (ii) a sequence encoding one or more heterologous pathogen, cancer, or allergy-related immunogens.

2. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said one or more deletions or mutations is within capsid (C) sequences of the flavivirus genome.

3. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said one or more deletions or mutations is within pre-membrane (prM) and/or envelope (E) sequences of the flavivirus genome.

4. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said one or more deletions or mutations is within capsid (C), pre-membrane (prM), and envelope (E) sequences of the flavivirus genome.

5. The replication-deficient pseudoinfectious flavivirus of claim 1, wherein said one or more deletions or mutations is within non-structural protein 1 (NS1) sequences of the flavivirus genome.

6. The replication-deficient pseudoinfectious flavivirus of any of claims 1-5, wherein said heterologous immunogen is from a pathogen selected from the group consisting of a rabies virus, *Borrelia burgdorferi*, a tick, an influenza virus, a human immunodeficiency virus, a simian immunodeficiency virus, a human papilloma virus, a respiratory syncytial virus, malaria parasite, and *Mycobacterium tuberculosis*.

7. The replication-deficient pseudoinfectious flavivirus of claim 6, wherein said heterologous immunogen comprises a rabies virus G protein immunogen or an immunogenic fragment thereof.

8. The replication-deficient pseudoinfectious flavivirus of claim 6, wherein said heterologous immunogen comprises *Borrelia burgdorferi* OspA immunogen or an immunogenic fragment thereof.

9. The replication-deficient pseudoinfectious flavivirus of claim 6, wherein said heterologous immunogen comprises a tick saliva protein selected from the group consisting of 64TRP, Isac, and Salp20, or an immunogenic fragment thereof.

10. The replication-deficient pseudoinfectious flavivirus of claim 6, wherein said heterologous immunogen comprises an influenza virus M2, hemagglutinin (HA), or neuraminidase (NA) epitope, or an immunogenic fragment thereof.

11. The replication-deficient pseudoinfectious flavivirus of claim 6, wherein said heterologous immunogen comprises a codon-optimized HIV gag, tat/nef, or gp120 protein, or an immunogenic fragment thereof.

12. The replication-deficient pseudoinfectious flavivirus of claim 6, wherein said heterologous immunogen comprises an HPV16 or HPV18 capsid protein L1 or L2, or an immunogenic fragment thereof.

13. The replication-deficient pseudoinfectious flavivirus of claim 6, wherein said heterologous immunogen comprises a respiratory syncytial virus F or G glycoprotein.

14. The replication-deficient pseudoinfectious flavivirus of any of claims 1-13, wherein said flavivirus genome comprises sequences encoding a pre-membrane (prM) and/or envelope (E) protein.

15. The replication-deficient pseudoinfectious flavivirus of any of claims 1-14, wherein the flavivirus genome is selected from that of yellow fever virus, West Nile virus, tick-borne encephalitis virus, Langat virus, Japanese encephalitis virus, dengue virus, and St. Louis encephalitis virus sequences, and chimeras thereof.

16. The replication-deficient pseudoinfectious flavivirus of claim 15, wherein said chimera comprises pre-membrane (prM) and envelope (E) sequences of a first flavivirus, and capsid (C) and non-structural sequences of a second, different flavivirus.

17. The replication-deficient pseudoinfectious flavivirus of claim 16, wherein said first flavivirus is a tick-borne encephalitis virus or a Langat virus.

18. The replication-deficient pseudoinfectious flavivirus of claim 16 or 17, wherein said second, different flavivirus is a yellow fever virus or a West Nile virus or Langat virus.

19. The replication-deficient pseudoinfectious flavivirus of any of claims 1-18, wherein said genome is packaged in a particle comprising pre-membrane (prM) and envelope (E) sequences from a flavivirus that is the same or different from that of the genome.

20. The replication-deficient pseudoinfectious flavivirus of any of claims 1-19, wherein sequences encoding said heterologous immunogen are inserted in the place of or in combination with the one or more deletions or mutations of the one or more proteins.

21. The replication-deficient pseudoinfectious flavivirus of any of claims 1-20, wherein sequences encoding said heterologous immunogen are inserted in the flavivirus genome within sequences encoding the envelope (E) protein, within sequences 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), or as a bicistronic insertion in the 3' untranslated region of the flavivirus genome.

22. A composition comprising a first replication-deficient pseudoinfectious flavivirus of any of claims 1-21 and a second, different replication-deficient pseudoinfectious flavivirus comprising a genome comprising 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 one or more proteins encoded by the sequences in which the one or more 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 one or more deletion(s) or mutation(s) occur in the first replication-deficient pseudoinfectious flavivirus.

23. A method of inducing an immune response to an immunogen in a subject, the method comprising administering to the subject one or more replication-deficient pseudoinfectious flaviviruses of any of claims 1-21 and/or a composition of claim 22 to the subject.

24. The method of claim 23, wherein the subject is at risk of but does not have an infection by said pathogen or a disease or condition associated with said cancer or allergy-related immunogen.

25. The method of claim 23, wherein the subject has an infection by said pathogen or a disease or condition associated with said cancer or allergy-related immunogen.

26. The method of any of claims 23-25, wherein the immunogen is from a pathogen selected from the group consisting of a rabies virus, *Borrelia burgdorferi*, a tick, an influenza virus, a human immunodeficiency virus, a simian immunodeficiency virus, a human papilloma virus, a respiratory syncytial virus, malaria parasite, and *Mycobacterium tuberculosis*.

27. The method of any of claims 23-26, wherein the method is for inducing an immune response against a protein encoded by the flavivirus genome, in addition to the source of the immunogen.

28. The method of claim 27, wherein the subject is at risk of but does not have an infection by the flavivirus corresponding to the genome of the pseudoinfectious flavivirus, which comprises sequences encoding a flavivirus pre-membrane and/or envelope protein.

29. The method of claim 27, wherein the subject has an infection by the flavivirus corresponding to the genome of the pseudoinfectious flavivirus which comprises sequences encoding a flavivirus pre-membrane and/or envelope protein.

30. A live, attenuated chimeric flavivirus comprising 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 comprises a hybrid of yellow fever virus and tick-borne encephalitis or Langat virus capsid/pre-membrane signal sequences, or a variant thereof.

31. The live, attenuated chimeric flavivirus of claim 30, wherein the capsid/pre-membrane signal sequence of the chimeric flavivirus comprises yellow fever virus

sequences in the amino terminal region and tick-borne encephalitis or Langat virus sequences in the carboxy terminal region.

32. A live, attenuated chimeric flavivirus comprising 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 comprises a tick-borne encephalitis or a Langat virus capsid/pre-membrane signal sequence, or a variant thereof.

33. A pharmaceutical composition comprising a pseudoinfectious flavivirus of any of claims 1-21, the composition of claim 22, or the live, attenuated flavivirus of any of claims 30-32 and a pharmaceutically acceptable carrier or diluent.

34. The pharmaceutical composition of claim 33, further comprising an adjuvant.

35. A replication-deficient pseudoinfectious flavivirus comprising a flavivirus genome comprising one or more deletions or mutations in nucleotide sequences encoding non-structural protein 1 (NS1), non-structural protein 3 (NS3), or non-structural protein 5 (NS5).

36. A nucleic acid molecule corresponding to the genome of a pseudoinfectious flavivirus of any of claims 1-21 or 35, or the genome the live, attenuated flavivirus of any of claims 30-32.

37. A method of making a replication-deficient pseudoinfectious flavivirus of any of claims 1-21 or 35, the method comprising introducing a nucleic acid molecule of claim 36 into a cell that expresses the protein corresponding to any sequences deleted from the flavivirus genome of the replication-deficient pseudoinfectious flavivirus.

38. The method of claim 37, wherein the protein is expressed in the cell from the genome of a second, different, replication-deficient pseudoinfectious flavivirus.

39. The method of claim 37, wherein the protein is expressed from a replicon.

40. The method of claim 39, wherein the replicon is an alphavirus replicon.

41. The method of claim 40, wherein the alphavirus is a Venezuelan Equine Encephalitis virus.

42. A composition comprising two or more replication-deficient pseudoinfectious flaviviruses, wherein two of the replication-deficient pseudoinfectious flaviviruses are selected from the groups consisting of:

(a) a replication-deficient pseudoinfectious flavivirus comprising a genome comprising Japanese encephalitis virus sequences, and a replication-deficient pseudoinfectious flavivirus comprising a genome comprising dengue virus sequences;

(b) a replication-deficient pseudoinfectious flavivirus comprising a genome comprising yellow fever virus sequences, and a replication-deficient pseudoinfectious flavivirus comprising a genome comprising dengue virus sequences; and

(c) a replication-deficient pseudoinfectious flavivirus comprising a genome comprising tick-borne encephalitis or Langat virus sequences and an inserted sequence encoding a *Borrelia burgdorferi* immunogen, and a replication-deficient pseudoinfectious flavivirus comprising a genome comprising tick-borne encephalitis or Langat virus sequences and an inserted sequence encoding a tick saliva protein immunogen, or a replication-deficient pseudoinfectious flavivirus comprising a genome comprising tick-borne encephalitis or Langat virus sequences and inserted sequences encoding a *Borrelia burgdorferi* immunogen and a tick saliva protein immunogen.

43. A pharmaceutical composition comprising the live, attenuated chimeric flavivirus of any of claims 30-32.

44. A method of inducing an immune response to tick-borne encephalitis virus or Langat virus in a subject, the method comprising administering to the subject the pharmaceutical composition of claim 43.

45. The method of claim 44, wherein the subject does not have but is at risk of developing infection by tick-borne encephalitis virus or Langat virus.

46. The method of claim 44, wherein the subject is infected with tick-borne encephalitis virus or Langat virus.

47. A replication-deficient pseudoinfectious flavivirus comprising a flavivirus genome comprising 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 comprises 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 comprise a hybrid of sequences encoding yellow fever virus and tick-borne encephalitis or Langat virus capsid/pre-membrane signal sequences, or a variant thereof.

48. The replication-deficient pseudoinfectious flavivirus of claim 47, wherein the sequences encoding the capsid/pre-membrane signal sequence of the flavivirus genome comprise yellow fever virus sequences in the 5' region and tick-borne encephalitis or Langat virus sequences in the 3' region.

49. A replication-deficient pseudoinfectious flavivirus comprising a flavivirus genome comprising 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 comprises 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 comprise sequences encoding a tick-borne encephalitis or a Langat virus capsid/pre-membrane signal sequence, or a variant thereof.

50. A replication-deficient pseudoinfectious flavivirus comprising a flavivirus genome comprising 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) and non-structural (NS) proteins in said flavivirus genome are from Langat virus and any pre-membrane (prM) and envelope (E) proteins are from a tick-borne encephalitis virus.