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(54) Title: LIVE ATTENUATED FLAVIVIRUS VACCINES AND METHODS OF USING AND MAKING SAME

(57) Abstract: Recombinant flaviviruses and flavivirus genomes which have been attenuated by using a dual approach involving (a) the insertion of miRNA targeting sequences and (b) insertion of an internal ribosome entry site (IRES) upstream of a viral protein, thereby forming a bicistronic virus construct, causing manipulation of the level or amount of virus protein(s) under translational control of the IRES as a separate cistronic element are provided. Recombinant flaviviruses and flavivirus genomes that have been attenuated by an approach involving insertion of an IRES upstream of a viral protein and altering the arrangement of flavivirus protein genes in the genome are also disclosed. Methods for constructing such attenuated flaviviruses, immunogenic compositions comprising same, and methods for inducing an immune response against a flavivirus by administering an effective amount of compositions comprising the attenuated flaviviruses are also provided.



LIVE ATTENUATED FLAVIVIRUS VACCINES AND METHODS OF USING AND MAKING SAME**CROSS REFERENCE TO RELATED APPLICATIONS**

This application claims the benefit of U.S. Provisional Patent Application No. 62/443,214, filed January 6, 2017, which is incorporated herein by reference in its entirety.

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[01] Research supporting this application was carried out by the United States of America as represented by the Secretary, Department of Health and Human Services.

INCORPORATION BY REFERENCE

[02] All publications, patent applications, patents, figures and other references cited or referenced herein and all documents cited or referenced in the herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated by reference. In addition, all database accession numbers are incorporated by reference in their entirety as present in the respective database at least as of January 6, 2017.

BACKGROUND**[03] 1. Field of the disclosure**

[04] The present disclosure relates to live attenuated flavivirus vaccines and attenuated chimeric flavivirus vaccines that are immunogenic against one or more flaviviruses, including Zika virus and West Nile virus, among others. The disclosure further relates to methods for attenuating a flavivirus or a chimeric flavivirus using a synergistic dual strategy involving inserting miRNA-targeting sequences to restrict virus replication in target hosts, cells and/or tissues and placing one or more flavivirus genes under translational control of an internal ribosome entry site (IRES). Still further, the disclosure relates to immunogenic or vaccine compositions comprising the attenuated flaviviruses described herein, and to multivalent immunogenic or vaccine compositions comprising the attenuated flaviviruses described here. Further still, the disclosure relates to methods for treating and/or preventing and/or immunizing against flaviviruses, or flavivirus infections as the case may be, comprising administering an effective amount of an immunogenic composition described herein.

[05] 2. Background

[06] Many members of the *Flaviviridae* family (e.g., Dengue (DEN), Japanese encephalitis (JEV), Saint Louis encephalitis (SLEV), tick-borne encephalitis (TBEV), West Nile, and Zika viruses) are emerging and re-emerging human pathogens that have caused outbreaks of devastating and often fatal diseases and represent a serious public health problem on a global scale. Human flavivirus-associated disease ranges from mild febrile illness to severe fatal neurologic infection. Both mosquito- and tick-borne flaviviruses are typically characterized as enveloped viruses having an icosahedral and/or spherical

geometry with a diameter of around 50 nm per virion. The flavivirus genomes comprise a linear positive-sense RNA genome (see FIG. 1A) of about 10-11 kilobases in length and containing a single long open reading frame that encodes three major viral structural proteins (capsid (C), premembrane/membrane (prM), and envelope (E) proteins) and at least seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The polyprotein is processed by cellular and viral proteases to form the individual viral proteins. In addition, flavivirus genomes also contain the 5' noncoding region (NCR or untranslated region (5' UTR)) of about 100 nucleotides (nt) in length and the 3' UTR of about 400-800 nucleotides in length containing various conserved stem and loop structures that are, in part, involved in virus genome replication.

[07] Zika virus (ZIKV), in particular, has recently given rise to worldwide concern. Infection by ZIKV has historically only been known to cause mild symptoms in humans. In addition, ZIKV infections were generally observed in limited geographic regions localized near the equator between Africa and Asia. However, the virus is now thought to be linked to infant microcephaly and miscarriage in pregnant women, and has expanded its geographic reach. Zika has now spread to Mexico, Central and South America, and the Caribbean. The Centers for Disease Control (CDC) have now reported that Zika infections in South America have reached nearly pandemic levels.

[08] Like other flaviviruses, ZIKV is primarily transmitted from person to person by mosquitoes as a vector. In particular, ZIKV is transmitted by the female species known as *Aedes aegypti*, but has been detected in numerous other mosquito species in the *Aedes* genus, including *A. africanus*, *A. furcifer*, and *A. hensilli*. Unlike other well-studied mosquito-borne flaviviruses, ZIKV is able to establish long-term persistent infection in humans and utilizes non-vector transmission routes, including sexual transmission and vertical transmission from pregnant woman to the fetus.

[09] While effective vaccines exist for other flaviviruses, such as yellow fever, tick-borne encephalitis, and Japanese encephalitis, a human vaccine against ZIKV is not yet available. In addition, there is no single attenuation strategy that yet exists which is sufficient to prepare a safe (*e.g.*, non-virulent), but immunogenic and effective attenuated live-virus vaccine that will work for any flavivirus, including the most important pathogens including Zika, West Nile, and dengue viruses. Given the current state of global outbreak of Zika, there is a need in the art to develop effective vaccines which will be of significant benefit in prevention of human diseases caused by the virulent pathogens of Flaviviridae family including a vaccine against Zika infections.

SUMMARY

[010] The present disclosure relates in part to new strategies for attenuating a flavivirus or flavivirus chimera that ensures the safety of the flavivirus or their chimeras for use as a live-virus vaccine to treat and/or immunize and/or vaccinate against a flavivirus infection, including infection by a Zika virus (ZIKV), a West Nile virus (WNV), a Saint Louis encephalitis virus (SLEV), a tick-borne encephalitis virus (TBEV), a yellow fever virus (YFV), Langat virus (LGTV), a Japanese encephalitis virus (JEV), a dengue virus (DENV), a Powassan virus (POWV), and an Usutu virus (USUV).

[011] In some embodiments, the disclosure relates to recombinant flavivirus genomes which have been attenuated by using a dual approach involving (a) the insertion of one or more microRNA (miRNA) targeting sequences (*e.g.*, target sequences for brain-expressed miRNAs (*e.g.*, mir-9, mir-124 and/or mir-128) thereby mitigating against neurotropism and encephalitis; reproductive tissue-specific miRNAs (*e.g.*, mir-34, mir-141, mir-202, mir-148); and/or placenta-specific miRNAs (*e.g.*, mir-517a, mir-518e, mir-515, mir-519)) and (b) insertion of an internal ribosome entry site (IRES) (*e.g.*, IRES from encephalomyocarditis virus) upstream of a viral protein gene(s) (*e.g.*, viral capsid (C) protein), thereby forming a bicistronic virus construct, causing manipulation of the level or amount of virus protein(s) under translational control of the IRES as a separate cistronic element.

[012] The attenuation effects include the restriction of replication of the miRNA-targeting virus in those cells, tissues, or hosts (*e.g.*, human versus insect host) since the cell- or tissue-specific and highly expressed miRNAs would recognize the introduced complementary target sequences in the viral RNA genome and limit its translation, replication, and assembly into a virion. For instance, in one embodiment, an attenuated flavivirus that comprises one or more target sequences for brain-specific mir-124 will have an inability or a reduced ability to replicate in the brain or other neuronal tissue which expresses the corresponding mir-124 miRNA. Thus, in the case of a Zika virus for instance, this is expected to result in an attenuated Zika flavivirus which is impaired in its ability to replicate in the brain, thereby avoiding neurotropism and encephalitis or microcephaly. In another example embodiment, an attenuated Zika virus that comprises one or more target miRNA sequences that are characteristically expressed in *Aedes aegypti* but not laboratory/clinical cells (*e.g.*, Vero cells) will have an inability or a reduced ability to replicate in a mosquito host which expresses the corresponding miRNA molecule. Thus, in the case of a Zika virus for instance, this is expected to result in an attenuated Zika flavivirus which is a safer vaccine candidate since its ability to replicate and consequently spread via its mosquito host would be diminished or entirely vanquished.

[013] The attenuation effects also stem from the conversion of the flavivirus genome from a monocistronic genome (a ssRNA plus sense strand that is translated into a single polycistronic polypeptide which is then proteolytically processed to individual viral proteins) to a bicistronic genome on account of the inserted IRES. In other words, the IRES insertion divides the flavivirus genome into two separate open reading frames: a first open reading frame (*e.g.*, ORF1) beginning at the 5' end of the virus genome which is under wild type 5'-cap-dependent translation control to produce a first polyprotein; and a second open reading (*e.g.*, ORF2) that is under IRES-dependent translational control. This bicistronic configuration—without being bound by theory—is thought to disrupt the optimal levels and/or balanced relative amounts of viral proteins that are achieved or produced normally under monocistronic genomic structure, which in turn impacts typical viral-related processes such as replication, assembly, packaging, infection, and/or cell-to-cell spreading, resulting in virus attenuation.

[014] Further, it has been surprisingly discovered that introducing a combination of IRES-dependent translational controls of certain viral proteins and miRNA-based replication controls into a flavivirus genome produce synergistic levels of attenuation that are not observed or predicted by either approach standing alone.

[015] As demonstrated in the Examples with respect to various embodiments, the attenuated flaviviruses described herein provide for safe (*e.g.*, nonvirulent and environmentally safe), yet immunogenic live virus vaccines for use in treating and/or immunizing or vaccinating against a flavivirus, such as Zika, West Nile, or Japanese encephalitis virus.

[016] As such, the present disclosure relates to attenuated flaviviruses based on the dual IRES-based and miRNA based approaches for attenuation, flavivirus genomes comprising said attenuating elements, immunogenic compositions comprising the attenuated flaviviruses described herein, multivalent immunogenic compositions that are immunogenic against more than one flavivirus, vaccine compositions comprising the attenuated flaviviruses described herein, methods for constructing the attenuated flaviviruses using the dual approach to attenuation, methods for inducing an immune reaction in a subject against a flavivirus by administering an effective amount of an immunogenic composition described herein, and methods for immunizing or vaccinating against a flavivirus (or multiple flaviviruses) by administering an effective amount of an immunogenic flavivirus composition or vaccine (or a multivalent composition or vaccine) to a subject in need thereof.

[017] In other embodiments, the present disclosure relates to an attenuated flavivirus or chimeric flavivirus comprising a recombinant flavivirus genome that encodes (a) a first open reading frame (ORF1) that is under 5'-cap-dependent translational control and which codes for at least a truncated C protein (*e.g.*, about 1-100N-terminal amino acids of the C protein), a prM/E protein, and an NS1 protein and (b) a second open reading frame (ORF2) that is under IRES-dependent translational control and which codes for the another portion of the C protein (such as a cytoplasmic portion) and the other flavivirus proteins not encoded by ORF1. In some embodiments, the portion of the C protein encoded in ORF2 is a codon-optimized sequence. Such attenuated flaviviruses or chimeric flaviviruses are both bicistronic and have a rearrangement of the genome. In some embodiments, the bicistronic, rearranged flavivirus also includes at least one miRNA target sequence that is complementary to a host-specific miRNA, and thus may be a dual-approach, rearranged flavivirus or chimeric flavivirus.

[018] Thus, in one aspect, the present disclosure provides a nucleic acid molecule comprising an attenuated flavivirus or chimeric flavivirus genome comprising one or more target nucleotide sequences complementary to a host-specific micro-RNA (miRNA) and a nucleotide sequence encoding one or more flavivirus proteins that are under translational control of an internal ribosome entry site (IRES).

[019] In an embodiment, the one or more target nucleotide sequences are effective in restricting replication of the flavivirus in the presence of the complementary host-specific miRNA.

[020] In another embodiment, the IRES is effective in converting the flavivirus genome into a bicistronic flavivirus genome, thereby causing attenuation of the flavivirus.

[021] In various embodiments, the flavivirus genome which becomes attenuated can be a Zika virus (ZIKV) genome, a West Nile virus (WNV) genome, a Saint Louis encephalitis virus (SLEV) genome, a tick-borne encephalitis virus (TBEV) genome, a yellow fever virus (YFV) genome, Langat virus (LGTV), a Japanese encephalitis virus (JEV) genome, a dengue type 1 virus (DEN1), a dengue type 2 virus (DEN2), a dengue type 3 virus (DEN3), a dengue type 4 virus (DEN4) genome, a Powassan virus (POWV) genome, or an Usutu genome (USUV) genome.

[022] In one embodiment, the genome is a Zika virus (ZIKV) genome. The Zika virus (ZIKV) genome can be a previously identified or sequenced Zika virus isolate, such as, GenBank Accession No. AY632535, LC002520, KU055503, KU055505, KU055502, KU955591, or KU955594. In a particular embodiment, the genome is KX280026 – Paraiba_01/2015 isolate (an infectious cDNA clone of ZIKV from the 2015 epidemic in Brazil).

[023] In another embodiment, the genome is a West Nile virus (WNV) genome, such as GenBank Accession No. KC601756.

[024] In various embodiments, the attenuated flaviviruses or chimeric flaviviruses include at least one target nucleotide sequence complementary to a host-specific micro-RNA (miRNA), or at least two such sequences, or at least three such sequences, or at least four such sequences, or at least five such sequences, or at least six such sequences, or at least seven such sequences, or at least eight such sequences, or at least nine such sequences, or at least ten or more such sequences. In certain embodiments, these so-called miRNA target or microRNA target sequences can be all the same sequence, or they can be different sequences. The flaviviruses may include miRNA target sequences that are complementary to one or more human miRNAs produced/expressed in a specific cell or tissue (*e.g.*, neuronal tissue, reproductive tissue, or placenta) and/or arthropod vector-specific miRNAs that are expressed/produced only in mosquitoes or ticks. The miRNA target sequences may be designed to correspond to (*e.g.*, are complementary to) any known or yet-to-be discovered microRNA sequence.

[025] In certain embodiments, the attenuated flavivirus or chimeric flavivirus includes at least three target nucleotide sequences complementary to a host-specific micro-RNA (miRNA).

[026] In other embodiments, the attenuated flavivirus or chimeric flaviviruses include target sequences that bind to host-specific micro-RNA (miRNA) that are expressed only in humans. The miRNA may be selectively expressed in an epithelial tissue, connective tissue, nervous system tissue, or muscle tissue. The miRNA may also be selectively expressed in the cardiovascular system, digestive system, endocrine system, excretory system, lymphatic system, integumentary system, muscular system, nervous system, reproductive system, respiratory system, or skeletal system.

[027] In various embodiments, the human-specific miRNAs that are complementary to the miRNA target sequences may include, but are not limited to, mir-124 (brain/neuronal specific), mir-9 (brain/neuronal specific), mir-34, mir-141, mir-202, mir-148 (reproductive organ specific), and mir-517a, mir-518e, mir-515, and mir-519 (placenta specific).

[028] In other embodiments, the attenuated flaviviruses or chimeric flaviviruses may include one or more target sequences that specifically bind to invertebrate vector-specific micro-RNA (miRNA). Such invertebrate vector-specific miRNA may be expressed in arthropods, such as mosquitoes or ticks.

[029] In various embodiments, the vector-specific miRNAs that are complementary to the miRNA target sequences may include mir-184, mir-275, and mir-1.

[030] In various embodiments, the one or more miRNA target sequences may be inserted or introduced in any location in a flavivirus genome (*e.g.*, at any location in a Zika virus genome), including in the 5'UTR, the portion of the genome encoding the structural genes (C/prM/E genes), the portion of the genome encoding the nonstructural genes (NS1-NS5), or the 3'UTR. In some examples, the insertion of the one or more miRNA target sequences are introduced in a manner that does not disrupt the proper translation or proteolytic processing of the viral proteins encoded by the genome. In certain embodiments, the one or more miRNA target sequences are introduced into the 5'UTR or the 3'UTR or both regions. In certain other embodiments, the one or more miRNA target sequences can be introduced into the open reading frame of a flavivirus polyprotein but in a manner that does not disrupt translation or proteolytic processing. Still further, insertion of the miRNA target elements into the flavivirus genomes may be conducted in accordance with Tsetsarkin *et al.*, *PLOS Pathogens* 11(4):e1004852 (2015), the contents of which are incorporated herein by reference in its entirety.

[031] In certain embodiments, the flaviviruses and flavivirus compositions described herein involve a genome having a single-stranded RNA molecule. In certain other embodiments, the flaviviruses and flavivirus compositions described herein involve a genome having a double-stranded cDNA molecule, which may be transcribed to form a plus-sense single strand RNA that may be translated to a flavivirus polyprotein and then processed proteolytically to form viral proteins.

[032] In other embodiments, the dual attenuation approach (IRES + miRNA target sequences) may be used to attenuate a chimeric flavivirus, which due its chimeric nature, may already be initially attenuated (*e.g.*, WNV/DENV chimeras in US 8,778,671, incorporated by reference). The chimeric flavivirus genome may include a first nucleic acid sequence encoding at least one structural protein from a first flavivirus and a second nucleic acid sequence encoding the remaining structural and nonstructural proteins from a second flavivirus.

[033] In one embodiment, the first nucleic acid sequence can be from a Zika virus genome. The second nucleic acid sequence can be from another flavivirus, such as a dengue virus, West Nile virus, Yellow fever virus, or Japanese encephalitis virus.

[034] In another embodiment, the second nucleic acid sequence is from a Zika virus genome. The first nucleic acid sequence can be from another flavivirus, such as a dengue virus, West Nile virus, or Japanese encephalitis virus.

[035] In another aspect, the present disclosure relates to an attenuated live flavivirus or attenuated live chimeric flavivirus comprising a nucleic acid molecule derived from an attenuated flavivirus or chimeric flavivirus genome. The live attenuated flavivirus can be a Zika virus (ZIKV), a West Nile virus (WNV), a tick-borne encephalitis virus (TBEV), a yellow fever virus (YFV), Langat virus (LGTV), a Japanese encephalitis virus (JEV), a dengue virus (DENV), a Powassan virus (POWV), an Usutu virus (USUV), or a chimeric flavivirus thereof that comprises a chimeric genome having at least one portion from one flavivirus (*e.g.*, Zika virus) and the remaining portion from another flavivirus (*e.g.*, dengue virus).

[036] In yet another aspect, the present disclosure relates to an attenuated flavivirus or chimeric flavivirus comprising a recombinant flavivirus genome that encodes (a) a first open reading frame (ORF1) that is under 5'-cap-dependent translational control and which codes for at least one flavivirus protein and (b) a second open reading frame (ORF2) that is under IRES-dependent translational control and which codes for the remaining flavivirus proteins not encoded by ORF1, and wherein said genome further comprises at least one miRNA target sequence that is complementary to a host-specific miRNA.

[037] In still another aspect, the present disclosure relates to a bicistronic attenuated flavivirus comprising a recombinant flavivirus genome comprising a first nucleotide sequence encoding a first open reading frame (ORF1) coding for at least one flavivirus protein or functional variant thereof and a second nucleotide sequence encoding a second open reading frame (ORF2) coding for the remaining flavivirus proteins not coded for by ORF1, wherein the first open reading frame is under IRES-dependent translational control, and where said genome further comprises at least one miRNA-target sequence.

[038] In still another aspect, the present disclosure relates to an immunogenic composition comprising a live attenuated virus or live chimeric attenuated flavivirus that has been attenuated using the dual approach attenuation of introducing an IRES element and one or more miRNA targeting sequences into the genome.

[039] The present disclosure further relates to attenuated flaviviruses comprising the dual attenuating elements of an IRES and one or more miRNA targeting sequences, wherein the attenuated flaviviruses comprise one or more additional attenuating mutations, such as attenuating point mutations, deletions, insertions, chimeric portions, or other attenuating elements that may be introduced into the flaviviruses described herein.

[040] In various embodiments, the additional attenuating mutations can be introduced into one or more of the genes encoding the three major viral structural proteins (capsid (C), premembrane/membrane (prM) and envelope (E) proteins) or into one or more of the genes encoding the at least seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins.

[041] In other embodiments, the attenuating mutations and/or deletions can be introduced into the 5' UTR.

[042] In still other embodiments, the attenuating mutations and/or deletions can be introduced into the 3' UTR.

[043] In still further embodiments, the further attenuating mutations and/or deletions can be introduced into any of the nonstructural protein genes, structural protein genes, the 5' UTR, the 3' UTR, or combinations thereof. In still other embodiments, the further attenuating mutations can be deletions introduced into the 3'UTR which removes, shortens, or otherwise disrupts one or more stem-loop structures of the 3'UTR of flavivirus genome (*e.g.*, a deletion that corresponds with a $\Delta 30$, $\Delta 31$, $\Delta 30/31$, or $\Delta 86$ mutation in the DENV background).

[044] In some embodiments, the live attenuated dual-approach flavivirus or chimeric flavivirus further includes at least one additional attenuating mutation selected from the group consisting of: (a) an attenuating deletion in the 3'UTR or (b) an attenuating point mutation.

[045] In other embodiments, the live attenuated dual-approach flavivirus or chimeric flavivirus described herein, includes an attenuating deletion in the 3'UTR that corresponds with a $\Delta 30$, $\Delta 31$, $\Delta 30/31$, or $\Delta 86$ mutation in the DENV serotype 4 background.

[046] In other embodiments, the live attenuated dual approach flaviviruses or chimeric flaviviruses described herein include further attenuating point mutations that correspond with a mutation at nucleotide 4861 or 4995 of the NS3 gene in the DENV serotype 4 background.

[047] In various embodiments, the IRES sequence is from a poliovirus (PV) or an encephalomyocarditis virus (EMCV). In certain embodiments, the IRES sequence is from an encephalomyocarditis virus (EMCV).

[048] In certain embodiments, the dual approach attenuated flaviviruses described herein comprise a genome that includes the insertion of an IRES in or just before the gene encoding the viral capsid (C) gene or a functional fragment thereof.

[049] In other embodiments, the dual approach attenuated flaviviruses described herein comprise a genome that includes the insertion of an IRES in or just before the gene encoding the viral prM gene or a functional fragment thereof.

[050] In other embodiments, the dual approach attenuated flaviviruses described herein comprise a genome that includes the insertion of an IRES in or just before the gene encoding the viral E gene or a functional fragment thereof.

[051] In other embodiments, the dual approach attenuated flaviviruses described herein comprise a genome that includes the insertion of an IRES in or just before one of the genes encoding a viral nonstructural protein or a functional fragment thereof.

[052] In one embodiment, the IRES-based attenuation is introduced into a flavivirus genome by inserting a termination codon followed by an IRES downstream of the coding region of the capsid (C) protein but upstream of the coding region for prM. In accordance with this exemplary embodiment, the capsid protein is under translational control of a 5'-cap-dependent mechanism, whereas translation of all other viral proteins (prM to NS5) is under control of IRES-dependent translation. This embodiment may be carried out in accordance with Frese *et al.*, 88:2056-2070 (2014), which is incorporated herein by reference.

[053] In further embodiments, the attenuated flaviviruses described herein may also include one or more interferon genes, *e.g.*, interferon beta, interferon alpha, or interferon gamma, or any subtypes thereof. This embodiment may be carried out in accordance with Frese *et al.*, *Journal of Virology*, 88:2056-2070 (2014), which is incorporated herein by reference.

[054] Also disclosed is a method of inducing an immune response in a subject infected with a flavivirus comprising administering an effective amount of an attenuated flavivirus described herein, such as a dual-approach attenuated flavivirus described herein, a bicistronic, rearranged flavivirus, a dual-approach, rearranged flavivirus, or an immunogenic composition comprising same.

[055] According to another aspect, the present disclosure provides a pharmaceutical kit comprising an immunogenic composition or vaccine. The vaccine may comprise an attenuated flavivirus that is attenuated by the dual approach of inserting an IRES and one or more miRNA targets into the genome of a flavivirus or an attenuated virus that is attenuated by the bicistronic rearrangement approach described herein. Any flavivirus genome or chimeric flavivirus genome may be used as a starting point to introduce attenuation using the approaches described herein. Additional attenuating mutations may be introduced in any of the flaviviruses described herein as well, including point mutations, deletions, and insertions. Said kits may be included with a set of instructions for using the composition to vaccinate a subject.

[056] According to an aspect, the present disclosure provides a method for vaccinating a subject to increase or provide immunity against a flavivirus, such as a ZIKV. The method comprises administering a pharmaceutically acceptable dose of a flavivirus vaccine described herein. The vaccine comprises an attenuated flavivirus that has been attenuated using the dual approach of inserting an IRES and one or more target miRNA sequences or a flavivirus that has been attenuated using the bicistronic rearrangement approach.

[057] According to a further aspect, the present disclosure provides a method for manufacturing a vaccine comprising an attenuated flavivirus, wherein said attenuation is the result of the genome of the flavivirus comprising an IRES inserted at the 5' end of a coding region of one flavivirus gene, *e.g.*, C, prM, E, or NS1-NS5, and also is the result of the insertion of one or more target miRNA sequences. The methods for manufacturing include introducing an IRES into the 5' end of a coding region of a flavivirus gene and also introducing at least one target miRNA sequence into the genome into the 5'NCR, a coding region, or the 3'NCR location. In other examples, the disclosure provides a method for manufacturing a vaccine

comprising an attenuated flavivirus, wherein said attenuation is the result of the insertion of an IRES and rearrangement of one or more of the protein coding genes of the flavivirus in the flavivirus genome. The manufacturing method further comprises combining the attenuated flavivirus with one or more pharmaceutical excipients to provide said vaccine.

[058] These and other embodiments are disclosed or are contemplated variations from and encompassed by the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[059] The above and other features of the present disclosure will now be described in detail with reference to certain exemplary embodiments thereof illustrated the accompanying drawings which are given herein below by way of illustration only, and thus are not limiting, and wherein:

[060] FIG. 1A provides a map of a flavivirus.

[061] FIG. 1B depicts a wildtype flavivirus genome showing the C/prM/E structural protein gene region, the nonstructural protein gene region, and the 5'UTR and 3'UTR regions. The genome, a single stranded positive sense RNA, contains a single open reading frame (or "monocistronic coding region") that is translated based on a 5'-cap-dependent mechanism to form a single polyprotein, which is then cleaved by proteolytic processing into the individual viral proteins C, prM, E, and NS1-NS5. Viral assembly, replication, and further infection would subsequently occur.

[062] FIG. 1C is a schematic depicting certain embodiments of the attenuated flaviviruses described herein which are attenuated by the dual approach of inserting an IRES upstream of a coding region of a viral protein and inserting one or more miRNA target sequences into the genome. As depicted in the schematic, construction of the attenuated flavivirus begins with a flavivirus genome. The flavivirus can be any flavivirus, including Zika, West Nile, or dengue virus. The flavivirus may also be a chimeric flavivirus that comprises a genome having a portion from one flavivirus and the remaining portion from a second flavivirus. The flavivirus genome is modified by inserting an IRES into the genome. The insertion may be made in any location, including in the 5'UTR, the coding region, and the 3'UTR; however, in certain embodiments the IRES insertion is made in the coding region and at a position upstream of the coding region of a gene coding for a viral gene. In a specific embodiment, the insertion is made upstream the gene encoding capsid (C) protein. The attenuation strategy also includes inserting one or more miRNA target sequences into one or more locations into the genome. The locations may be the same or different. A combination of locations may be used. The configuration of miRNA target sequences may also include placing the sequences in the same location in tandem. These modifications form a bicistronic genome, as depicted, which includes a first open reading frame (ORF1) under translational control of a 5' cap-dependent mechanism, and a second open reading frame (ORF2) which is under translational control of the IRES. Two separate proteins or polyproteins are translated, a product corresponding to each of the ORFs. The products are then cleaved into individual viral proteins via proteolytic processing. However, the bicistronic

nature of the modified genome results in suboptimal levels and/or imbalanced relative or molar amounts of viral proteins, thereby impacting normal viral functions such as replication, assembly, packaging, release, and cell to cell spreading. In addition, the miRNA target sequences restrict replication of the flavivirus in cells or tissues that express the cognate or corresponding miRNA molecules.

[063] Not shown in the general model of FIG. 1C are additional embodiments also contemplated herein which include other modifications such as constructing an IRES-controlled coding region or unit that comprises at least one gene encoding a viral protein or a functional fragment thereof and insertion of said IRES-dependent coding region or unit into a non-native location in the genome, such as downstream or upstream of the normal location of the viral gene, or in the 5' or 3' UTR (or NCR). The viral gene now under control of IRES translation may be deleted or otherwise inactivated at its native location. Examples of these types of embodiments can be seen in FIG. 1D.

[064] FIG. 1D relates to the development of certain embodiments of the bicistronic flaviviruses described herein in an LGTV genetic background. Schematic representation of the viral genomes constructed for Example 1. C trn (48AA) denotes replication promoter region of C gene. ORF-shifting insertion (asterisk, position 151 nt of LGTV genome) of a single A nucleotide (Fr Sh +1) and ORF restoration (-1) are indicated. Boxes denote 2A protease gene of FMDV (2A) and mir-124 target (T) sequences (solid boxes), respectively. Shaded boxes denote codon-optimized sequence of C gene.

[065] FIG. 2 relates to the development of bicistronic LGTV. Graph shows the growth kinetics of recovered viruses (cap-C, cap-ΔC, IRES-C, and IRES-124 of FIG. 1) in Vero cells. Individual samples for each time point were titrated in Vero cells in duplicate. Results are presented as an average ± standard deviation (shown as error bars). The dashed line indicates the limit of virus detection (0.7 log₁₀ pfu/mL).

[066] FIGS. 3A and 3B depict that relocation of structural genes (C/prM/E) into the 3'NCR under control of IRES impairs growth of bicistronic LGTV in Vero cells. FIG. 3A is a schematic representation of the viral genomes used in the study. To generate IRES-C/prM/E, ΔC/prM/E genes were deleted in IRES-C preserving 34 C-terminal AA of E protein (ΔE). prM/E genes were inserted downstream of C-opt gene of IRES-C. Vero cell monolayers in 12.5 cm² flasks were transfected with 5 μg of IRES-C or IRES-C/prM/E constructs. At 4 dpi cell culture medium was collected and titrated in Vero cells (FIG. 3B). Limit of virus detection is 0.7 log₁₀ pfu/mL.

[067] FIGS. 4A and 4B demonstrates that the insertion of an additional copy of mir-124(T) sequence between NS5 gene and 5' end of IRES in IRES-C reduces mortality of newborn SW mice after IC infection with bicistronic LGTV. FIG. 4A is a schematic representation of the viral genomes used in the study. FIG. 4B shows survival of newborn SW mice (n=10) inoculated IC with 10³ pfu/mouse of rLGTVs. Differences in survival curves were compared using log-rank (Mantel-Cox) test.

[068] FIG. 5 is a table showing substitutions identified in IRES-124 after 10 passages in Vero cells.

[069] FIG. 6 relates to the development of bicistronic LGTV. Graph shows the growth kinetics of recovered viruses (IRES-124, IRES-124(3m), and IRES-124(4m) of FIG. 1D) in Vero cells. Individual samples for each time point were titrated in Vero cells in duplicate. Results are presented as an average $\pm$ standard deviation (shown as error bars). The dashed line indicates the limit of virus detection ($0.7 \log_{10}$ pfu/mL).

[070] FIG. 7 is a table showing the immunogenicity and protective efficacy of IRES-124(3m) virus in 3-week old C3H mice. Three-week-old C3H mice (female) were infected intraperitoneally with 10^5 pfu of IRES-124(3m) or mock-inoculated with L-15 medium supplemented with 1x SPG and monitored for neurological symptoms daily until 28 dpi. At 29 dpi mice were challenged with 10^4 pfu of LGTV (strain TP-21) and monitored for morbidity for an additional 28 days. Mice were bled on 1 and 30 dpi for detection of virus in the serum and on 28 and 56 dpi for measurement of neutralizing antibody titer (presented as a geometrical mean) using the 50% plaque reduction neutralization assay (PRNT₅₀) against LGTV TP-21 strain as described previously (Pletnev *et al.*, *J. Virol.* 75:8259-8267, 2001).

[071] FIG. 8 is a table showing the substitutions identified in IRES-124(3m) after 10 passages in Vero cells.

[072] FIG. 9 is a schematic representation of recombinant (r) LGTV genomes used in this study. Boxes denote mir-124(T) and mir-9(T) sequences, respectively. Striped boxes indicate scrambled (synonymous) sequence for mir-124(T) and mir-9(T).

[073] FIG. 10 shows the growth kinetics of rLGTVs in the brains of newborn Swiss Webster (SW) mice, after IC inoculation with 10^2 pfu. Viral load in the brain ($n=3$) $\pm$ standard deviation (SD) were determined by titration in Vero. The dashed line indicates the limit of virus detection ($1.7 \log_{10}$ pfu/mL of serum). These results help demonstrate a synergistic effect between IRES- and miRNA- based strategies attenuates neurovirulence of bicistronic LGTVs in mice.

[074] FIG. 11 shows growth in Vero cells of viruses used in mice studies. Vero cell monolayers in 12.5 cm^2 flasks were transfected with 5 μg of plasmid DNA constructs depicted in FIG. 9. Cell culture medium aliquots collected at indicated time points were titrated in Vero cells in duplicate. Mean virus titers $\pm$ SD are shown. The dashed line indicates the limit of virus detection ($0.7 \log_{10}$ pfu/mL).

[075] FIG. 12 shows the survival of newborn SW mice ($n=10$) inoculated IC with 10^4 pfu/mouse of rLGTVs. These results help demonstrate a synergistic effect between IRES- and miRNA- based strategies attenuates neuropathogenesis of bicistronic LGTVs in mice.

[076] FIG. 13 shows the mean titer in the serum of SCID mice after IP infection with 10^5 pfu/mouse. Due to the death or paralysis of the animals, mouse serum samples from IRES-124/9 (4m)* and cap-124/9 infected groups were not collected after days 7 and 23, respectively. These results help demonstrate a synergistic effect between IRES- and miRNA- based strategies attenuates neuropathogenesis and neuroinvasiveness of bicistronic LGTVs in mice.

[077] FIG. 14 shows the mean titer in the brain of SCID mice after IP infection with 10^5 pfu/mouse. Brains from healthy mice infected with IRES-124 (4m) and IRES-124/9 (4m) were isolated on day 52. Brains from paralyzed/dead mice were isolated on day 23 (IRES-124/9 (4m)* group) or from day 26 to 31 (cap-124/9 group). The dashed line indicates the limit of virus detection ($1.7 \log_{10}$ pfu/g of brain). These results help demonstrate a synergistic effect between IRES- and miRNA- based strategies attenuates neuropathogenesis and neuroinvasiveness of bicistronic LGTVs in mice.

[078] FIG. 15 shows the survival of SCID mice ($n = 4$ or 5) inoculated IP with 10^5 pfu/mouse of rLGTVs. These results help demonstrate a synergistic effect between IRES- and miRNA- based strategies attenuates neuropathogenesis and neuroinvasiveness of bicistronic LGTVs in mice.

[079] FIG. 16 shows the sequence analysis of cap-124/9 viruses recovered from the brain of morbid SCID mice. SCID mice ($n = 5$) were infected IP with 10^5 pfu/mouse. Mice succumbed to encephalitis between dpi 26-31. Brains from paralyzed mice were collected and used for viral isolation and sequencing analysis. Double arrows schematically indicate the location of deleted sequences in the cap-124/9 genome.

[080] FIG. 17 demonstrates that adult B6 IFNRI^{-/-} mice immunized with bicistronic LGTVs are protected against lethal challenge with wt LGTV. Graph shows survival of B6 IFNRI^{-/-} mice ($n=5$) after immunization. Mice were inoculated IP with 10^5 pfu/mouse of rLGTVs or with diluent alone (mock).

[081] FIG. 18 demonstrates that adult B6 IFNRI^{-/-} mice immunized with bicistronic LGTVs are protected against lethal challenge with wt LGTV. The graph shows survival of vaccinated mice after IP challenge with 10^2 pfu of wt LGTV. At 31 days post vaccination, animals were challenged with wt LGTV and monitored for neurological signs of disease.

[082] FIG. 19 demonstrates that adult B6 IFNRI^{-/-} mice immunized with bicistronic LGTVs are protected against lethal challenge with wt LGTV. B6 IFNRI^{-/-} mice immunized with bicistronic LGTVs or mock-infected animals were challenged on day 32 with 10^2 pfu of wt LGTV. Graph shows titer of wt LGTV in the serum of mice on days 1 and 4 post challenge. Virus titer in the serum was determined by titration in LLC-MK2 cells.

[083] FIG. 20 demonstrates that adult B6 IFNRI^{-/-} mice immunized with bicistronic LGTVs are protected against lethal challenge with wt LGTV. Graph shows neutralizing antibody titer in the serum of B6 IFNRI^{-/-} mice on 28 and 56 days post inoculation with bicistronic LGTVs. Neutralizing antibody titer was determined using the 50% plaque reduction neutralization assay (PRNT₅₀) against wt LGTV.

[084] FIGS. 21A-21C demonstrate that bicistronic LGTVs are growth restricted in tick-derived cells. FIG. 21A is a schematic representation of the viral genomes used in the study. Tick-derived ISE6 cells (FIG. 21B) or Vero cells (FIG. 21C) were infected with mono- or bicistronic LGTVs at an MOI of 0.1 in duplicate wells of 6-well plates. Virus titer in cell supernatant was determined on day 5 p.i. Mean virus titers $\pm$ SD are shown. The dashed line indicates the limit of virus detection ($0.7 \log_{10}$ pfu/mL).

[085] FIGS. 22A-22C provides maps of attenuated viruses. ZV-IRES-version 1 is shown in FIG. 22A. ZV-IRES version 2 is shown in FIG. 22B. See Example 2 for further description of the constructs. FIG. 22C is a schematic representation of the JV-IRES-miR or WNV-IRES-miR genome. C trn (68AA) denotes replication promoter region of C gene. ORF-shifting insertion (asterisk) of a single A nucleotide (Fr Sh +1) and ORF restoration (-1) are indicated. Boxes denote mir-124 target (T) and mir-9 (T) sequences. ECMV IRES is indicated as stem-loop structure located between ORF 1 and ORF 2. Δ C, Δ pr and Δ NS1 indicate truncated C, pr, and NS1 genes respectively.

[086] FIGS. 23A-23F provides the complete nucleotide sequence of the attenuated flavivirus IRES-124/9 (SEQ ID NO: 13). A map of this flavivirus is provided in FIG. 9.

[087] FIGS. 24A-24F provides the complete nucleotide sequence of the attenuated flavivirus IRES-124 (SEQ ID NO: 14). A map of this flavivirus is provided in FIG. 1D.

[088] FIGS. 25A-25G provides the complete nucleotide sequence of the attenuated flavivirus ZV-IRES version 2 (SEQ ID NO: 15). A map of this flavivirus is provided in FIG. 22B.

[089] FIGS. 26A-G are a schematic diagram showing the ZIKV-IRES version 3 construct (FIG. 26A) and the complete nucleotide sequence of the ZV-IRES version 3 construct (FIGS. 26B-26G; SEQ ID NO: 16).

[090] FIGS. 27A-27C are a series of graphs showing survival of mice vaccinated with ZIKV-IRES version 3 or mock-vaccinated following challenge with 10^5 pfu of ZIKV-wt (FIG. 27A), neutralizing antibody titer in serum of mice 28 days post-inoculation with ZIKV-IRES version 3 and 27 days post-challenge (dpi 56) with ZIKV-wt (FIG. 27B), and titer of ZIKV-wt in serum, brain, testis, and epididymis of mice post-challenge (FIG. 27C). Serum titer was measured 2 days post-challenge and brain, testis, and epididymis titer was measured 10-12 days post-challenge. The dashed line indicates limit of virus detection in mouse organs ($1.7 \log_{10}$ pfu/g) and the dashed line indicates the limit of virus detection in mouse serum ($1.5 \log_{10}$ pfu/g).

[091] FIG. 28 is a graph showing growth of ZIKV-IRES version 3 in *Aedes albopictus*-derived C6-36 cells. Mean virus titers $\pm$ are shown. The dashed line indicates the limit of virus detection ($0.7 \log_{10}$ pfu/ml).

[092] FIG. 29 is a schematic diagram of an exemplary ZIKV-IRES “rearranged” bicistronic construct. This construct does not include any miRNA targets. C trn (50AA) denotes replication promoter region of C gene. ORF-shifting insertion (asterisk) of a single A nucleotide (Fr Sh +1) and ORF restoration (-1) are indicated. ECMV IRES is indicated as stem-loop structure located between ORF 1 and ORF 2. pr/ Δ NS1 indicates truncated prM and NS1 genes.

SEQUENCE LISTING

[093] Any nucleic acid and amino acid sequences listed herein or in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases and amino acids, as defined in 37 C.F.R. § 1.822. In at least some cases, only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

[094] SEQ ID NOs: 1-12 and 17 are exemplary miRNA sequences.

[095] SEQ ID NO: 13 is the nucleic acid sequence of IRES-124/9 virus.

[096] SEQ ID NO: 14 is the nucleic acid sequence of IRES-124 virus.

[097] SEQ ID NO: 15 is the nucleic acid sequence of ZIKV-IRES version 2.

[098] SEQ ID NO: 16 is the nucleic acid sequence of ZIKV-IRES version 3.

DETAILED DESCRIPTION

[099] Flaviviruses include significant human pathogens, including Zika, dengue, West Nile, yellow fever, and Japanese encephalitis viruses. While effective vaccines exist for some flaviviruses, safe and effective vaccines against certain flaviviruses—including Zika virus—are not yet available. In addition, no single attenuation strategy exists that is sufficient to prepare a safe (*e.g.*, non-virulent) but immunogenic and effective attenuated live-virus vaccine that will work for any flavivirus, including the most important pathogens include Zika, West Nile, and dengue virus. Given the current state of global outbreak of Zika, there is an immediate need in the art to develop an effective vaccine that will induce durable immunity and afford an effective and rapid protection against Zika virus infection, as well as against any other flavivirus pathogen.

[0100] The present disclosure relates in part to a strategies for attenuating a flavivirus or flavivirus chimeras that ensures the safety of them for use as live-virus vaccines to treat and/or immunize and/or vaccinate against a flavivirus infection, including Zika virus (ZIKV), a West Nile virus (WNV), a tick-borne encephalitis virus (TBEV), a yellow fever virus (YFV), Langat virus (LGTV), a Japanese encephalitis virus (JEV), a dengue virus (DENV), a Powassan virus (POWV), and an Usutu virus (USUV). In some embodiments, the strategy involves the introducing an internal ribosome entry site (IRES) into a genome of a flavivirus or a chimeric flavivirus to produce a bicistronic flavivirus genome in combination with introducing one or more miRNA-target sequences into the genome. This dual approach thus involves (a) the insertion of miRNA target sequences (*e.g.*, target sequences for brain/neuronal specific miRNAs, mir-124 and/or mir-9, thereby mitigating against neurotropism and encephalitis, or target sequences for reproductive organ-specific miRNAs, mir-34, mir-141, mir-148, and/or mir-202, or placenta-specific miRNAs, mir-518e, mir-515, mir-517a, and/or mir-519, to restrict virus replication in reproductive organs or placenta) and (b) insertion of an internal ribosome entry site (IRES) (*e.g.*, IRES from encephalomyocarditis virus) upstream of a viral protein gene (*e.g.*, viral capsid (C) protein). Overall

attenuation is achieved in a synergistic manner by (a) the restriction of virus replication in certain hosts, cells, or tissues which express the corresponding microRNA (miRNA), and (b) disruption of the normal balanced levels or molar amounts of viral proteins by conversion of the genome from a monocistronic genome to a bicistronic genome due to the presence of the IRES. This bicistronic configuration—without being bound by theory—is thought to disrupt the optimal levels and/or balanced relative amounts of viral proteins that are achieved or produced normally under monocistronic genomic structure, which in turn impacts typical viral-related processes such as replication, assembly, packaging, infection, and/or cell-to-cell spreading, resulting in virus attenuation.

[0101] For instance, in one embodiment, an attenuated flavivirus that comprises one or more mir-124 target sequences will have an inability or a reduced ability to replicate in the brain or other neuronal tissue which expresses the corresponding mir-124 microRNA molecule. Thus, in the case of a Zika virus for instance, this is expected to result in an attenuated Zika flavivirus which is impaired in its ability to replicate in the brain, thereby avoiding neurotropism and neuropathogenesis. In another example embodiment, an attenuated Zika virus that comprises one or more miRNA target sequences that are characteristically expressed in *Aedes* species of mosquitoes but not in vertebrate cells (e.g., Vero cells) will have an inability or a reduced ability to replicate in a mosquito host which expresses the corresponding miRNA molecule. Thus, in the case of a Zika virus for instance, this is expected to result in an attenuated Zika flavivirus which is a safer vaccine candidate since its ability to replicate and consequently spread via its mosquito vector transmission would be diminished or entirely vanquished.

[0102] Further, it has been surprisingly discovered that combining the IRES-dependent translational control of viral proteins with miRNA-based suppression of virus replication has synergistic effect on flavivirus attenuation that was not observed or predicted by either approach standing alone. As demonstrated in the Examples with respect to various embodiments, the attenuated flaviviruses described herein provide for safe (e.g., nonvirulent and environmentally safe) yet immunogenic live virus vaccine candidates for use in treating and/or immunizing or vaccinating against a flavivirus, such as Zika, West Nile, or Japanese encephalitis virus.

[0103] In other embodiments, the present disclosure relates to an attenuated flavivirus or chimeric flavivirus comprising a recombinant flavivirus genome that encodes (a) a first open reading frame (ORF1) that is under 5'-cap-dependent translational control and which codes for at least a truncated C protein (e.g., about 1-100N-terminal amino acids of the C protein), a prM/E protein, and an NS1 protein and (b) a second open reading frame (ORF2) that is under IRES-dependent translational control and which codes for the another portion of the C protein (such as a cytoplasmic portion) and the other flavivirus proteins not encoded by ORF1. In some embodiments, the portion of the C protein encoded in ORF2 is a codon-optimized sequence. Such attenuated flaviviruses or chimeric flaviviruses are both bicistronic and have a rearrangement of the genome. In some embodiments, the bicistronic, rearranged flavivirus also includes at

least one miRNA target sequence that is complementary to a host-specific miRNA, and thus may be a dual-approach, rearranged flavivirus or chimeric flavivirus.

[0104] As such, the present disclosure relates to attenuated flaviviruses, flavivirus genomes comprising said attenuating elements, immunogenic compositions comprising the attenuated flaviviruses described herein, multivalent immunogenic compositions that are immunogenic against more than one flavivirus, vaccine compositions comprising the attenuated flaviviruses described herein, methods for constructing the attenuated flaviviruses using the dual approach to attenuation, methods for inducing an immune reaction in a subject against a flavivirus by administering an effective amount of an immunogenic composition described herein, and methods for immunizing or vaccinating against a flavivirus (or multiple flaviviruses) by administering an effective amount of an immunogenic flavivirus composition or vaccine (or a multivalent composition or vaccine) to a subject in need.

[0105] All publications, patent applications, patents, figures and other references cited or referenced herein and all documents cited or referenced in the herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated by reference. For example, the present disclosure is related to U.S. Patent Application Publications 2009/0028900 A1, 2010/0316670 A1, 2005/0010043 A1, 2004/0033594 A1, 2005/0100886 A1, 2007/0134256, 2010/0104598 A1, 2007/0009552 A1, WO 2008/157136 A1, WO 2006/036233 A1, WO 03/092592 A1, WO 03/059384 A1, and WO 01/59093 A1, all of which are incorporated by reference for all purposes.

[0106] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. *See, e.g.*, Singleton P and Sainsbury D., *Dictionary of Microbiology and Molecular Biology* 3rd ed., J. Wiley & Sons, Chichester, New York, 2001, and Fields *Virology* 4th ed., Knipe D.M. and Howley P.M. eds, Lippincott Williams & Wilkins, Philadelphia 2001.

[0107] The term "about" means within 1, 2, or 3 nucleotides.

[0108] The articles "a" and "an" as used herein and in the appended claims are used herein to refer to one or to more than one (*e.g.*, to at least one) of the grammatical object of the article unless the context clearly indicates otherwise. By way of example, "an element" means one element or more than one element.

[0109] The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, *e.g.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, *e.g.*, "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B," when

used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

[0110] As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, *e.g.*, the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (*e.g.*, “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.”

[0111] In the claims, as well as in the specification, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, *e.g.*, to mean including but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

[0112] As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a nonlimiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

[0113] It should also be understood that, in certain methods described herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited unless the context indicates otherwise.

[0114] The terms “co-administration” and “co-administering” or “combination therapy” refer to both concurrent administration (administration of two or more therapeutic agents at the same time) and time

varied administration (administration of one or more therapeutic agents at a time different from that of the administration of an additional therapeutic agent or agents), as long as the therapeutic agents are present in the patient to some extent, preferably at effective amounts, at the same time. In certain preferred aspects, one or more of the present compounds described herein, are coadministered in combination with at least one additional bioactive agent. In particular aspects, the co-administration of compounds results in synergistic activity and/or therapy.

[0115] The term “patient” or “subject” is used throughout the specification to describe an animal, preferably a human or a domesticated animal, to whom treatment, including prophylactic treatment, with the compositions according to the present disclosure is provided. For treatment of those infections, conditions or disease states which are specific for a specific animal such as a human patient, the term patient refers to that specific animal, including a domesticated animal such as a dog or cat or a farm animal such as a horse, cow, sheep, etc. In general, in the present disclosure, the term patient refers to a human patient unless otherwise stated or implied from the context of the use of the term.

[0116] The term “effective” is used to describe an amount of a compound, composition (*e.g.*, attenuated flavivirus immunogenic composition) or component which, when used within the context of its intended use, effects an intended result. The term effective subsumes all other effective amount or effective concentration terms, which are otherwise described or used in the present application.

[0117] The term “residue” is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a peptide by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (*e.g.*, amino acid mimetics). Moreover, an amide bond mimetic includes peptide backbone modifications well known to those skilled in the art.

[0118] Individual substitutions, deletions or additions in the amino acid sequence, or in the nucleotide sequence encoding for the amino acids, which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are conservatively modified variations, wherein the alterations result in the substitution of an amino acid with a chemically similar amino acid. The following exemplary six groups each contain amino acids that are conservative substitutions for one another: (1) Alanine (A), Serine (S), Threonine (T); (2) Aspartic acid (D), Glutamic acid (E); (3) Asparagine (N), Glutamine (Q); (4) Arginine (R), Lysine (K); (5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and (6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[0119] As used herein, the term “attenuated” as in “attenuated flavivirus” refers to the reduction, inhibition, or otherwise impairment (including the complete removal) of one or more otherwise normal functions of a biological agent, such as a flavivirus. Normal functions of a flavivirus can include, for instance, viral replication, viral protein translation and post-translation proteolytic processing, viral protein assembly, virus particle packaging, virus release from infected cells, cell-to-cell spreading or reinfection, and host-to-vector transmission.

[0120] As used herein, the term “microRNA” or “miRNA” refers to a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals and some viruses, that functions in RNA silencing and post-transcriptional regulation of gene expression. miRNA are often tissue/cell-specific (*e.g.*, neuronal-specific miRNAs) or species-specific (*e.g.*, Arthropod-specific miRNAs).

[0121] As used herein, the term “translational control” refers to the molecular mechanism that drives translation of a mRNA or otherwise translation of a single stranded RNA molecule (such as a flavivirus genome). The flaviviruses herein described generally comprise at least two open reading frames (ORFs) each with a separate translational control element, including the eukaryotic 5'-cap-translational control element (the typical mechanism utilized by cells to translate mRNA) and the IRES-dependent translational control element.

[0122] As used herein, the “internal ribosome entry site” or (“IRES”) refers to a naturally occurring genetic element found in certain viruses (including the poliovirus and the encephalomyocarditis virus (EMCV)) that initiates protein translation from RNA in an end-independent manner, as part of the greater process of protein synthesis. In eukaryotic translation, initiation typically occurs at the 5' end of mRNA molecules, since 5' cap recognition is required for the assembly of the initiation complex. The location for IRES elements is often in the 5'UTR, but can also occur elsewhere in mRNAs. In the present disclosure, the IRES may be inserted in the 5'UTR, the 3'UTR, or anywhere in the coding region upstream from a gene encoding a virus protein including C, prM, E, and NS1-NS5. In various embodiments, it is contemplated that a translation stop signal can be inserted just upstream of (*e.g.*, prior to, or 5' of) the IRES element.

[0123] As used herein, the term “monocistronic,” as in monocistronic flavivirus genome, refers to the naturally occurring flaviviruses which comprise a single stranded RNA genome having a single 5'-cap translation initiation point and a single open reading frame which is translated to form a single polyprotein containing each of the viral proteins C, prM, E, and NS1-NS5. The polyprotein is proteolytically processed to produce the individual viral proteins.

[0124] As used herein, the term “bicistronic,” as in bicistronic flavivirus genome, refers to a modified flavivirus genome that contains not a single open reading frame but two open reading frames, each with its own independent point of translation initiation. The attenuated flaviviruses described herein are bicistronic due to insertion of an IRES into the genome, thereby resulting in a genome having the normal 5'-cap-dependent translation initiation site and the IRES-dependent translation initiation site. In various embodiments, it is contemplated that a translation stop signal can be inserted just upstream of (*e.g.*, prior to or 5' of) the IRES element.

[0125] As used herein, reference to a “dual approach” for attenuation of a flavivirus refers to the modification of a flavivirus in at least two ways: (a) the insertion of miRNA targeting sequences (*e.g.*, target sequences for one or more miRNAs) and (b) insertion of an internal ribosome entry site (IRES) (*e.g.*, IRES from encephalomyocarditis virus) upstream of a viral protein (*e.g.*, viral capsid (C) protein), thereby forming

a bicistronic virus construct, causing manipulation of the levels or molar amounts of virus protein(s) under translational control of the IRES as a separate cistronic element. In certain embodiments, the attenuated bicistronic flavivirus may also be achieved by constructing a translation unit that comprises the IRES inserted upstream of one or more viral genes (*e.g.*, C, prM, E, NS1-NS5) which is then inserted as a translation unit into the 5'UTR or the 3'UTR, and with a concomitant deletion or inactivation of the corresponding viral gene(s) at their native location in the genome, thereby creating a bicistronic system whereby some of the viral genes are under normal 5'-cap translation control and the remaining viral genes are translated under control of the IRES.

[0126] As used herein, reference to a “bicistronic, rearranged” flavivirus refers to modification of a flavivirus by insertion of an internal ribosome entry site (IRES) (*e.g.*, IRES from encephalomyocarditis virus) upstream of a viral protein (*e.g.*, viral capsid (C) protein), thereby forming a bicistronic virus construct and modification of the order of the encoded proteins (or at least a portion of an encoded protein) in the flavivirus genome compared to a wild type flavivirus genome. In certain embodiments, the attenuated bicistronic flavivirus may also be achieved by constructing a translation unit that comprises the IRES inserted upstream of one or more viral genes (*e.g.*, C, prM, E, NS1-NS5) which is then inserted as a translation unit into the 5'UTR or the 3'UTR, and with a concomitant deletion or inactivation of the corresponding viral gene(s) at their native location in the genome, thereby creating a bicistronic system whereby some of the viral genes are under normal 5'-cap translation control and the remaining viral genes are translated under control of the IRES. In one example, at least a portion of the C protein coding sequence is located downstream of the prM/E/NS1 coding sequence, as compared to upstream of the prM/E coding sequence in a wild type flavivirus genome.

[0127] As used herein, “mir-124” is a host-specific miRNA. A target sequence for mir-124 is a sequence complementary to mir-124 which may have the following sequence: 5'-TTCGAATCATACAGCTAGATAACCAAAGACTCGAG-3' (SEQ ID NO: 1).

[0128] As used herein, “mir-9” is a host-specific miRNA. A target sequence for mir-9 is a sequence complementary to mir-9 which may have the following sequence: 5'-TTCGAATGGCATTACCGCGTGCCTTAAGTCGAG-3' (SEQ ID NO: 2).

[0129] As used herein, “mir-34” is a host-specific miRNA. A target sequence for mir-34 is a sequence complementary to mir-34 which may have the following sequence: 5'-TAGGCAGTGTCATTAGCTGATTG-3' (SEQ ID NO: 6).

[0130] As used herein, “mir-141” is a host-specific miRNA. A target sequence for mir-141 is a sequence complementary to mir-141 which may have the following sequence: 5'-TAACACTGTCTGGTAAAGATGG-3' (SEQ ID NO: 7).

[0131] As used herein, “mir-202” is a host-specific miRNA. A target sequence for mir-202 is a sequence complementary to mir-202 which may have the following sequence: 5’-TTCCTATGCATATACTTCTTTG-3’ (SEQ ID NO: 8).

[0132] As used herein, “mir-148” is a host-specific miRNA. A target sequence for mir-148 is a sequence complementary to mir-148 which may have the following sequence: 5’-TCAGTGCACCTACAGAACTTTGT-3’ (SEQ ID NO: 9).

[0133] As used herein, “mir-517a” is a host-specific miRNA. A target sequence for mir-517a is a sequence complementary to mir-517a which may have the following sequence: 5’-ATCGTGCATCCCTTTAGAGTGT -3’ (SEQ ID NO: 10).

[0134] As used herein, “mir-518e” is a host-specific miRNA. A target sequence for mir-518e is a sequence complementary to mir-518e which may have the following sequence: 5’-AAAGCGCTTCCCTTCAGAGTG-3’ (SEQ ID NO: 11).

[0135] As used herein, “mir-515” is a host-specific miRNA. A target sequence for mir-515 is a sequence complementary to mir-515 which may have the following sequence: 5’-TTCTCCAAAAGAAAGCACTTTCTG-3’. (SEQ ID NO: 12).

[0136] As used herein, “mir-519” is a host-specific miRNA. A target sequence for mir-519 is a sequence complementary to mir-519 which may have the following sequence: 5’-AAAGTGCATCCTTTTAGAGTGT -3’ (SEQ ID NO: 17).

[0137] As used herein, “mir-184,” “mir-275,” and “mir-1” are mosquito-specific miRNAs. Target sequences for mir-184, mir-275, and mir-1 are 5’-GCCCTTATCAGTTCTCCGTCCA-3’ (SEQ ID NO: 3), 5’-GCGCTACTTCAGGTACCTGA-3’ (SEQ ID NO: 4), and 5’-CTCCATACTTCTTTACATTCCA-3’ (SEQ ID NO: 5), respectively.

[0138] Recombinant Attenuated Bicistronic Flaviviruses

[0139] The present disclosure relates in part to strategies for attenuating a flavivirus or flavivirus chimera that ensures the safety of the flavivirus or their chimeras for use as a live-virus vaccine to treat and/or immunize and/or vaccinate against a flavivirus infection, including infection by a Zika virus (ZIKV), a West Nile virus (WNV), a tick-borne encephalitis virus (TBEV), a yellow fever virus (YFV), Langat virus (LGTV), a Japanese encephalitis virus (JEV), dengue viruses (DENV), a Powassan virus (POWV), and an Usutu virus (USUV).

[0140] The disclosure relates to recombinant attenuated flavivirus genomes which have been attenuated by using a dual approach involving (a) the insertion of one or more miRNA targeting sequences and (b) insertion of an internal ribosome entry site (IRES) (*e.g.*, IRES from encephalomyocarditis virus) upstream of a viral protein (*e.g.*, viral capsid (C) protein), thereby forming a bicistronic virus construct, causing manipulation of the levels or molar amounts of virus protein(s) under translational control of the IRES as a

separate cistronic element. In some non-limiting examples, the recombinant attenuated flavivirus genome comprises or consists of a nucleic acid sequence with at least 95% sequence identity (such as at least 96%, 97%, 98%, 99%, or 100% identity) to any one of SEQ ID NOs: 13-16.

[0141] The disclosure also relates to recombinant attenuated flavivirus genomes which have been attenuated using a bicistronic and rearrangement approach involving a recombinant flavivirus genome that encodes (a) a first open reading frame (ORF1) that is under 5'-cap-dependent translational control and which codes for at least a truncated C protein (*e.g.*, a transmembrane portion of a C protein), a prM/E protein, and an NS1 protein and (b) a second open reading frame (ORF2) that is under IRES-dependent translational control (*e.g.*, IRES from encephalomyocarditis virus) and which codes for the remaining portion of the C protein (*e.g.*, a cytoplasmic portion of a C protein) and the other flavivirus proteins not encoded by ORF1. In some examples, ORF2 also includes truncated prM and NS1 genes. The portion of the C protein encoded in ORF2 may be 5' to NS2A-NS5, or may be 3' to NS2A-NS5. In some examples, the attenuated flavivirus genome has the organization shown schematically in FIG. 29. In some embodiments, the portion of the C protein encoded in ORF2 is a codon-optimized sequence. In additional embodiments, the bicistronic, rearranged flavivirus also includes at least one miRNA target sequence that is complementary to a host-specific miRNA, and thus may be a "dual-approach, rearranged" flavivirus or chimeric flavivirus.

[0142] Any flavivirus stain, serotype, or otherwise isolate may be used as a starting point for attenuation using the dual-approach attenuation scheme or the bicistronic rearrangement scheme. The flaviviruses that may be modified include, but are not limited to, a Zika virus (ZIKV), a West Nile virus (WNV), a tick-borne encephalitis virus (TBEV), a yellow fever virus (YFV), Langat virus (LGTV), a Japanese encephalitis virus (JEV), dengue viruses (DENV; *e.g.*, dengue serotype 1, dengue serotype 2, dengue serotype 3, or dengue serotype 4), a Powassan virus (POWV), and an Usutu virus (USUV).

[0143] In addition, any flavivirus that has been pre-attenuated by another mechanism (*e.g.*, by chimerization or by introduction of another attenuating mutation) may be used as a starting point for further attenuation using the dual-approach strategy or the bicistronic rearrangement strategy to attenuation described herein. Numerous attenuated flaviviruses and chimeric attenuated flavivirus are described in the following references and are incorporated herein by reference: U.S. Patent No. 8,778,671 and U.S. Patent Application Publications 2009/0028900 A1, 2010/0316670 A1, 2005/0010043 A1, 2004/0033594 A1, 2005/0100886 A1, 2007/0134256, 2010/0104598 A1, 2007/0009552 A1, WO 2008/157136 A1, WO 2006/036233 A1, WO 03/092592 A1, WO 03/059384 A1, and WO 01/59093 A1.

[0144] In certain embodiments, the disclosure relates to attenuated Zika viruses that are attenuated using the dual approach for attenuation or the bicistronic rearrangement scheme described herein. The Zika virus may be further attenuated (before or after applying an attenuation approach described herein) as a result of (a) the introduction of one or more attenuating mutations in the Zika viral genome, or (b) converting a ZIKV to a chimeric virus by modifying a first flavivirus "backbone" genome (*e.g.*, DEN1, DEN2, DEN3, or

DEN4, tick-borne encephalitis virus, or West Nile virus) to include one or more ZIKV genes encoding immunogenic components (*e.g.*, genes encoding Zika capsid, envelope, or pre-membrane proteins).

[0145] In the case of any flavivirus used herein, including Zika viruses, the further attenuating mutations can include any point mutation, insertion, deletion, or any combinations thereof, or any such mutation which reduces or eliminates the virulence of the flavivirus, but which do not block the ability of the virus to replicate and otherwise allow its immunogenic components to be expressed. The attenuating mutations may be introduced anywhere in the genome. For example, mutations may be introduced into one or more nonstructural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 genes), or one or more structural genes (capsid (C), premembrane/membrane (prM) and envelope (E) protein genes), or the '5' UTR or the 3' UTR, or combinations thereof.

[0146] In the case of the chimeric flaviviruses (*e.g.*, used as a starting point for the dual attenuation approach or the bicistronic rearrangement approach), the structure of the chimeric virus may vary considerably. In certain embodiments, a flavivirus genome (*e.g.*, a wildtype strain of ZIKV) can be modified by replacing or substituting one or more genetic components (*e.g.*, a nonstructural gene, a structural gene, or a 5' or 3' UTR) in the flavivirus genome with the same genetic component from another flavivirus (*e.g.*, from DEN1, DEN2, DEN3, or DEN4). In this embodiment, the first flavivirus can be considered as a backbone genome into which certain genetic components therein are replaced with corresponding genetic components from another flavivirus to form a chimeric virus. The resulting chimeric viruses are attenuated. In certain other embodiments, a flavivirus genome other than Zika (*e.g.*, DEN1, DEN2, DEN3, or DEN4) can be modified by replacing or substituting one or more genetic components (*e.g.*, a nonstructural gene, a structural gene, or a 5' or 3' UTR) in the flavivirus genome with the corresponding genetic component from a ZIKV genome. In this embodiment, the flavivirus genome can be considered as a backbone genome into which certain genetic components therein are replaced with corresponding genetic components from a ZIKV to form a chimeric virus. The resulting chimeric viruses are attenuated.

[0147] In one embodiment, the disclosure provides a chimeric ZIKV constructed from a flavivirus backbone wherein one or more structural genes (flavivirus C, prM, or E) therein have been replaced with the corresponding one or more structural genes from a ZIKV. This virus may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation.

[0148] In another embodiment, the disclosure provides a chimeric ZIKV constructed from a dengue virus backbone (*e.g.*, DEN1, DEN2, DEN3, or DEN4, or a chimeric thereof) wherein one or more structural genes (dengue C, prM, or E) therein have been replaced with the corresponding one or more structural genes from a ZIKV. This virus may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation.

[0149] In still another embodiment, the disclosure provides a chimeric ZIKV constructed from a dengue serotype 2 virus backbone, wherein one or more structural genes (dengue serotype 2 C, prM, or E)

therein have been replaced with the corresponding one or more structural genes from a ZIKV. This virus may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation.

[0150] In yet another embodiment, the disclosure provides a chimeric ZIKV constructed from a dengue serotype 4 virus backbone, wherein one or more structural genes (dengue serotype 4 C, prM, or E) therein have been replaced with the corresponding one or more structural genes from a ZIKV. This virus may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation.

[0151] In another embodiment, the disclosure provides a chimeric ZIKV constructed from a dengue serotype 1 virus backbone, wherein one or more structural genes (dengue serotype 1 C, prM, or E) therein have been replaced with the corresponding one or more structural genes from a ZIKV. This virus may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation.

[0152] In yet another embodiment, the disclosure provides a chimeric ZIKV constructed from a dengue serotype 3 virus backbone, wherein one or more structural genes (dengue serotype 3 C, prM, or E) therein have been replaced with the corresponding one or more structural genes from a ZIKV. This virus may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation.

[0153] In any of the chimeric ZIKV embodiments, the backbone virus used to form the chimeric ZIKV (*e.g.*, a ZIKV in certain embodiments, or another flavivirus in other embodiments) can comprise, in addition, one or more attenuating mutations as described above. These additional attenuating mutations may be introduced anywhere in the backbone genome. For example, mutations may be introduced into one or more nonstructural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 genes), or one or more structural genes (C, prM, and E protein genes), or the 5' UTR or the 3' UTR, or combinations thereof. For example, a chimeric ZIKV comprising a DEN2 backbone or a DEN4 backbone into which one or more structural protein genes therein were substituted with the corresponding Zika structural protein genes may further comprise a $\Delta 30$, $\Delta 30/31$, or $\Delta 86$, or any other attenuating mutation in the 3'UTR in addition to the $\Delta 30$, $\Delta 30/31$, or $\Delta 86$ mutations. This virus may be further attenuated using the dual approach to attenuation.

[0154] Immunogenic flaviviruses and flavivirus chimeras and methods for preparing the same are provided herein. The immunogenic flaviviruses and chimeras are useful, alone or in combination, in a pharmaceutically acceptable carrier as immunogenic compositions to immunize and protect individuals and animals against infection by a flavivirus. In certain embodiments, the flaviviruses should induce a humoral (antibody) response to a flavivirus, while the non-structural proteins of dengue virus should include a T-cell response.

[0155] Zika chimeras of the present disclosure can comprise nucleotide sequences encoding the immunogenic structural proteins of a ZIKV and further nucleotide sequences selected from the backbone of a dengue virus. Zika chimeras of the present disclosure can comprise nucleotide sequences encoding the immunogenic structural proteins and the nonstructural proteins of ZIKV and the 3'UTR of a dengue virus (*e.g.*, serotype 1, serotype 2, serotype 3, or serotype 4). In an embodiment, the 3'UTR of the dengue virus contains an attenuating deletion. The present disclosure also contemplates an attenuated ZIKV that includes an attenuating deletion or mutations, as described below with regard to dengue virus attenuation. Zika chimeric viruses derived from the nucleotide sequences can be used to induce an immunogenic response against ZIKV. Such viruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation.

[0156] In another embodiment, the chimera is a Zika nucleic acid chimera comprising a first nucleotide sequence encoding at least one structural protein from a ZIKV, and a second nucleotide sequence encoding nonstructural proteins from a dengue virus. In another embodiment, the dengue virus is attenuated. In another embodiment, the dengue virus is DEN2. In another embodiment, the dengue virus is DEN4. In yet another embodiment, the dengue virus is DEN3. In a further embodiment, the dengue virus is DEN1. In particular embodiments, the structural protein can be the C protein of a ZIKV, the prM protein of a ZIKV, the E protein of a ZIKV, or any combination thereof. Such viruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation.

[0157] As used herein, the terms “Zika chimera,” “Zika chimeric virus,” and “chimeric ZIKV” mean an infectious construct comprising nucleotide sequences encoding the immunogenicity of a ZIKV and further nucleotide sequences derived from the backbone of a flavivirus, such as, but not limited to, dengue virus, or an attenuated ZIKV. Similar definitions apply where another flavivirus is concerned, *e.g.*, “dengue chimera” or “West Nile chimera” or the like.

[0158] As used herein, “infectious construct” indicates a virus, a viral construct, a viral chimera, a nucleic acid derived from a virus, or any portion thereof, which may be used to infect a cell.

[0159] As used herein, “structural and nonstructural proteins” can mean or include any protein comprising or any gene encoding the sequence of the complete protein, an epitope of the protein, or any fragment comprising, for example, three or more amino acid residues thereof. The structural and nonstructural proteins can include functional variants or fragments, *e.g.*, truncated variants or codon-optimized variants.

[0160] In the case of ZIKV/DEN chimeras, the flavivirus chimeras of the disclosure are constructs formed by fusing structural protein genes from a ZIKV with non-structural protein genes from a flavivirus, such as, but not limited to, dengue virus, *e.g.*, DEN1, DEN2, DEN3, or DEN4. The use of any dengue strain is contemplated, such as those dengue strains of Table 1.

Table 1. Examples of Dengue Virus Strains and their Associated Accession Numbers

Serotype	Strain	Accession No.
1	02-20	AB178040
1	16007	AF180817
1	16007 PDK-13	AF180818
1	259par00	AF514883
1	280par00	AF514878
1	293arg00	AY206457
1	295arg00	AF514885
1	297arg00	AF514889
1	301arg00	AF514876
1	98901518	AB189120
1	98901530	AB189121
1	A88	AB074761
1	Abidjan	AF298807
1	ARG0028	AY277665
1	ARG0048	AY277666
1	ARG9920	AY277664
1	BR-90	AF226685
1	BR-01-MR	AF513110
1	BR-97-111	AF311956
1	BR-97-233	AF311958
1	BR-97-409	AF311957
1	Cambodia	AF309641
1	FGA-89	AF226687
1	FGA-NA d1d	AF226686
1	Fj231-04	DQ193572
1	GD05-99	AY376738
1	GD23-95	AY373427
1	GZ-80	AF350498
1	D1-hu-Yap-NIID27-2004	AB204803

Serotype	Strain	Accession No.
1	D1-H-IMTSSA-98-606	AF298808
1	Mochizuki	AB074760
1	D1.Myanmar.059-01	AY708047
1	D1.Myanmar.194-01	AY713474
1	D1.Myanmar.206-01	AY713475
1	D1.Myanmar.23819-96	AY722802
1	D1.Myanmar.305-01	AY713476
1	D1.Myanmar.31459-98	AY726555
1	D1.Myanmar.31987-98	AY726554
1	D1.Myanmar.32514-98	AY722803
1	D1.Myanmar.37726-01	AY726549
1	D1.Myanmar.38862-01	AY726550
1	D1.Myanmar.40553-71	AY713473
1	D1.Myanmar.40568-76	AY722801
1	D1.Myanmar.44168-01	AY726551
1	D1.Myanmar.44988-02	AY726552
1	D1.Myanmar.49440-02	AY726553
1	rWestern Pacific-delta30	AY145123
1	Western Pacific rDEN1mutF	AY145122
1	S275-90	A75711
1	D1-hu-Seychelles-NIID41-2003	AB195673
1	Singapore 8114-93	AY762084
1	Singapore S275-90	M87512
1	ThD1_0008_81	AY732483
1	ThD1_0049_01	AY732482
1	ThD1_0081_82	AY732481
1	ThD1_0097_94	AY732480
1	ThD1_0102_01	AY732479
1	ThD1_0323_91	AY732478

Serotype	Strain	Accession No.
1	ThD1_0336_91	AY732477
1	ThD1_0442_80	AY732476
1	ThD1_0488_94	AY732475
1	ThD1_0673_80	AY732474
1	Recombinant Western Pacific	AY145121
1	Nauru Island Western Pacific 45AZ5	NC_001477
1	Nauru Island, Western Pacific Bethesda	U88535
1	Nauru Island Western Pacific 45AZ5-PDK27	U88537
2	131	AF100469
2	16681-PDK53	M84728
2	16681 Blok	M84727
2	16681 Kinney	U87411
2	43	AF204178
2	44	AF204177
2	98900663	AB189122
2	98900665	AB189123
2	98900666	AB189124
2	BA05i	AY858035
2	Bangkok 1974	AJ487271
2	BR64022	AF489932
2	C0166	AF100463
2	C0167	AF100464
2	C0371	AF100461
2	C0390	AF100462
2	China 04	AF119661
2	Cuba115-97	AY702036
2	Cuba13-97	AY702034
2	Cuba165-97	AY702038
2	Cuba205-97	AY702039

Serotype	Strain	Accession No.
2	Cuba58-97	AY702035
2	Cuba89-97	AY702037
2	DR23-01	AB122020
2	DR31-01	AB122021
2	DR59-01	AB122022
2	FJ-10	AF276619
2	FJ11-99	AF359579
2	I348600	AY702040
2	IQT1797	AF100467
2	IQT2913	AF100468
2	Jamaica-N 1409	M20558
2	K0008	AF100459
2	K0010	AF100460
2	Mara4	AF100466
2	DEN2-H-IMTSSA-MART-98-703	AF208496
2	New Guinea C	AF038403
2	New Guinea C-PUO-218 hybrid	AF038402
2	New Guinea-C	M29095
2	PDK-53	U87412
2	S1 vaccine	NC_001474
2	TB16i	AY858036
2	ThD2_0017_98	DQ181799
2	ThD2_0026_88	DQ181802
2	ThD2_0038_74	DQ181806
2	ThD2_0055_99	DQ181798
2	ThD2_0078_01	DQ181797
2	ThD2_0168_79	DQ181805
2	ThD2_0263_95	DQ181800
2	ThD2_0284_90	DQ181801

Serotype	Strain	Accession No.
2	ThD2_0433_85	DQ181803
2	ThD2_0498_84	DQ181804
2	ThNH-28-93	AF022435
2	ThNH29-93	AF169678
2	ThNH36-93	AF169679
2	ThNH45-93	AF169680
2	ThNH-52-93	AF022436
2	ThNH54-93	AF169682
2	ThNH55-93	AF169681
2	ThNH62-93	AF169683
2	ThNH63-93	AF169684
2	ThNH69-93	AF169685
2	ThNH73-93	AF169686
2	ThNH76-93	AF169687
2	ThNH81-93	AF169688
2	ThNH-p36-93	AF022441
2	ThNH-7-93	AF022434
2	ThNH-p11-93	AF022437
2	ThNH-p12-93	AF022438
2	ThNH-p14-93	AF022439
2	ThNH-p16-93	AF022440
2	Tonga-74	AY744147
2	TSV01	AY037116
2	Taiwan-1008DHF	AY776328
2	Ven2	AF100465
3	D3-H-IMTSSA-MART-1999-1243	AY099337
3	D3-H-IMTSSA-SRI-2000-1266	AY099336
3	80-2	AF317645
3	98901403	AB189125

Serotype	Strain	Accession No.
3	98901437	AB189126
3	98901517	A6189127
3	98902890	AB189128
3	BA51	AY858037
3	BDH02-1	AY496871
3	BDH02-3	AY496873
3	BDH02-4	AY496874
3	BDH02-7	AY496877
3	BR74886-02	AY679147
3	C0331-94	AY876494
3	C0360-94	AY923865
3	den3_88	AY858038
3	den3_98	AY858039
3	FW01	AY858040
3	FW06	AY858041
3	H87	NC_001475
3	D3-Hu-TL018NIID-2005	AB214879
3	D3-Hu-TL029NIID-2005	AB214880
3	D3-Hu-TL109NIID-2005	AB214881
3	D3-Hu-TL129NIID-2005	AB214882
3	InJ_16_82	DQ401690
3	KJ30i	AY858042
3	kJ46	AY858043
3	kJ71	AY858044
3	mutant BDH02_01	DQ401689
3	mutant BDH02_03	DQ401691
3	mutant BDH02_04	DQ401692
3	mutant BDH02_07	DQ401693
3	mutant InJ_I6_82	DQ401694

Serotype	Strain	Accession No.
3	mutant PhMH_J1_97	DQ401695
3	PF89-27643	AY744677
3	PF89-320219	AY744678
3	PF90-3050	AY744679
3	PF90-3056	AY744680
3	PF90-6056	AY744681
3	PF92-2956	AY744682
3	PF92-2986	AY744683
3	PH86	AY858045
3	PhMH-J1-97	AY496879
3	PI64	AY858046
3	Singapore	AY662691
3	Singapore 8120-95	AY766104
3	Sleman-78	AY648961
3	TB16	AY858047
3	TB55i	AY858048
3	ThD3_0007_87	AY676353
3	ThD3_0010_87	AY676353
3	ThD3_0055_93	AY676351
3	ThD3_0104_93	AY676350
3	ThD3_1283_98	AY676349
3	ThD3_1687_98	AY676348
3	PF92-4190	AY744684
3	PF94-136116	AY744685
3	Taiwan-739079A	AY776329
4	2A	AF375822
4	Recombinant clone rDEN4	AF326825
4	2AdeI30	AF326826
4	814669	AF326573

Serotype	Strain	Accession No.
4	B5	AF289029
4	rDEN4del30	AF326827
4	H241	AY947539
4	rDEN4	NC_002640
4	Singapore 8976-95	AY762085
4	SW38i	AY858050
4	ThD4_0017_97	AY618989
4	ThD4_0087_77	AY618991
4	ThD4_0348_91	AY618990
4	ThD4_0476_97	AY618988
4	ThD4_0485_01	AY618992
4	ThD4_0734_00	AY618993
4	Taiwan-2K0713	AY776330
4	Unknown	M14931

[0161] The genome of any flavivirus can be used as the backbone in the attenuated chimeras described herein, which may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation. The backbone can contain mutations that contribute to the attenuation phenotype of the flavivirus or that facilitate replication in the cell substrate used for manufacture, *e.g.*, Vero cells. The mutations can be in the nucleotide sequence encoding nonstructural proteins, the 5' untranslated region or the 3' untranslated region. The backbone can also contain further mutations to maintain the stability of the attenuation phenotype and to reduce the possibility that the attenuated virus or chimera might revert back to the virulent wild-type virus.

[0162] In certain embodiments, the genome of any dengue virus can be used as the backbone in the attenuated chimeras described herein. The backbone can contain mutations that contribute to the attenuation phenotype of the dengue virus or that facilitate replication in the cell substrate used for manufacture, *e.g.*, Vero cells. The mutations can be in the nucleotide sequence encoding nonstructural proteins, the 5' untranslated region or the 3' untranslated region. The backbone can also contain further mutations to maintain the stability of the attenuation phenotype and to reduce the possibility that the attenuated virus or chimera might revert back to the virulent wild-type virus.

[0163] The attenuated flaviviruses described herein that have been attenuated by the dual approach or the bicistronic rearrangement approach may additionally carry one or more attenuating deletions in the 3'UTR. In particular, a mutation corresponding to a deletion of 30 ("Δ30") nucleotides from the 3'

untranslated region of the DEN4 genome between nucleotides 10478-10507 results in attenuation of the DEN4 virus. Furthermore, other dengue virus genomes containing an analogous deletion mutation in the 3' untranslated region of the genomes of other dengue virus serotypes may also be used as the backbone structure of the chimera of the present disclosure. For example, a mutation at this locus can be used in the genome of dengue type 1 (deletion of 30 nucleotides between 10562-10591 of DEN1; DEN1 Δ 30), dengue type 2 (deletion of 30 nucleotides between 10541-10570 of DEN2 Tonga/74; DEN2 Δ 30), dengue type 3 (deletion of 30 nucleotides between 10535-10565 of DEN3 Sleman/78; DEN3 Δ 30), and/or dengue type 4 (deletion of 30 nucleotides between 10478-10507 of DEN4; DEN4 Δ 30) as a backbone structure of the chimera of the present disclosure. The Δ 30 deletion removes the TL-2 homologous structure and sequence up to the TL-3 homologous structure (see *e.g.*, International Pat. Publ. No. WO 2017/156511, incorporated herein by reference). The analogous structures or regions may be deleted from any starting point flavivirus to further introduce attenuation characteristics.

[0164] In another embodiment, a mutation that is a deletion of 31 (" Δ 31") nucleotides from the TL-3 of the dengue genome (see *e.g.*, International Pat. Publ. No. WO 2017/156511, incorporated herein by reference) attenuates the backbone structure of the chimera of the present disclosure. Therefore, the genome of any dengue type 2 virus containing such a mutation at this locus can be used as the backbone in the attenuated chimeras described herein. Furthermore, other dengue virus genomes containing an analogous deletion mutation in the TL-3 of the genomes of other dengue virus serotypes may also be used as the backbone structure of the chimera of the present disclosure.

[0165] In some embodiments, the dengue backbone structure of the Zika chimera of the present disclosure includes both the Δ 30 and Δ 31 mutations (*e.g.*, DEN1 Δ 30/31, DEN2 Δ 30/31 Δ , DEN3 Δ 30/31, and/or DEN4 Δ 30/31).

[0166] In another embodiment, a mutation that is a deletion of 86 (" Δ 86") nucleotides removes the TL-2 homologous structure and the sequence up to the TL-3 homologous structure of a dengue virus (*e.g.*, DEN1, DEN2, DEN3 and/or DEN4) (see *e.g.*, International Pat. Publ. No. WO 2017/156511, incorporated herein by reference). Therefore, the genome of any dengue type 1, 2, 3, and/or 4 virus containing such a mutation at this locus can be used as the backbone in the attenuated chimeras described herein.

[0167] In a particular embodiment, the Zika chimera includes the DEN2 Δ 30 as the backbone structure of the chimera. In another embodiment, the Zika chimera includes the DEN4 Δ 30 as the backbone structure of the chimera. In other embodiments, the Zika chimera includes the DEN3 Δ 30/31 as the backbone structure of the chimera.

[0168] In various embodiments, the Zika chimeras of the disclosure can include mutations and/or deletions in the 3' UTR and/or 5' UTR that are in addition to the Δ 30, Δ 31, and Δ 86 deletions, including those described in International Patent Publication No. WO 2008/022196, which is incorporated herein by reference.

[0169] The mutations described above may be achieved by site-directed mutagenesis using techniques known to those skilled in the art. It will be understood by those skilled in the art that the virulence screening assays, as described herein and as are well known in the art, can be used to distinguish between virulent and attenuated backbone structures. Any of the mutagenesis techniques discussed in International Patent Publication No. WO 2008/022196 are contemplated.

[0170] Any of the 3'UTR mutations that are described above for dengue virus genome or Zika virus genomes may be applied to other flavivirus genomes at the corresponding or analogous sequence locations as the 3'UTR structures are widely conserved in flaviviruses. These 3'UTR deletions may be used to introduce further attenuating characteristics above and beyond the attenuation introduced using the dual strategy approach described herein. In other words, the disclosure contemplates a combination of attenuation strategies that include the dual approach strategy or the bicistronic rearranged strategy in combination with chimerism, point mutations, and attenuating 3'UTR deletions.

[0171] Dual Approach Attenuation Strategy/Virus Construction

[0172] Referring to FIG. 1A, a map of a typical flavivirus is provided.

[0173] Referring to FIGS. 1B and 1C, the concept of the dual attenuation approach is illustrated. FIG. 1B depicts a wildtype flavivirus genome showing the C/prM/E structural gene region, the nonstructural gene region, and the 5'UTR and 3'UTR regions. The genome, a single stranded + sense RNA, contains a single open reading frame (or "monocistronic coding region") that is translated based on a 3'-cap-dependent mechanism to form a single polypeptide, which is then cleaved by proteolytic processing into the individual viral proteins C, prM, E, and NS1-NS5. Viral assembly, replication, and further infection would subsequently occur.

[0174] FIG. 1C is a schematic depicting certain embodiments of the attenuated flaviviruses described herein which are attenuated by the dual approach of inserting an IRES upstream of a coding region of a viral protein and inserting one or more miRNA target sequences into the genome. As depicted in the schematic, construction of the attenuated flavivirus begins with a flavivirus genome. The flavivirus can be any flavivirus, including Zika, West Nile, or dengue virus. The flavivirus may also be a chimeric flavivirus that comprises a genome having a portion from one flavivirus and the remaining portion from a second flavivirus. The flavivirus genome is modified by inserting an IRES into the genome. The insertion may be made in any location, including in the 5'UTR, the coding region, and the 3'UTR; however, in certain embodiments the IRES insertion is made in the coding region and at a position upstream of the coding region of a gene coding for a viral gene. In a specific embodiment, the insertion is made upstream the gene encoding capsid (C) protein. The attenuation strategy also includes inserting one or more miRNA target sequences into one or more locations into the genome. The locations may be the same or different. A combination of locations may be used. The configuration of miRNA target sequences may also include placing the sequences in the same location in tandem. These modifications form a bicistronic genome, as depicted, which includes a first open reading frame (ORF1) under translational control of a 5'cap-dependent

mechanism, and a second open reading frame (ORF2) which is under translational control of the IRES. Two separate proteins or polyproteins are translated, *e.g.*, a product corresponding to each of the ORFs. The products are then cleaved into individual viral proteins via proteolytic processing. However, the bicistronic nature of the modified genome results in suboptimal levels and/or imbalanced relative or molar amounts of viral proteins, thereby impacting normal viral functions such as replication, assembly, packaging, release, and cell to cell spreading. In addition, the miRNA target sequences restrict replication of the flavivirus in cells or tissues that express the cognate or corresponding miRNA molecules.

[0175] Not shown in the general model of FIG. 1C are additional embodiments also contemplated herein which include other modifications such as constructing an IRES-controlled coding region or unit that comprises at least one gene encoding a viral protein or a functional fragment thereof and insertion of said IRES-dependent coding region or unit into a non-native location in the genome, such as downstream or upstream of the normal location of the viral gene, or in the 5' or 3' UTR (or NCR). The viral gene now under control of IRES translation may be deleted or otherwise inactivated at its native location. Examples of these types of embodiments can be seen in FIG. 1D.

[0176] FIG. 1D relates to the development of certain embodiments of the bicistronic flaviviruses described herein in an LGTV genetic background. Schematic representation of the viral genomes constructed for Example 1. C trn (48AA) denotes replication promoter region of C gene. ORF-shifting insertion (asterisk, position 151 nt of LGTV genome) of a single A nucleotide (Fr Sh +1) and ORF restoration (-1) are indicated. Boxes denote 2A protease gene of FMDV (2A) and mir-124 target (T) sequences. Shaded boxes denote codon-optimized sequence of C gene.

[0177] Bicistronic Rearrangement Approach Attenuation Strategy/Virus Construction

[0178] FIG. 29 is a schematic depicting certain embodiments of the bicistronic rearranged flaviviruses described herein. The flavivirus can be any flavivirus, including Zika, West Nile, or dengue virus. The flavivirus may also be a chimeric flavivirus that comprises a genome having a portion from one flavivirus and the remaining portion from a second flavivirus. The flavivirus genome is modified by inserting an IRES into the genome. The insertion may be made in any location, including in the 5'UTR, the coding region, and the 3'UTR; however, in certain embodiments the IRES insertion is made in the coding region and at a position downstream of the coding region of a gene coding for the NS1 viral protein. In a specific embodiment, the insertion is made downstream of the gene encoding the NS1 viral protein and upstream of a gene encoding a portion of the C protein, which in some examples is a codon-optimized sequence. The C protein may be codon-optimized for *Homo sapiens*. In addition, to increase heterogeneity between original and optimized sequences of C gene, all codons that remain intact after sequence optimization for *Homo sapiens* may be substituted with synonymous codons.

[0179] These modifications form a bicistronic genome, as depicted in FIG. 29, which includes a first open reading frame (ORF1) under translational control of a 5' cap-dependent mechanism, which encodes the a first portion of the C protein (in this example, a transmembrane portion of the C protein), prM protein, E

protein, and NS1 protein, and a second open reading frame (ORF2) which is under translational control of the IRES and encodes a second portion of the C protein (*e.g.*, the cytoplasmic domain of the C protein) and the NS2A-NS5 proteins. Two separate proteins or polyproteins are translated, *e.g.*, a product corresponding to each of the ORFs. The products are then cleaved into individual viral proteins via proteolytic processing. However, the bicistronic nature of the modified genome results in suboptimal levels and/or imbalanced relative or molar amounts of viral proteins, thereby impacting normal viral functions such as replication, assembly, packaging, release, and cell to cell spreading. In addition, the insertion of IRES sequence itself may result in substantial attenuation of viral replication. The IRES sequence contains multiple stem-loop structures (double-stranded RNAs) which could be targeted by various mechanisms of innate immunity. Being critical for translation of second ORF, the IRES sequence therefore could not be deleted in bicistronic viruses, ensuring the high genetic stability of attenuated phenotype.

[0180] In some embodiments, the bicistronic rearranged attenuation approach is combined with the dual approach attenuation described herein, for example, one or more miRNA targeting sequences are also inserted in the flavivirus genome. One or more miRNA target sequences may be inserted into one or more locations into the genome. The locations may be the same or different. A combination of locations may be used. The configuration of miRNA target sequences may also include placing the sequences in the same location in tandem. An example of this approach is shown in FIG. 26A, in the context of a ZIKV genome. This approach may be utilized with any flavivirus, including Zika, West Nile, or dengue virus or with any chimeric flavivirus that comprises a genome having a portion from one flavivirus and the remaining portion from a second flavivirus.

[0181] Live Attenuated Flavivirus Vaccines

[0182] The dual approach or bicistronic rearrangement approach attenuated flaviviruses described herein provide live, attenuated viruses useful as immunogens or vaccines. In some embodiments, the attenuated flaviviruses exhibit high immunogenicity while at the same time not producing dangerous pathogenic or lethal effects. In addition, the miRNA targeting sequence(s) (if present) allow for a means to restrict replication in a host cell or tissue, *e.g.*, restricted replication in the brain or reproductive tissue of vertebrate host or in arthropod vector (mosquito or tick). Restriction replication in a vector host provides for an environmentally safe virus whose arthropod-based vector transmission is eliminated or greatly diminished.

[0183] Viruses described herein are typically grown using techniques known in the art. Virus plaque or focus forming unit (PFU or FFU) titrations are then performed and plaques or FFU are counted in order to assess the viability, titer and phenotypic characteristics of the virus grown in cell culture. Wild type viruses are mutagenized to derive attenuated candidate starting materials.

[0184] Infectious viral clones are constructed from various flavivirus strains. The cloning of virus-specific cDNA fragments can also be accomplished, if desired. The cDNA fragments containing the structural or nonstructural protein genes may be amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from flavivirus RNA with various primers. Amplified fragments are cloned into the

cleavage sites of other intermediate clones. Intermediate, chimeric flavivirus clones are then sequenced to verify the sequence of the inserted flavivirus-specific cDNA.

[0185] Multivalent and Pentavalent Flavivirus Chimera Vaccine

[0186] The present disclosure not only relates to single virus attenuated flavivirus vaccines, but also to multivalent vaccines comprising the combination of at least two different vaccines, wherein at least one vaccine can be a vaccine against ZIKV. In other words, the disclosure contemplates combining one or more Zika vaccines (*e.g.*, an attenuated ZIKV, a chimeric attenuated ZIKV, or both) with one or more additional vaccines to other pathogens. In certain embodiments, the one or more additional vaccines are flavivirus vaccines. The one or more additional vaccines can be selected from any flavivirus vaccine, such as, but not limited to, a dengue vaccine (against DEN1, DEN2, DEN3, DEN4, or combinations thereof), yellow fever virus vaccine, JEV vaccine, TBEV vaccine, and West Nile virus vaccine.

[0187] In certain embodiments, a multivalent vaccine comprises:

[0188] (a) one or more Zika vaccines (*e.g.*, attenuated ZIKV or chimeric attenuated ZIKV), combined with one, two, three, four, or five additional flavivirus vaccines;

[0189] (b) one or more Zika vaccines (*e.g.*, attenuated ZIKV or chimeric attenuated ZIKV), combined with one, two, three, four, or five additional dengue vaccines (against DEN1, DEN2, DEN3, DEN4, or chimeras thereof);

[0190] (c) a chimeric attenuated ZIKV vaccine combined one or more dengue virus vaccines, said dengue virus vaccines each comprising one of a DEN1, DEN2, DEN3, or DEN4 virus, or chimerics thereof; and

[0191] (d) a chimeric attenuated ZIKV vaccine combined a DEN1 virus vaccine, a DEN2 virus vaccine, a DEN3 virus vaccine, and a DEN4 virus vaccine, or chimerics thereof, *e.g.*, to provide a pentavalent vaccine, wherein any of the flaviviruses may be attenuated using the dual approach to attenuation or bicistronic rearranged approach to attenuation described herein.

[0192] In embodiments relating to multivalent and/or pentavalent vaccines, the one or more additional flavivirus vaccines may comprise flaviviruses which comprise one or more attenuating mutations, including deletions and/or mutations in the 3'UTR, *e.g.*, $\Delta 30$, $\Delta 30/31$, and $\Delta 86$ attenuating mutations.

[0193] In certain embodiments, the description provides flavivirus (*e.g.*, dengue viruses) and chimeric flaviviruses (*e.g.*, dengue viruses) having one or more mutations that result in attenuation, methods of making such dengue viruses, and methods for using these flaviviruses to prevent or treat flavivirus infection (*e.g.*, dengue virus infection). In some examples, the mutation (or mutations) in the dengue virus is present in the 3' untranslated region (3'-UTR) formed by the most downstream approximately 384 nucleotides of the viral RNA, which have been shown to play a role in determining attenuation. The viruses and methods disclosed herein are described further, as follows. A molecular approach is used to develop a genetically stable live attenuated multivalent (*e.g.*, pentavalent) Zika, flavivirus virus immunogenic composition or vaccine. The multivalent immunogenic composition comprises: at least one first attenuated virus that is immunogenic against a flavivirus, and a second attenuated virus that is immunogenic against ZIKV. In a

particular embodiment, the at least one first attenuated virus is immunogenic against a virus selected from the group consisting of: dengue virus (*e.g.*, DEN1, DEN2, DEN3, DEN4, or a combination thereof), West Nile virus, yellow fever virus, Japanese encephalitis virus, and tick-borne encephalitis virus. In another embodiment, the second attenuated virus is a Zika nucleic acid chimera in accordance with the present disclosure. In another particular embodiment, the second attenuated virus is a ZIKV comprising one or more attenuating mutations in the genome. Each component of the multivalent vaccine is attenuated, genetically stable, and immunogenic. Any of the flaviviruses may be attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0194] For example, each component of a pentavalent vaccine, *e.g.*, DEN1, DEN2, DEN3, DEN4, and ZIKV, is attenuated, genetically stable, and immunogenic. The pentavalent vaccine provides simultaneous protection against each of the four dengue viruses, thereby precluding the possibility of developing the more serious illnesses dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which occurs in humans during secondary infection with a heterotypic wild-type dengue virus. Since dengue viruses may undergo genetic recombination in nature, the pentavalent vaccine will be genetically incapable of undergoing a recombination event between its five virus components that could lead to the generation of viruses lacking attenuating mutations. Previous approaches to develop a tetravalent dengue virus vaccine have been based on independently deriving each of the four virus components through separate mutagenic procedures, such as passage in tissue culture cells derived from a heterologous host. This strategy has yielded attenuated vaccine candidates previously. However, it is possible that gene exchanges among the four components of these independently derived tetravalent vaccines could occur in vaccinees, possibly creating a virulent recombinant virus. Virulent polioviruses derived from recombination have been generated in vaccinees following administration of a trivalent poliovirus vaccine. Any of these flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0195] In certain aspects, the present disclosure provides for a pentavalent vaccine that can include: (1) attenuated Zika chimera according to the present disclosure, rDEN4 Δ 30, rDEN1 Δ 30, rDEN2 Δ 30, and rDEN3 Δ 30 recombinant viruses containing a 30 nucleotide deletion (Δ 30) in a section of the 3' untranslated region (UTR) that is homologous to that in the rDEN4 Δ 30 recombinant virus; (2) attenuated nucleic acid Zika chimera according to the present disclosure, rDEN1 Δ 30, rDEN2 Δ 30, rDEN3 Δ 30, and rDEN4 Δ 30; (3) attenuated antigenic chimeric viruses, rDEN1/4 Δ 30, rDEN2/4 Δ 30, and rDEN3/4 Δ 30, for which the CME, ME, or E gene regions of rDEN4 Δ 30 have been replaced with those derived from DEN1, DEN2, or DEN3, rDEN4 Δ 30, and Zika chimera; alternatively rDEN1/3 Δ 30, rDEN2/3 Δ 30, and rDEN4/3 Δ 30 for which CME, ME, or E gene regions of rDEN3 Δ 30 have been replaced with those derived from DEN1, 2, or 4, rDEN3 Δ 30, and Zika chimera; alternatively rDEN1/2 Δ 30, rDEN3/2 Δ 30, and rDEN4/2 Δ 30 for which CME, ME, or E gene regions of rDEN2 Δ 30 have been replaced with those derived from DEN1, 3, or 4, rDEN2 Δ 30, and Zika chimera; and alternatively rDEN2/1 Δ 30, rDEN3/1 Δ 30, and rDEN4/1 Δ 30 for which

CME, ME, or E gene regions of rDEN1 Δ 30 have been replaced with those derived from DEN2, 3, or 4, rDEN1 Δ 30, and Zika chimera; and (4) attenuated rDEN1/4 Δ 30, rDEN2/4 Δ 30, rDEN3/4 Δ 30, rDEN4 Δ 30, and Zika chimera; alternatively rDEN1/3 Δ 30, rDEN2/3 Δ 30, rDEN4/3 Δ 30, rDEN3 Δ 30, and Zika chimera; alternatively rDEN1/2 Δ 30, rDEN3/2 Δ 30, rDEN4/2 Δ 30, rDEN2 Δ 30, and Zika chimera; and alternatively rDEN2/1 Δ 30, rDEN3/1 Δ 30, rDEN4/1 Δ 30, rDEN1 Δ 30, and Zika chimera. These pentavalent vaccines are unique since they contain a common shared attenuating mutation which eliminates the possibility of generating a virulent wild-type virus in a vaccinee since each component of the vaccine possesses the same Δ 30 attenuating deletion mutation. In addition, the rDEN1 Δ 30, rDEN2 Δ 30, rDEN3 Δ 30, rDEN4 Δ 30, Zika chimera pentavalent vaccine is the first to combine the stability of the Δ 30 mutation with broad antigenicity. Alternatively, utilizing the same scheme, the Δ 31, Δ 30/31, or Δ 86 deletions of the 3'UTR may be utilized in the chimera schemes described above, or within DEN1, DEN2, DEN3, DEN4, and Zika chimera. Since the Δ 30, Δ 31, Δ 30/31, and Δ 86 deletion mutation is in the 3' UTR of each virus, all of the proteins of the five component viruses are available to induce a protective immune response. Thus, the method provides a mechanism of attenuation that maintains each of the proteins of DEN1, DEN2, DEN3, DEN4, and Zika chimera viruses in a state that preserves the full capability of each of the proteins of the five viruses to induce humoral and cellular immune responses against all of the structural and non-structural proteins present in each dengue virus serotype and ZIKV. Any of the flaviviruses may be attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0196] As previously described, the DEN4 recombinant virus, rDEN4 Δ 30 (previously referred to as 2A Δ 30), was engineered to contain a 30 nucleotide deletion in the 3' UTR of the viral genome (Durbin *et al.* 2001 *Am J Trop Med Hyg* 65:405-13; Men *et al.* 1996 *J Virol* 70:3930-7). Evaluation in rhesus monkeys showed the virus to be significantly attenuated relative to wild-type parental virus, yet highly immunogenic and completely protective. Also, a phase I clinical trial with adult human volunteers showed the rDEN4 Δ 30 recombinant virus to be safe and satisfactorily immunogenic (Durbin *et al.* 2001 *Am J Trop Med Hyg* 65:405-13). To develop a pentavalent vaccine bearing a shared attenuating mutation in a untranslated region, the Δ 30, Δ 31, Δ 30/31, or Δ 86 deletion to attenuate wild-type dengue viruses of serotypes 1, 2, and 3 since it attenuated wild-type DEN4 virus for rhesus monkeys and was safe in humans, may be used. U.S. Patent Publication Application 2007/0009552. Any of these flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0197] According to an aspect, the present disclosure provides for a pentavalent immunogenic composition comprising: a first attenuated virus that is immunogenic against dengue serotype 1 (DEN1), a second attenuated virus that is immunogenic against dengue serotype 2 (DEN2), a third attenuated virus that is immunogenic against dengue serotype 3 (DEN3), a fourth attenuated virus that is immunogenic against dengue serotype 4 (DEN4), and a fifth attenuated virus that is immunogenic against ZIKV. In a particular embodiment, the fifth attenuated virus is the Zika nucleic acid chimera in accordance with the present

disclosure. Any of these flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0198] In an embodiment, the first, second, third, and fourth attenuated viruses are selected from the group consisting of: (1) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4Δ30, (2) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/1Δ30, (3) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/2Δ30, (4) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/3Δ30, (5) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4Δ30, (6) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (7) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (8) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (9) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4Δ30, (10) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (11) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (12) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (13) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4Δ30, (14) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (15) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (16) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (17) rDEN1Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4Δ30, (18) rDEN1Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/1Δ30, (19) rDEN1Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/2Δ30, (20) rDEN1Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/3Δ30, (21) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4Δ30, (22) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (23) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (24) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (25) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4Δ30, (26) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (27) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (28) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (29) rDEN1Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4Δ30, (30) rDEN1Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (31) rDEN1Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (32) rDEN1Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (33) rDEN1Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4Δ30, (34) rDEN1Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/1Δ30, (35) rDEN1Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/2Δ30, (36) rDEN1Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/3Δ30, (37) rDEN1Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4Δ30, (38) rDEN1Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (39) rDEN1Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (40) rDEN1Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (41) rDEN1Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4Δ30, (42) rDEN1Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (43) rDEN1Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (44) rDEN1Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (45) rDEN1Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4Δ30, (46) rDEN1Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (47) rDEN1Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (48) rDEN1Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (49) rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4Δ30, (50) rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/1Δ30, (51) rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/2Δ30, (52) rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/3Δ30, (53) rDEN1Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4Δ30, (54) rDEN1Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (55) rDEN1Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (56) rDEN1Δ30, rDEN2/4Δ30, rDEN3/1Δ30,

rDEN4/3Δ30, (57) rDEN1Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4Δ30, (58) rDEN1Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (59) rDEN1Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (60) rDEN1Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (61) rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4Δ30, (62) rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (63) rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (64) rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (65) rDEN1/2Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4Δ30, (66) rDEN1/2Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/1Δ30, (67) rDEN1/2Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/2Δ30, (68) rDEN1/2Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/3Δ30, (69) rDEN1/2Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4Δ30, (70) rDEN1/2Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (71) rDEN1/2Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (72) rDEN1/2Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (73) rDEN1/2Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4Δ30, (74) rDEN1/2Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (75) rDEN1/2Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (76) rDEN1/2Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (77) rDEN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4Δ30, (78) rDEN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (79) rDEN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (80) rDEN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (81) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4Δ30, (82) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/1Δ30, (83) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/2Δ30, (84) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/3Δ30, (85) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4Δ30, (86) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (87) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (88) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (89) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4Δ30, (90) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (91) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (92) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (93) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4Δ30, (94) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (95) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (96) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (97) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4Δ30, (98) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/1Δ30, (99) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/2Δ30, (100) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/3Δ30, (101) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4Δ30, (102) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (103) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (104) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (105) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4Δ30, (106) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (107) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (108) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (109) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4Δ30, (110) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (111) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (112) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (113) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4Δ30, (114) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/1Δ30, (115) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/2Δ30, (116)

rDEN1/2Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/3Δ30, (117) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4Δ30, (118) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (119) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (120) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (121) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4Δ30, (122) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (123) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (124) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (125) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4Δ30, (126) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (127) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (128) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (129) rDEN1/3Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4Δ30, (130) rDEN1/3Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/1Δ30, (131) rDEN1/3Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/2Δ30, (132) rDEN1/3Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/3Δ30, (133) rDEN1/3Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4Δ30, (134) rDEN1/3Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (135) rDEN1/3Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (136) rDEN1/3Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (137) rDEN1/3Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4Δ30, (138) rDEN1/3Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (139) rDEN1/3Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (140) rDEN1/3Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (141) rDEN1/3Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4Δ30, (142) rDEN1/3Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (143) rDEN1/3Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (144) rDEN1/3Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (145) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4Δ30, (146) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/1Δ30, (147) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/2Δ30, (148) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/3Δ30, (149) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4Δ30, (150) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (151) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (152) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (153) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4Δ30, (154) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (155) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (156) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (157) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4Δ30, (158) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (159) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (160) rDEN1/3Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (161) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4Δ30, (162) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/1Δ30, (163) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/2Δ30, (164) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/3Δ30, (165) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4Δ30, (166) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (167) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (168) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (169) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4Δ30, (170) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (171) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (172) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (173) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4Δ30, (174)

rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (175) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (176) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (177) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4Δ30, (178) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/1Δ30, (179) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/2Δ30, (180) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/3Δ30, (181) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4Δ30, (182) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (183) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (184) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (185) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4Δ30, (186) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (187) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (188) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (189) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4Δ30, (190) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (191) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (192) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (193) rDEN1/4Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4Δ30, (194) rDEN1/4Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/1Δ30, (195) rDEN1/4Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/2Δ30, (196) rDEN1/4Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/3Δ30, (197) rDEN1/4Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4Δ30, (198) rDEN1/4Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (199) rDEN1/4Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (200) rDEN1/4Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (201) rDEN1/4Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4Δ30, (202) rDEN1/4Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (203) rDEN1/4Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (204) rDEN1/4Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (205) rDEN1/4Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4Δ30, (206) rDEN1/4Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (207) rDEN1/4Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (208) rDEN1/4Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (209) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4Δ30, (210) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/1Δ30, (211) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/2Δ30, (212) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/3Δ30, (213) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4Δ30, (214) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (215) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (216) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (217) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4Δ30, (218) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (219) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (220) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (221) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4Δ30, (222) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (223) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (224) rDEN1/4Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (225) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4Δ30, (226) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/1Δ30, (227) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/2Δ30, (228) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/3Δ30, (229) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4Δ30, (230) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (231) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/2Δ30,

(232) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (233) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4Δ30, (234) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (235) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (236) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (237) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4Δ30, (238) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (239) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/2Δ30, (240) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/3Δ30, (241) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4Δ30, (242) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/1Δ30, (243) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/2Δ30, (244) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/3Δ30, (245) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4Δ30, (246) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/1Δ30, (247) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/2Δ30, (248) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/3Δ30, (249) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4Δ30, (250) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/1Δ30, (251) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/2Δ30, (252) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/3Δ30, (253) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4Δ30, (254) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/1Δ30, (255) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/2Δ30, and (256) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/3Δ30. In another embodiment, the fifth attenuated virus of the pentavalent immunogenic composition of any of the combinations of the first, second, third and fourth attenuated viruses described above is a Zika chimera of the present disclosure, as described in greater detail above. In an embodiment, each of the attenuated viruses comprises the same dengue backbone. In another embodiment, the fifth attenuated virus comprises a different dengue virus backbone than the first, second, third, and fourth attenuated viruses. Any of these flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0199] In an embodiment, the first, second, third, and fourth attenuated viruses are selected from the group consisting of: (1) rDEN1Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4Δ31, (2) rDEN1Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/1Δ31, (3) rDEN1Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/2Δ31, (4) rDEN1Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/3Δ31, (5) rDEN1Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4Δ31, (6) rDEN1Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (7) rDEN1Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (8) rDEN1Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (9) rDEN1Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4Δ31, (10) rDEN1Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (11) rDEN1Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (12) rDEN1Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (13) rDEN1Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4Δ31, (14) rDEN1Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (15) rDEN1Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (16) rDEN1Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (17) rDEN1Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4Δ31, (18) rDEN1Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/1Δ31, (19) rDEN1Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/2Δ31, (20) rDEN1Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/3Δ31, (21) rDEN1Δ31, rDEN2/1Δ31, rDEN3/1Δ31,

rDEN4Δ31, (22) rDEN1Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (23) rDEN1Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (24) rDEN1Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (25) rDEN1Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4Δ31, (26) rDEN1Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (27) rDEN1Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (28) rDEN1Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (29) rDEN1Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4Δ31, (30) rDEN1Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (31) rDEN1Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (32) rDEN1Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (33) rDEN1Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4Δ31, (34) rDEN1Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/1Δ31, (35) rDEN1Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/2Δ31, (36) rDEN1Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/3Δ31, (37) rDEN1Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4Δ31, (38) rDEN1Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (39) rDEN1Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (40) rDEN1Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (41) rDEN1Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4Δ31, (42) rDEN1Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (43) rDEN1Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (44) rDEN1Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (45) rDEN1Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4Δ31, (46) rDEN1Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (47) rDEN1Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (48) rDEN1Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (49) rDEN1Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4Δ31, (50) rDEN1Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/1Δ31, (51) rDEN1Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/2Δ31, (52) rDEN1Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/3Δ31, (53) rDEN1Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4Δ31, (54) rDEN1Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (55) rDEN1Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (56) rDEN1Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (57) rDEN1Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4Δ31, (58) rDEN1Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (59) rDEN1Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (60) rDEN1Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (61) rDEN1Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4Δ31, (62) rDEN1Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (63) rDEN1Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (64) rDEN1Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (65) rDEN1/2Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4Δ31, (66) rDEN1/2Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/1Δ31, (67) rDEN1/2Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/2Δ31, (68) rDEN1/2Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/3Δ31, (69) rDEN1/2Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4Δ31, (70) rDEN1/2Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (71) rDEN1/2Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (72) rDEN1/2Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (73) rDEN1/2Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4Δ31, (74) rDEN1/2Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (75) rDEN1/2Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (76) rDEN1/2Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (77) rDEN1/2Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4Δ31, (78) rDEN1/2Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (79) rDEN1/2Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (80) rDEN1/2Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (81) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4Δ31, (82) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/1Δ31, (83)

rDEN1/2Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/2Δ31, (84) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/3Δ31, (85) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4Δ31, (86) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (87) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (88) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (89) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4Δ31, (90) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (91) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (92) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (93) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4Δ31, (94) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (95) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (96) rDEN1/2Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (97) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4Δ31, (98) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/1Δ31, (99) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/2Δ31, (100) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/3Δ31, (101) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4Δ31, (102) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (103) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (104) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (105) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4Δ31, (106) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (107) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (108) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (109) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4Δ31, (110) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (111) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (112) rDEN1/2Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (113) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4Δ31, (114) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/1Δ31, (115) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/2Δ31, (116) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/3Δ31, (117) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4Δ31, (118) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (119) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (120) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (121) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4Δ31, (122) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (123) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (124) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (125) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4Δ31, (126) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (127) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (128) rDEN1/2Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (129) rDEN1/3Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4Δ31, (130) rDEN1/3Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/1Δ31, (131) rDEN1/3Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/2Δ31, (132) rDEN1/3Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/3Δ31, (133) rDEN1/3Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4Δ31, (134) rDEN1/3Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (135) rDEN1/3Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (136) rDEN1/3Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (137) rDEN1/3Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4Δ31, (138) rDEN1/3Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (139) rDEN1/3Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (140) rDEN1/3Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (141)

rDEN1/3Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4Δ31, (142) rDEN1/3Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (143) rDEN1/3Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (144) rDEN1/3Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (145) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4Δ31, (146) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/1Δ31, (147) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/2Δ31, (148) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/3Δ31, (149) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4Δ31, (150) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (151) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (152) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (153) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4Δ31, (154) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (155) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (156) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (157) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4Δ31, (158) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (159) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (160) rDEN1/3Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (161) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4Δ31, (162) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/1Δ31, (163) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/2Δ31, (164) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/3Δ31, (165) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4Δ31, (166) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (167) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (168) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (169) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4Δ31, (170) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (171) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (172) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (173) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4Δ31, (174) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (175) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (176) rDEN1/3Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (177) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4Δ31, (178) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/1Δ31, (179) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/2Δ31, (180) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/3Δ31, (181) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4Δ31, (182) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (183) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (184) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (185) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4Δ31, (186) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (187) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (188) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (189) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4Δ31, (190) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (191) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (192) rDEN1/3Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (193) rDEN1/4Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4Δ31, (194) rDEN1/4Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/1Δ31, (195) rDEN1/4Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/2Δ31, (196) rDEN1/4Δ31, rDEN2Δ31, rDEN3Δ31, rDEN4/3Δ31, (197) rDEN1/4Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4Δ31, (198) rDEN1/4Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (199)

rDEN1/4Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (200) rDEN1/4Δ31, rDEN2Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (201) rDEN1/4Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4Δ31, (202) rDEN1/4Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (203) rDEN1/4Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (204) rDEN1/4Δ31, rDEN2Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (205) rDEN1/4Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4Δ31, (206) rDEN1/4Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (207) rDEN1/4Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (208) rDEN1/4Δ31, rDEN2Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (209) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4Δ31, (210) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/1Δ31, (211) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/2Δ31, (212) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3Δ31, rDEN4/3Δ31, (213) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4Δ31, (214) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (215) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (216) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (217) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4Δ31, (218) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (219) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (220) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (221) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4Δ31, (222) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (223) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (224) rDEN1/4Δ31, rDEN2/1Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (225) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4Δ31, (226) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/1Δ31, (227) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/2Δ31, (228) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3Δ31, rDEN4/3Δ31, (229) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4Δ31, (230) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (231) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (232) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (233) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4Δ31, (234) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (235) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (236) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (237) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4Δ31, (238) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (239) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/2Δ31, (240) rDEN1/4Δ31, rDEN2/3Δ31, rDEN3/4Δ31, rDEN4/3Δ31, (241) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4Δ31, (242) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/1Δ31, (243) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/2Δ31, (244) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3Δ31, rDEN4/3Δ31, (245) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4Δ31, (246) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/1Δ31, (247) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/2Δ31, (248) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/1Δ31, rDEN4/3Δ31, (249) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4Δ31, (250) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/1Δ31, (251) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/2Δ31, (252) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/2Δ31, rDEN4/3Δ31, (253) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4Δ31, (254) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/1Δ31, (255) rDEN1/4Δ31, rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/2Δ31, and (256) rDEN1/4Δ31,

rDEN2/4Δ31, rDEN3/4Δ31, rDEN4/3Δ31. In another embodiment, the fifth attenuated virus of the pentavalent immunogenic composition of any of the combinations of the first, second, third and fourth attenuated viruses described above is a Zika chimera of the present disclosure, as described in greater detail above. In an embodiment, each of the attenuated viruses comprises the same dengue backbone. In another embodiment, the fifth attenuated virus comprises a different dengue virus backbone than the first, second, third, and fourth attenuated viruses. Any of these flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0200] In an embodiment, the first, second, third, and fourth attenuated viruses are selected from the group consisting of: (1) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3Δ30/31, rDEN4Δ30/31, (2) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3Δ30/31, rDEN4/1Δ30/31, (3) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3Δ30/31, rDEN4/2Δ30/31, (4) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3Δ30/31, rDEN4/3Δ30/31, (5) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/1Δ30/31, rDEN4Δ30/31, (6) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/1Δ30/31, rDEN4/1Δ30/31, (7) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/1Δ30/31, rDEN4/2Δ30/31, (8) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/1Δ30/31, rDEN4/3Δ30/31, (9) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/2Δ30/31, rDEN4Δ30/31, (10) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/2Δ30/31, rDEN4/1Δ30/31, (11) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/2Δ30/31, rDEN4/2Δ30/31, (12) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/2Δ30/31, rDEN4/3Δ30/31, (13) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/4Δ30/31, rDEN4Δ30/31, (14) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/4Δ30/31, rDEN4/1Δ30/31, (15) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/4Δ30/31, rDEN4/2Δ30/31, (16) rDEN1Δ30/31, rDEN2Δ30/31, rDEN3/4Δ30/31, rDEN4/3Δ30/31, (17) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3Δ30/31, rDEN4Δ30/31, (18) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3Δ30/31, rDEN4/1Δ30/31, (19) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3Δ30/31, rDEN4/2Δ30/31, (20) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3Δ30/31, rDEN4/3Δ30/31, (21) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/1Δ30/31, rDEN4Δ30/31, (22) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/1Δ30/31, rDEN4/1Δ30/31, (23) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/1Δ30/31, rDEN4/2Δ30/31, (24) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/1Δ30/31, rDEN4/3Δ30/31, (25) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/2Δ30/31, rDEN4Δ30/31, (26) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/2Δ30/31, rDEN4/1Δ30/31, (27) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/2Δ30/31, rDEN4/2Δ30/31, (28) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/2Δ30/31, rDEN4/3Δ30/31, (29) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/4Δ30/31, rDEN4Δ30/31, (30) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/4Δ30/31, rDEN4/1Δ30/31, (31) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/4Δ30/31, rDEN4/2Δ30/31, (32) rDEN1Δ30/31, rDEN2/1Δ30/31, rDEN3/4Δ30/31, rDEN4/3Δ30/31, (33) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3Δ30/31, rDEN4Δ30/31, (34) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3Δ30/31, rDEN4/1Δ30/31, (35) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3Δ30/31, rDEN4/2Δ30/31, (36) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3Δ30/31, rDEN4/3Δ30/31, (37) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3/1Δ30/31, rDEN4Δ30/31, (38) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3/1Δ30/31, rDEN4/1Δ30/31, (39) rDEN1Δ30/31, rDEN2/3Δ30/31,

rDEN3/1Δ30/31, rDEN4/2Δ30/31, (40) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3/1Δ30/31, rDEN4/3Δ30/31,
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 rDEN2/3Δ30/31, rDEN3/2Δ30/31, rDEN4/1Δ30/31, (43) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3/2Δ30/31,
 rDEN4/2Δ30/31, (44) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3/2Δ30/31, rDEN4/3Δ30/31, (45)
 rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3/4Δ30/31, rDEN4Δ30/31, (46) rDEN1Δ30/31, rDEN2/3Δ30/31,
 rDEN3/4Δ30/31, rDEN4/1Δ30/31, (47) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3/4Δ30/31, rDEN4/2Δ30/31,
 (48) rDEN1Δ30/31, rDEN2/3Δ30/31, rDEN3/4Δ30/31, rDEN4/3Δ30/31, (49) rDEN1Δ30/31,
 rDEN2/4Δ30/31, rDEN3Δ30/31, rDEN4Δ30/31, (50) rDEN1Δ30/31, rDEN2/4Δ30/31, rDEN3Δ30/31,
 rDEN4/1Δ30/31, (51) rDEN1Δ30/31, rDEN2/4Δ30/31, rDEN3Δ30/31, rDEN4/2Δ30/31, (52)
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rDEN2/3Δ30/31, rDEN3Δ30/31, rDEN4Δ30/31, (226) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3Δ30/31, rDEN4/1Δ30/31, (227) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3Δ30/31, rDEN4/2Δ30/31, (228) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3Δ30/31, rDEN4/3Δ30/31, (229) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/1Δ30/31, rDEN4Δ30/31, (230) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/1Δ30/31, rDEN4/1Δ30/31, (231) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/1Δ30/31, rDEN4/2Δ30/31, (232) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/1Δ30/31, rDEN4/3Δ30/31, (233) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/2Δ30/31, rDEN4Δ30/31, (234) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/2Δ30/31, rDEN4/1Δ30/31, (235) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/2Δ30/31, rDEN4/2Δ30/31, (236) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/2Δ30/31, rDEN4/3Δ30/31, (237) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/4Δ30/31, rDEN4Δ30/31, (238) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/4Δ30/31, rDEN4/1Δ30/31, (239) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/4Δ30/31, rDEN4/2Δ30/31, (240) rDEN1/4Δ30/31, rDEN2/3Δ30/31, rDEN3/4Δ30/31, rDEN4/3Δ30/31, (241) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3Δ30/31, rDEN4Δ30/31, (242) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3Δ30/31, rDEN4/1Δ30/31, (243) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3Δ30/31, rDEN4/2Δ30/31, (244) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3Δ30/31, rDEN4/3Δ30/31, (245) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/1Δ30/31, rDEN4Δ30/31, (246) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/1Δ30/31, rDEN4/1Δ30/31, (247) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/1Δ30/31, rDEN4/2Δ30/31, (248) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/1Δ30/31, rDEN4/3Δ30/31, (249) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/2Δ30/31, rDEN4Δ30/31, (250) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/2Δ30/31, rDEN4/1Δ30/31, (251) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/2Δ30/31, rDEN4/2Δ30/31, (252) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/2Δ30/31, rDEN4/3Δ30/31, (253) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/4Δ30/31, rDEN4Δ30/31, (254) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/4Δ30/31, rDEN4/1Δ30/31, (255) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/4Δ30/31, rDEN4/2Δ30/31, and (256) rDEN1/4Δ30/31, rDEN2/4Δ30/31, rDEN3/4Δ30/31, rDEN4/3Δ30/31. In another embodiment, the fifth attenuated virus of the pentavalent immunogenic composition of any of the combinations of the first, second, third and fourth attenuated viruses described above is a Zika chimera of the present disclosure, as described in greater detail above. In an embodiment, each of the attenuated viruses comprises the same dengue backbone. In another embodiment, the fifth attenuated virus comprises a different dengue virus backbone than the first, second, third, and fourth attenuated viruses. Any of these flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0201] In an embodiment, the first, second, third, and fourth attenuated viruses are selected from the group consisting of: (1) rDEN1Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4Δ86, (2) rDEN1Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/1Δ86, (3) rDEN1Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/2Δ86, (4) rDEN1Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/3Δ86, (5) rDEN1Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4Δ86, (6)

rDEN1Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (7) rDEN1Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (8) rDEN1Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (9) rDEN1Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4Δ86, (10) rDEN1Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (11) rDEN1Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (12) rDEN1Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (13) rDEN1Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4Δ86, (14) rDEN1Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (15) rDEN1Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (16) rDEN1Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (17) rDEN1Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4Δ86, (18) rDEN1Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/1Δ86, (19) rDEN1Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/2Δ86, (20) rDEN1Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/3Δ86, (21) rDEN1Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4Δ86, (22) rDEN1Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (23) rDEN1Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (24) rDEN1Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (25) rDEN1Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4Δ86, (26) rDEN1Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (27) rDEN1Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (28) rDEN1Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (29) rDEN1Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4Δ86, (30) rDEN1Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (31) rDEN1Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (32) rDEN1Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (33) rDEN1Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4Δ86, (34) rDEN1Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/1Δ86, (35) rDEN1Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/2Δ86, (36) rDEN1Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/3Δ86, (37) rDEN1Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4Δ86, (38) rDEN1Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (39) rDEN1Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (40) rDEN1Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (41) rDEN1Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4Δ86, (42) rDEN1Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (43) rDEN1Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (44) rDEN1Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (45) rDEN1Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4Δ86, (46) rDEN1Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (47) rDEN1Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (48) rDEN1Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (49) rDEN1Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4Δ86, (50) rDEN1Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/1Δ86, (51) rDEN1Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/2Δ86, (52) rDEN1Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/3Δ86, (53) rDEN1Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4Δ86, (54) rDEN1Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (55) rDEN1Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (56) rDEN1Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (57) rDEN1Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4Δ86, (58) rDEN1Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (59) rDEN1Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (60) rDEN1Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (61) rDEN1Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4Δ86, (62) rDEN1Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (63) rDEN1Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (64) rDEN1Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (65) rDEN1/2Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4Δ86, (66) rDEN1/2Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/1Δ86, (67) rDEN1/2Δ86,

rDEN2Δ86, rDEN3Δ86, rDEN4/2Δ86, (68) rDEN1/2Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/3Δ86, (69)
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 rDEN3/2Δ86, rDEN4/2Δ86, (92) rDEN1/2Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (93)
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 rDEN4/1Δ86, (95) rDEN1/2Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (96) rDEN1/2Δ86,
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 (103) rDEN1/2Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (104) rDEN1/2Δ86, rDEN2/3Δ86,
 rDEN3/1Δ86, rDEN4/3Δ86, (105) rDEN1/2Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4Δ86, (106)
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 rDEN4/2Δ86, (108) rDEN1/2Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (109) rDEN1/2Δ86,
 rDEN2/3Δ86, rDEN3/4Δ86, rDEN4Δ86, (110) rDEN1/2Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/1Δ86,
 (111) rDEN1/2Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (112) rDEN1/2Δ86, rDEN2/3Δ86,
 rDEN3/4Δ86, rDEN4/3Δ86, (113) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4Δ86, (114) rDEN1/2Δ86,
 rDEN2/4Δ86, rDEN3Δ86, rDEN4/1Δ86, (115) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/2Δ86, (116)
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 rDEN4Δ86, (118) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (119) rDEN1/2Δ86,
 rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (120) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/3Δ86,
 (121) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4Δ86, (122) rDEN1/2Δ86, rDEN2/4Δ86,
 rDEN3/2Δ86, rDEN4/1Δ86, (123) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (124)
 rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (125) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/4Δ86,

rDEN4Δ86, (126) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (127) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (128) rDEN1/2Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (129) rDEN1/3Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4Δ86, (130) rDEN1/3Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/1Δ86, (131) rDEN1/3Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/2Δ86, (132) rDEN1/3Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/3Δ86, (133) rDEN1/3Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4Δ86, (134) rDEN1/3Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (135) rDEN1/3Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (136) rDEN1/3Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (137) rDEN1/3Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4Δ86, (138) rDEN1/3Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (139) rDEN1/3Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (140) rDEN1/3Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (141) rDEN1/3Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4Δ86, (142) rDEN1/3Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (143) rDEN1/3Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (144) rDEN1/3Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (145) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4Δ86, (146) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/1Δ86, (147) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/2Δ86, (148) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/3Δ86, (149) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4Δ86, (150) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (151) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (152) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (153) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4Δ86, (154) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (155) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (156) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (157) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4Δ86, (158) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (159) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (160) rDEN1/3Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (161) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4Δ86, (162) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/1Δ86, (163) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/2Δ86, (164) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/3Δ86, (165) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4Δ86, (166) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (167) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (168) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (169) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4Δ86, (170) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (171) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (172) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (173) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4Δ86, (174) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (175) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (176) rDEN1/3Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (177) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4Δ86, (178) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/1Δ86, (179) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/2Δ86, (180) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/3Δ86, (181) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4Δ86, (182) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (183) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/2Δ86,

(184) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (185) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4Δ86, (186) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (187) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (188) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (189) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4Δ86, (190) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (191) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (192) rDEN1/3Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (193) rDEN1/4Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4Δ86, (194) rDEN1/4Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/1Δ86, (195) rDEN1/4Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/2Δ86, (196) rDEN1/4Δ86, rDEN2Δ86, rDEN3Δ86, rDEN4/3Δ86, (197) rDEN1/4Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4Δ86, (198) rDEN1/4Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (199) rDEN1/4Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (200) rDEN1/4Δ86, rDEN2Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (201) rDEN1/4Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4Δ86, (202) rDEN1/4Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (203) rDEN1/4Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (204) rDEN1/4Δ86, rDEN2Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (205) rDEN1/4Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4Δ86, (206) rDEN1/4Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (207) rDEN1/4Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (208) rDEN1/4Δ86, rDEN2Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (209) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4Δ86, (210) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/1Δ86, (211) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/2Δ86, (212) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3Δ86, rDEN4/3Δ86, (213) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4Δ86, (214) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (215) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (216) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (217) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4Δ86, (218) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (219) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (220) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (221) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4Δ86, (222) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (223) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (224) rDEN1/4Δ86, rDEN2/1Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (225) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4Δ86, (226) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/1Δ86, (227) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/2Δ86, (228) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3Δ86, rDEN4/3Δ86, (229) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4Δ86, (230) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (231) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (232) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (233) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4Δ86, (234) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (235) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (236) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (237) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4Δ86, (238) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (239) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/2Δ86, (240) rDEN1/4Δ86, rDEN2/3Δ86, rDEN3/4Δ86, rDEN4/3Δ86, (241) rDEN1/4Δ86, rDEN2/4Δ86,

rDEN3Δ86, rDEN4Δ86, (242) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/1Δ86, (243) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/2Δ86, (244) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3Δ86, rDEN4/3Δ86, (245) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4Δ86, (246) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/1Δ86, (247) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/2Δ86, (248) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/1Δ86, rDEN4/3Δ86, (249) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4Δ86, (250) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/1Δ86, (251) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/2Δ86, (252) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/2Δ86, rDEN4/3Δ86, (253) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4Δ86, (254) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/1Δ86, (255) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/2Δ86, and (256) rDEN1/4Δ86, rDEN2/4Δ86, rDEN3/4Δ86, rDEN4/3Δ86. In another embodiment, the fifth attenuated virus of the pentavalent immunogenic composition of any of the combinations of the first, second, third and fourth attenuated viruses described above is a Zika chimera of the present disclosure, as described in greater detail above. In an embodiment, each of the attenuated viruses comprises the same dengue backbone. In another embodiment, the fifth attenuated virus comprises a different dengue virus backbone than the first, second, third, and fourth attenuated viruses. Any of these flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0202] In other embodiments, the first, second, third and fourth attenuated viruses are selected independently from the first, second, third and fourth attenuated viruses articulated above, and the fifth attenuated virus is an attenuated ZIKV or chimeric ZIKV of the present disclosure, as described in greater detail above. For example, in an embodiment, the first attenuated virus is rDEN1Δ30, the second attenuated virus is rDEN2/4Δ30, the third attenuated virus is rDEN3Δ30/31, and the fourth attenuated virus is rDEN4Δ30. In an embodiment, the first attenuate virus is rDEN1Δ30, the second attenuated virus is rDEN2/4Δ30, the third attenuated virus is rDEN3Δ30/31, the fourth attenuated virus is rDEN4Δ30, and the fifth attenuated virus is ZIKV/DEN2Δ30 or ZIKV/DEN3Δ30. Any of these flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0203] Dengue/Zika Chimeric Vaccines Including the Dual Approach or Bicistronic Rearrangement Approach to Attenuation

[0204] The disclosed dengue and Zika chimeric viruses and nucleic acid chimeras provide live, attenuated viruses useful as immunogens or vaccines. In one embodiment, the chimeras exhibit high immunogenicity while at the same time not producing dangerous pathogenic or lethal effects.

[0205] The dengue chimeric viruses or nucleic acid chimeras of the present disclosure can comprise the structural genes of a dengue virus of one serotype in a wild-type or an attenuated dengue virus backbone of a different serotype, while the Zika-dengue chimeric viruses or nucleic acid chimeras of the present disclosure comprise the structural genes of a ZIKV in a wild-type or an attenuated dengue virus backbone.

For example, the dengue chimera may express the structural protein genes of a dengue virus of one serotype in either of a dengue virus or an attenuated dengue virus background of a different serotype.

[0206] The strategy described herein of using a genetic background that contains nonstructural regions of a dengue virus genome of one serotype, and, by chimerization, the properties of attenuation, to express the structural protein genes of a dengue virus of a different serotype and a ZIKV has led to the development of live, attenuated Zika, dengue vaccine candidates that express structural protein genes of desired immunogenicity. Thus, vaccine candidates for control of dengue and Zika pathogens can be designed.

[0207] Viruses used in the chimeras described herein are typically grown using techniques known in the art. Virus plaque or focus forming unit (PFU or FFU) titrations are then performed and plaques or FFU are counted in order to assess the viability, titer and phenotypic characteristics of the virus grown in cell culture. Wild type viruses are mutagenized to derive attenuated candidate starting materials.

[0208] Chimeric infectious clones are constructed from various dengue serotypes. The cloning of virus-specific cDNA fragments can also be accomplished, if desired. The cDNA fragments containing the structural protein or nonstructural protein genes may be amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from dengue RNA with various primers. Amplified fragments are cloned into the cleavage sites of other intermediate clones. Intermediate, chimeric dengue clones are then sequenced to verify the sequence of the inserted dengue-specific cDNA.

[0209] Full genome-length chimeric plasmids constructed by inserting the structural or nonstructural protein gene region of dengue viruses into vectors are obtainable using recombinant techniques well known to those skilled in the art.

[0210] Any of these dengue/Zika chimera flaviviruses may be further attenuated using the dual approach to attenuation or the bicistronic rearrangement approach to attenuation described herein.

[0211] Method of Administration

[0212] The flaviviruses described herein are individually or jointly combined with a pharmaceutically acceptable carrier or vehicle for administration as an immunogen or vaccine to humans or animals. The terms “pharmaceutically acceptable carrier” or “pharmaceutically acceptable vehicle” are used herein to mean any composition or compound including, but not limited to, water or saline, a gel, salve, solvent, diluent, fluid ointment base, liposome, micelle, giant micelle, and the like, which is suitable for use in contact with living animal or human tissue without causing adverse physiological responses, and which does not interact with the other components of the composition in a deleterious manner.

[0213] The immunogenic or vaccine formulations may be conveniently presented in viral plaque forming unit (PFU) unit or focus forming unit (FFU) dosage form and prepared by using conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers. Formulations

suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets commonly used by one of ordinary skill in the art.

[0214] Unit dosage formulations are those containing a dose or unit, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the present disclosure may include other agents.

[0215] The immunogenic or vaccine composition may be administered through different routes, such as oral or parenteral, including, but not limited to, buccal and sublingual, rectal, aerosol, nasal, intramuscular, subcutaneous, intradermal, and topical. The composition may be administered in different forms, including, but not limited to, solutions, emulsions and suspensions, microspheres, particles, microparticles, nanoparticles and liposomes. It is expected that from about 1 to about 5 doses may be required per immunization schedule. Initial doses may range from about 100 to about 100,000 PFU or FFU, such as about 500 to about 20,000 PFU or FFU, about 750 to about 12,000 PFU or FFU, and about 750 to about 4000 PFU or FFU. Booster injections may range in dosage from about 100 to about 20,000 PFU or FFU, such as about 500 to about 15,000, about 500 to about 10,000 PFU or FFU, and about 500 to about 5000 PFU or FFU. For example, the volume of administration will vary depending on the route of administration. Intramuscular injections may range in volume from about 0.1 ml to 1.0 ml.

[0216] The composition may be stored at temperatures of from about -100°C to about 4°C. The composition may also be stored in a lyophilized state at different temperatures including room temperature. The composition may be sterilized through conventional means known to one of ordinary skill in the art. Such means include, but are not limited to, filtration. The composition may also be combined with bacteriostatic agents to inhibit bacterial growth.

[0217] Administration Schedule

[0218] The immunogenic or vaccine composition described herein may be administered to humans or domestic animals, such as horses or birds, especially individuals travelling to regions where ZIKV infection is present, and also to inhabitants of those regions. The optimal time for administration of the composition is about one to three months before the initial exposure to the ZIKV. However, the composition may also be administered after initial infection to ameliorate disease progression, or after initial infection to treat the disease.

[0219] Adjuvants

[0220] A variety of adjuvants known to one of ordinary skill in the art may be administered in conjunction with the chimeric virus in the immunogen or vaccine composition of this disclosure. Such

adjuvants include, but are not limited to, the following: polymers, co-polymers such as polyoxyethylene-polyoxypropylene copolymers, including block co-polymers, polymer p 1005, Freund's complete adjuvant (for animals), Freund's incomplete adjuvant; sorbitan monooleate, squalene, CRL-8300 adjuvant, alum, QS 21, muramyl dipeptide, CpG oligonucleotide motifs and combinations of CpG oligonucleotide motifs, trehalose, bacterial extracts, including mycobacterial extracts, detoxified endotoxins, membrane lipids, or combinations thereof.

[0221] Nucleic Acid Sequences

[0222] Nucleic acid sequences of the flaviviruses described herein, including ZIKV and dengue viruses, are useful for designing nucleic acid probes and primers for the detection of ZIKV and dengue virus chimeras in a sample or specimen with high sensitivity and specificity. Probes or primers corresponding to flaviviruses, including ZIKV and dengue virus, can be used to detect the presence of a vaccine virus. The nucleic acid and corresponding amino acid sequences are useful as laboratory tools to study the organisms and diseases and to develop therapies and treatments for the diseases.

[0223] Nucleic acid probes and primers selectively hybridize with nucleic acid molecules encoding ZIKV and dengue virus or complementary sequences thereof. By “selective” or “selectively” is meant a sequence which does not hybridize with other nucleic acids to prevent adequate detection of the ZIKV sequence and dengue virus sequence. Therefore, in the design of hybridizing nucleic acids, selectivity may depend upon the other components present in the sample. The hybridizing nucleic acid should have at least 70% complementarity with the segment of the nucleic acid to which it hybridizes. As used herein to describe nucleic acids, the term “selectively hybridizes” excludes the occasional randomly hybridizing nucleic acids, and thus has the same meaning as “specifically hybridizing.” The selectively hybridizing nucleic acid probes and primers can have at least 70%, 80%, 85%, 90%, 95%, 97%, 98% and 99% complementarity with the segment of the sequence to which it hybridizes, for example, 85% or more.

[0224] The present disclosure also contemplates sequences, probes and primers that selectively hybridize to the encoding nucleic acid or the complementary, or opposite, strand of the nucleic acid. Specific hybridization with nucleic acid can occur with minor modifications or substitutions in the nucleic acid, so long as functional species-species hybridization capability is maintained. By “probe” or “primer” is meant nucleic acid sequences that can be used as probes or primers for selective hybridization with complementary nucleic acid sequences for their detection or amplification, which probes or primers can vary in length from about 5 to about 100 nucleotides, or from about 10 to about 50 nucleotides, or about 18 to about 24 nucleotides. Isolated nucleic acids that selectively hybridize with the species-specific nucleic acids under stringent conditions and should have at least five nucleotides complementary to the sequence of interest as described in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989.

[0225] If used as primers, in some examples the composition includes at least two nucleic acid molecules which hybridize to different regions of the target molecule so as to amplify a desired region. Depending on the length of the probe or primer, the target region can range between 70% complementary

bases and full complementarity and still hybridize under stringent conditions. For example, for the purpose of detecting the presence of ZIKV and dengue virus, the degree of complementarity between the hybridizing nucleic acid (probe or primer) and the sequence to which it hybridizes is at least enough to distinguish hybridization with a nucleic acid from other organisms.

[0226] In some examples, the nucleic acid sequences encoding ZIKV and dengue virus can be inserted into a vector, such as a plasmid, and recombinantly expressed in a living organism to produce recombinant ZIKV and dengue virus peptide and/or polypeptides.

[0227] In some examples, a diagnostic probe serves to report the detection of a cDNA amplicon amplified from the viral genomic RNA template by using a reverse-transcription/polymerase chain reaction (RT-PCR), as well as forward and reverse amplimers that are designed to amplify the cDNA amplicon. In certain instances, one of the amplimers is designed to contain a vaccine virus-specific mutation at the 3'-terminal end of the amplimer, which effectively makes the test even more specific for the vaccine strain because extension of the primer at the target site, and consequently amplification, will occur only if the viral RNA template contains that specific mutation.

[0228] Automated PCR-based nucleic acid sequence detection systems and TaqMan assays (Applied Biosystems) are widely used. A more recently developed strategy for diagnostic genetic testing makes use of molecular beacons (Tyagi and Kramer 1996 *Nature Biotechnology* 14:303-308). Molecular beacon assays employ quencher and reporter dyes that differ from those used in the TaqMan assay.

[0229] Flavivirus Production

[0230] As described herein, live attenuated flavivirus vaccines are developed using recombinant DNA technology. The techniques herein are facilitated by the conservation among flaviviruses of genome organization, number of viral proteins, replicative strategy, gene expression, virion structure and morphogenesis. All wild type flaviviruses have a positive sense non-segmented RNA genome that encodes a single long polyprotein that is processed to yield capsid (C), premembrane (prM) and envelope glycoprotein (E) structural proteins followed by nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 in that order. Due to these shared properties viable chimeric viruses are produced by replacing the genes for the viral structural proteins in a full-length infectious cDNA clone of a flavivirus with the corresponding viral genes (in cDNA form) of another flavivirus. When tested, this strategy was successful for chimeras that contained the sequence for viral structural proteins prM and E of tick-borne encephalitis virus (TBEV) or tick-borne Langat virus (LGTV), while all other sequences were derived from the full-length infectious cDNA of mosquito-borne dengue type 4 virus (DEN4). This indicated that viral structural proteins of a disparate flavivirus, TBEV or LGTV, could function in the context of cis-acting 5' and 3' sequences and nonstructural proteins of DEN4. Significantly, both chimeras were highly attenuated in mice with respect to peripheral virulence, namely, the ability of a virus to spread to the CNS from a peripheral site of inoculation and cause encephalitis. Nonetheless, the chimeras proved to be immunogenic and able to induce resistance in mice against challenge with TBEV or LGTV. It appeared that a favorable balance between reduction in virus replication in vivo (attenuation) and induction of protective immunity

had been achieved. This is interpreted to mean that tick-borne flavivirus prM and E can interact in the context of DEN4 nonstructural proteins and cis-acting 5' and 3' sequences at a level sufficient for infectivity and induction of immunity but not sufficient for full expression of virulence that requires a high level of replication *in vivo* and ability to spread into the CNS. Recently, a West Nile virus and dengue virus chimeras containing structural proteins from the West Nile virus and an attenuated dengue virus backbone were shown to be effective as immunogens or vaccines. See U.S. Patent Application Publication 2005/0100886A1, which is incorporated herein by reference.

[0231] EXAMPLES

[0232] Example 1. Synergistic IRES/micro-RNA based approach for development of an attenuated, bicistronic live-virus vaccine against a flavivirus

[0233] Abstract

[0234] Recent emergence of Zika virus (ZIKV) underscores the need for new strategies for a rapid development of safe flavivirus vaccines. Using another flavivirus (Langkat virus, LGTV) that belongs to the group of tick-borne flaviviruses as a model, a dual strategy for virus attenuation is described in this Example, which accesses the specificity of microRNA (miRNA) genome targeting and the effectiveness of internal ribosome entry site (IRES) insertion. The engineered bicistronic LGTVs, which express capsid (C) protein under translational control from an IRES and contain multiple target sequences for brain specific miRNAs, were stable in Vero cells and induced adaptive immunity in mice. Importantly, miRNA-targeted bicistronic LGTVs were not pathogenic for either newborn mice after intracranial inoculation or adult immunodeficient mice (SCID or type I interferon receptor knockout) after intraperitoneal injection. Moreover, bicistronic LGTVs were restricted for replication in tick-derived cells, suggesting an interruption of viral transmission in nature by arthropod vectors. This approach is suitable for reliable attenuation of many flaviviruses, and may enable development of live attenuated flavivirus vaccines.

[0235] Introduction

[0236] Tick-borne flaviviruses (TBF) constitute a monophyletic single group within the *Flavivirus* genus (family *Flaviviridae*) that harbors causative agents of severe encephalitic (and less frequently hemorrhagic) disease in humans (Lasala *et al.*, *Clinics in laboratory medicine* 30:221-235 (2010)). Today, inactivated vaccines are available only against the prototype member of the group, tick-borne encephalitis virus (TBEV). Hampered by lengthy vaccination schedules and reliance on additional booster immunizations, the inactivated vaccines appear to be impractical for use in many TBEV endemic countries (Kunze, *Tick Borne Dis.* 7:399-404 (2016); Rumyantsev *et al.*, *Proc. Natl. Acad. Sci. USA* 110:13103-13108 (2013)). However, despite decades of active research, a single-dose live attenuated vaccine against any member of the TBF group has yet to be developed. This lag in progress is largely due to the absence of an effective strategy for reliable attenuation of TBF pathogenesis (Rumyantsev *et al.*, *Proc. Natl. Acad. Sci. USA* 110:13103-13108 (2013)).

[0237] Recently we demonstrated that targeting genomes of several TBF, including Langat and chimeric TBEV/DEN4 and TBEV/Langat viruses, using CNS-specific microRNAs (miRNA) resulted in a selective attenuation of viral replication in the CNS but not in peripheral organs (Teterina *et al.*, *Antiviral research* 127, 57-67 (2016); Tsetsarkin *et al.*, *Nucleic Acids Res* 44, 3330-3350 (2016); Tsetsarkin *et al.*, *Sci Rep* 6, 33088 (2016); Heiss *et al.*, *J. Virol.* 85:1464-1472 (2011)). However, considerable instability of the inserted miRNA-target sequences in the TBF genome has been observed during viral infection of the CNS, indicating that reliance on miRNA targeting is not sufficient for strong and consistent virus attenuation necessary for a safe vaccine candidate. To overcome this pitfall, a dual strategy is described in this Example that combines the specificity of miRNA-targeting and the robustness of encephalomyocarditis virus (EMCV) internal ribosome entry site sequence (IRES) insertion to achieve a surprising and unexpected level of virus attenuation that is synergistic. The engineered bicistronic, miRNA-targeted LGTVs, which express C protein under control of an IRES, were not pathogenic to newborn Swiss, adult immunodeficient SCID, or type I interferon receptor deficient mice, while they induced strong adaptive immunity in normal C3H mice. Moreover, bicistronic LGTVs were restricted for replication in tick-derived cells, thus mitigating issues of their environmental safety (Seligman *et al.*, *Lancet* 363:2073-2075 (2004); Tsetsarkin *et al.*, *mBio* 8:e02326-16 (2017)) as vaccine viruses.

[0238] Results and Discussion

[0239] Development of a bicistronic miRNA targeted LGTV.

[0240] The E5 strain of LGTV was modified by inserting three target (T) copies of brain-specific mir-124 miRNA into duplicated C gene regions (DCGR) using a strategy previously described (Tsetsarkin *et al.*, *PLoS pathogens* 11:e1004852 (2015)). The resulting monocistronic virus (cap-C) independently expresses regulatory (C-trn [truncated]) and structural (C-opt [codon optimized]) functions of the C gene, using the 2A protease of FMDV (foot-and-mouth disease virus) for cleavage of full-length C protein from the nascent polypeptide (FIG. 1). Open reading frame (ORF)-shifting insertions (+1 nt) and deletions (-1 nt) were introduced into C-trn to ensure genetic stability of the cap-C virus by preventing recombination between two C gene sequences (Tsetsarkin *et al.*, *PLoS pathogens* 11:e1004852 (2015)). The cap-C virus replicated efficiently in Vero cells, however deletion of the 2A protease of FMDV and 89 N-terminal amino acids (AA) of C-opt completely abolished accumulation of the cap-ΔC virus in Vero cells (FIG. 2). Insertion of a full length C-opt gene under control of the IRES into the 3'NCR of the cap-ΔC restored accumulation of infectious virus (IRES-C) in Vero cells (FIG. 2). Interestingly, relocation of all structural genes (C/prM/E) from the N-terminus of LGTV ORF into the 3'NCR (non-coding region) under control of IRES resulted in IRES-C/prM/E virus, which did not form detectable infectious foci in Vero cells (FIG. 3). To increase safety and stability of bicistronic LGTV in the CNS, the IRES-C was modified by inserting an additional mir-124 (T) sequence in the region between the NS5 gene and 5' end of IRES to generate IRES-124. Both IRES-124 and IRES-C replicated similarly in Vero cells (FIG. 2), but only the IRES-124 failed to cause

mortality in newborn Swiss Webster (SW) mice infected intracranially (IC) with a dose of 10^3 pfu/mouse (FIG. 4). Therefore, IRES-124 was selected for additional evaluation.

[0241] To improve growth of bicistronic LGTV in vertebrate cells and identify adaptation mutations, IRES-124 was passaged 10 times in Vero cells, followed by genome sequencing. Four mutations were identified of which three were located outside of the prM/E gene region (FIG. 5). To minimize the effect of cell adaptive mutations on the immunogenicity of LGTV, only mutations located outside of the structural prM/E gene region were introduced into the IRES-124 virus cDNA. Titer of the resulted IRES-124(3m) virus in Vero cells was increased 50-fold compared to IRES-124 (FIG. 6). To assess immunogenicity of bicistronic LGTV, adult C3H mice were inoculated intraperitoneally (IP) with a dose of 10^5 pfu of IRES-124(3m). All animals (n=5) remained free of neurological disease, while developing a mean neutralizing antibody of 204 ± 161 (reciprocal PRNT₅₀) by day 28 (FIG. 7). Moreover, viremia was not detected in any of the immunized mice on day one post challenge with 10^4 pfu of wt LGTV, whereas all mock-inoculated mice became viremic (FIG. 7) with a mean virus titer of $2.7 \pm 0.4 \log_{10}$ pfu/mL.

[0242] To assess stability of IRES-124(3m) in vertebrate cells, we passaged the virus an additional 10 times in Vero cells. Sequence analysis identified accumulation of two novel mutations: one in the E and one in the NS5 gene region (FIG. 8). The latter (NS5-A724T) substitution was incorporated into IRES-124(3m). The resulting IRES-124(4m) virus replicated slightly more efficiently compared to IRES-124(3m) (FIG. 6), and was selected for further evaluation.

[0243] Characterization of bicistronic LGTV in animals.

[0244] Previously, it was shown that miRNA targeting of the LGTV genome with a combination of CNS-specific mir-124 and mir-9 targets was more effective for attenuation of virus neurovirulence compared to insertion of only mir-124 targets (Tsetsarkin *et al.*, *Nucleic Acids Res* 44,:3330-3350 (2016)). Therefore, an additional virus was developed in which one of the mir-124(T)s was substituted with mir-9(T) (see IRES-124/9(4m) in FIG. 9). To evaluate the effects of miRNA targeting and/or IRES insertion on LGTV growth in the CNS, newborn SW mice were infected in the brain with 100 pfu of either IRES-124/9(4m) or IRES-124(4m) viruses. Control mice were infected with IRES-124/9(4m)* virus, which has synonymous mutations in all miRNA(T)s, or with monocistronic cap-124/9 virus, which has the same combination of miRNA(T)s as bicistronic IRES-124/9(4m) (FIG. 9). Replication of IRES-124/9(4m) and IRES-124(4m) in mouse brain was dramatically reduced compared to IRES-124/9(4m)* and cap-124/9 viruses, indicating that both IRES- and miRNA-based approaches contribute to viral attenuation in the CNS (FIG. 10). The difference in titers attained by cap-124/9 and IRES-124/9(4m) in the brains was substantially greater compared to the difference in Vero cells (FIG. 10, FIG. 11), suggesting synergy between IRES- and miRNA-based mechanisms of LGTV attenuation in the CNS. The synergism was unexpected since, to the inventors' knowledge, there are no data that show interactions between eukaryotic miRNA machinery for regulation of gene expression and translation mechanism involved in ECMV IRES. Therefore, it was initially assumed that both attenuating mechanisms should work independently. To

corroborate these results we infected newborn SW mice IC with a high virus dose (10^4 pfu) and monitored survival of mice for 21 days. All animals infected with IRES-124/9(4m) and IRES-124(4m) survived infection, whereas all mice infected with IRES-124/9(4m)* or cap-124/9 died by day 12 (FIG. 12).

[0245] To evaluate ability of bicistronic LGTV to invade the CNS from the periphery, three-week-old SCID mice (n = 4 or 5 animals/group) were infected IP with 10^5 pfu of viruses shown in FIG. 9 and monitored for 52 days. SCID mice are severely deficient in development of functional B and T lymphocytes, permitting prolonged viral replication *in vivo* uninhibited by the development of humoral and cell-mediated adaptive immunity. All mice infected with IRES-124(4m) and IRES-124/9(4m) survived (FIG. 15) and developed moderate viremia (FIG. 13) that persisted for the duration of the experiment (52 days). The viral load in the brains at day 52 (FIG. 14) was approximately 200-400 fold lower compared to the load in the serum. In contrast, 100% of mice infected with cap-124/9 died by day 31 and developed substantial viremia (FIG. 15, FIG. 13). Moreover, a high titer of cap-124/9 virus was detected in the brains of paralyzed animals (approximately 100-fold higher compared to viral load in the serum). Sequence analysis of cap-124/9 recovered from the brain of morbid mice revealed accumulation of deletions in the miRNA(T) region (FIG. 16). Interestingly, bicistronic IRES-124/9(4m)* lacking functional miRNA(T) sequences was also highly neuroinvasive (FIG. 14), causing paralysis in 100% of SCID mice by day 23, even though viremia produced by this virus was substantially lower compared to cap-124/9 on day 7 (FIG. 13). Together these data demonstrate that cooperation between IRES- and miRNA-based attenuating mechanisms is instrumental to ensure safety of bicistronic LGTV in immunocompromised animals. Sequencing analyses of IRES-124(4m) and IRES-124/9(4m) viruses recovered from serum samples collected at day 52 did not identify mutations or deletions in the miRNA(T) region, confirming high genetic stability of bicistronic LGTV *in vivo*.

[0246] Immunization of C3H mice with bicistronic LGTV induced an adaptive immune response. However, immunocompetent C3H mice quickly developed resistance to wt LGTV challenge, which limits their use in lethal challenge studies (FIG. 7). To overcome this limitation we used adult type I interferon receptor deficient mice (B6 IFNRI^{-/-}), which have been shown to be sensitive to flavivirus infection (Weber *et al.*, *J. Virol.* 88:12202-12212 (2014); Samuel *et al.*, *J. Virol.* 79:13350-13361 (2005)). B6 IFNRI^{-/-} mice were inoculated IP with 10^5 pfu of viruses depicted in FIG. 9, or with diluent alone (mock), followed by challenge with 10^2 pfu of wt LGTV at day 32. All mice infected with bicistronic LGTVs survived the primary inoculation (FIG. 17), challenge with wt LGTV (FIG. 18) and developed LGTV-specific neutralizing antibodies (FIG. 20). In contrast, all cap-124/9-inoculated mice died by day 7, and all mock-inoculated mice succumbed to wt LGTV challenge by day 7. Sequence analysis of the viruses recovered from the brain and spleen of 2 morbid mice infected with cap-124/9, which died at day 7, showed that all miRNA(T) sequences remained stable. These data suggest that in contrast to 3-day-old SW and adult SCID mice, the IRES-based attenuation approach alone is sufficient to prevent pathogenesis of LGTV in B6 IFNRI^{-/-} mice. Viremia was not detected after the challenge of immunized mice with wt LGTV. In contrast, all mock -infected animals developed high viremia (approximately $9 \log_{10}$ pfu/mL (FIG. 19)). Challenge

with wt LGTV on day 32 did not increase neutralizing antibody titer (FIG. 20), suggesting that bicistronic LGTVs can potentially be used as a single dose vaccine candidate against neurotropic tick-borne flaviviruses.

[0247] Bicistronic LGTVs are growth restricted in tick-derived cells.

[0248] Like all tick-borne flaviviruses, LGTV uses ticks for natural transmission between vertebrate hosts. Since RNA translation from EMCV IRES is inhibited in cells of insect origin (Woolaway *et al.*, *J. Virol.* 75:10244-10249 (2001)), it was hypothesized that IRES-based C gene expression in bicistronic LGTVs might also restrict virus growth in tick-derived cells. To validate this hypothesis, ISE6 (derived from *Ixodes scapularis* ticks) or simian Vero cells were infected with IRES-124/9(4m), IRES-124/9(4m)* or cap-124/9 viruses at an MOI of 0.1 pfu/cell (FIG. 21). Both bicistronic viruses with and without functional miRNA(T)s failed to replicate in tick-derived cells, but not in vertebrate Vero cells. In contrast, cap-124/9 replicated efficiently in both cell lines. To determine if Vero cells adaptive mutations in bicistronic viruses affect LGTV host range, we incorporated 3 out of 4 mutations (the 4th mutation was part of the IRES, which is not present in monocistronic LGTV) into monocistronic cap-124/9 (FIG. 21A). The resulting virus, cap-124/9(3m), replicated efficiently in both ISE6 and Vero cell lines (FIG. 21B, FIG. 21C), indicating that IRES is directly responsible for growth restriction of bicistronic LGTVs in tick-derived cells. It was, therefore, concluded that bicistronic genome organization not only synergistically attenuates virulence of neurotropic flaviviruses in vertebrate host but also can ensure environmental safety of designed viruses by preventing their transmission in nature.

[0249] Attempts had been made earlier to develop bicistronic neurotropic flaviviruses, which utilized a genetic configuration substantially different to the one described here (Frese *et al.*, *J. Virol.* 88:2056-2070 (2014); Orlinger *et al.*, *J. Virol.* 80:12197-12208 (2006)). Viruses generated in those studies were either genetically unstable, quickly reverting to wt (monocistronic) configuration (Frese *et al.*, *J. Virol.* 88:2056-2070 (2014)), or replicated to low titers (Orlinger *et al.*, *J. Virol.* 80:12197-12208 (2006); Orlinger *et al.*, *Journal of virology* 81:12619-12629 (2007)). Importantly, the previously developed approaches did not incorporate a mechanism to selectively restrain virus neuropathogenesis in the CNS and were associated with significant neurovirulence in mice, limiting their application for vaccine development. The combination approach described here, which employs IRES-based attenuation and miRNA-mediated suppression of encephalitic flaviviruses replication, successfully overcomes such limitations providing a reliable attenuation platform without compromising virus stability or immunogenicity.

[0250] As a final note, it is believed that the developed approach should not be confined to TBF, but that it can also be applied for development of live vaccines against mosquito-borne flaviviruses such as Japanese encephalitis, West Nile virus and Zika virus. The latter has recently emerged as the world's most significant arbovirus (Fauci *et al.*, *N Engl J Med* 374:601-604 (2016)). Associated with development of severe birth defects in humans, the ongoing ZIKV outbreaks underscore the need for new strategies (exemplified by this study) for a rapid development of safe flavivirus vaccines.

[0251] Materials and Methods

[0252] All experimental protocols were approved by the NIH Institutional Biosafety Committee.

[0253] *Plasmids and viruses*

[0254] Infectious cDNA clone of attenuated E5 strain of LGTV under transcriptional control of Pol II promoter from *cytomegalovirus* and its derivative C48-124(2)/9/1-E5 have been described previously (Tsetsarkin *et al.*, *Nucl. Acids Res.* 44:3330-3350 (2016)).

[0255] cap-C

[0256] To generate cap-C, the C48-124(2)/9/1-E5 was modified by replacing a sequence for mir-9(T) with mir-124(T) and deleting the mir-1(T) in the duplicated C gene region (DCGR).

[0257] cap-ΔC

[0258] The cap-ΔC was generated by deleting of the 2A protease gene of foot and mouth disease virus (FMDV) and 89 N-terminal amino acids (AA) of C-opt gene in the cap-C.

[0259] IRES-C

[0260] To generate IRES-C, region encoding IRES sequence from pIRESpuro2 plasmid (*Clontech*) was fused with C-opt gene using PCR and inserted after 8th nt of cap-ΔC.

[0261] IRES-124

[0262] The IRES-124 was produced by inserting mir-124(T) after 6th nt located downstream of UAA stop codon of NS5 gene of the IRES-C.

[0263] IRES-124(3m)

[0264] To generate IRES-124(3m), the IRES-124 was subsequently modified by introducing substitutions: U→A at position 332 nt of NS4A gene (results in F₁₁₁→Y change) and G→A at position 263 of C-opt gene (results in G₈₈→D change), and by deletion of a single A residue (ΔA) at nt position 490 of IRES sequence, generating IRES-124(3m).

[0265] IRES-124(4m)

[0266] To generate IRES-124(4m), a G→A substitution was introduced into IRES-124(3m) at nt position 2170 of NS5 gene (results in A₇₂₄→T change). The IRES-124(4m) was modified by replacing 5' terminal copy of mir-124(T) sequence with that of mir-9(T), generating IRES-124/9(4m).

[0267] IRES-124/9(4m)*

[0268] The IRES-124/9(4m)* was constructed by introducing synonymous mutations into each AA codon within miRNA(T) sequences located in the ORF of the IRES-124/9(4m). The mir-124(T) located between NS5 and IRES was mutated using the identical 'scrambled' sequences as both mir-124(T)s located in the ORF. The cap-124/9 was constructed by introducing mir-124(T) sequence into the 3'NCR of the C48-124(2)/9/1-E5 (Tsetsarkin *et al.*, *Nucl. Acids Res.* 44:3330-3350 (2016)) at nt position 14 followed by deletion of mir-1(T) sequence in the DCGR. The NS4A-F111Y, C-G88D and NS5-A724T substitutions were introduced into cap-124/9 to generate cap-124/9(3m).

[0269] All intermediate plasmids were propagated in the MC1061 strain of *E. coli* and purified using

Endo Free® Plasmid Maxi Kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

[0270] *Cells*

[0271] Vero (*Cercopithecus aethiops*, African green monkey kidney) and LLC-MK2 (*Macaca mulatta* kidney) cells were maintained at 37°C and 5% CO₂ in Opti-Pro medium (Invitrogen) supplemented with 2% FBS and 50 µg/ml of gentamicin (Rumyantsev *et al.*, *Proc. Natl. Acad. Sci. USA* 110:13103-13108 (2013)). Tick ISE6 cells derived from *Ixodes scapularis* (Tsetsarkin *et al.*, *Sci. Rep.* 6:33088 (2016)) were maintained at 34°C in 66% Leibovitz's L-15 media (Invitrogen) supplemented with 3.3% FBS (Lonza), 6.6% Tryptose phosphate broth solution (MP Biomedicals, Irvine, CA), 0.66% Bovine Cholesterol Lipoprotein Concentrate (MP Biomedicals, Irvine, CA), 50 µg/ml of gentamicin (Invitrogen), 0.296 g/L of L-aspartic acid, 0.333 g/L of L-glutamine, 0.3 g/L of L-proline, 0.323 g/L of L-glutamic acid, 0.296 g/L of α-ketoglutaric acid, 11.9 g/L of D-glucose, and with 1x mineral and vitamin solution as described earlier (Tsetsarkin *et al.*, *Nucleic Acids Res* 44:3330-3350 (2016)).

[0272] *DNA transfection, virus recovery, and titration*

[0273] Viruses were rescued from plasmid DNA using Lipofectamine® 2000 transfection method as described previously (Tsetsarkin *et al. Sci Rep* 6:33088 (2016)). Briefly, 5 µg of plasmid DNA diluted in 0.25 mL of OptiMEM™ medium (Invitrogen) was mixed with 10 µg of Lipofectamine® 2000 (Invitrogen) diluted in 0.25 mL of OptiMEM™ medium. The 0.5 mL DNA/ Lipofectamine® 2000 solution was used for transfection of 1.5x10⁶ Vero cells seeded onto a 12.5-cm² flask for 4 h at 37°C and 5% CO₂. Cells were washed twice with 5 mL of Opti-MEM™ medium and maintained in 5 mL of DMEM (Invitrogen) supplemented with 10% FBS and 1x Penicillin-Streptomycin-Glutamine (PSG) solution for 5 days at 37°C and 5% CO₂. Aliquots of cell culture medium (0.5 mL) were stored at -80°C and titrated in Vero cells using an immunostaining plaque-forming assay described previously (Rumyantsev *et al.*, *Proc. Natl. Acad. Sci. USA* 110:13103-13108 (2013)). Infectious foci in methanol fixed Vero cells monolayers were visualized using immunostaining with TBEV-specific antibodies in hyperimmune mouse ascitic fluid and peroxidase-labeled anti-mouse IgG (Dako Co., Carpinteria, CA).

[0274] *Serial passaging and genetic stability of bicistronic LGTVs in Vero cells.*

[0275] Viruses in the supernatant of Vero cells were harvested 5 days post DNA transfection and were diluted in Opti-Pro medium to 2x10⁴ pfu/mL, and one milliliter was used to infect new Vero cells monolayers in 25-cm² flasks (MOI ~ 0.01). Cells were maintained in 5 mL of Opti-Pro medium at 37°C and 5% CO₂ for 5 days or until cytopathic effect (cpe) was observed (for IRES-124 it occurred at 7-10 dpi). Supernatant was harvested, diluted 1/10 with Opti-Pro medium, and 1 mL of inoculum was used to infect 25-cm² flasks of fresh Vero cells. The process was repeated 10 times. At the end of the 10th passage, viral RNA was extracted from 0.14 mL of supernatant using the QIAamp® Viral RNA Mini kit (Qiagen) and PCR-amplified using Transcriptor One-Step RT-PCR Kit (Roche), followed by sequence analysis of the complete viral genome.

[0276] *Mice study*

[0277] All mice were purchased from Taconic Inc. All animal study protocols were approved by the NIAID/NIH Institutional Animal Care and Use Committee. All animal experiments were performed in compliance with the guidelines of the NIAID/NIH Institutional Animal Care and Use Committee. The NIAID DIR Animal Care and Use Program acknowledges and accepts responsibility for the care and use of animals involved in activities covered by the NIH IRP's PHS Assurance #A4149-01, last issued 6/11/2011.

[0278] 1-Replication kinetics of LGT viruses in the brain of newborn Swiss Webster (SW) mice

[0279] Litters of 10 three-day-old SW mice (Taconic, Hudson, NY) were inoculated intracranially (IC) with 100 pfu of virus in 10 μ L of L-15 medium supplemented with 1x SPG (218 mM sucrose, 6 mM L-glutamic acid, 3.8 mM KH_2PO_4 , 7.2 mM K_2HPO_4 , pH 7.2) as described previously (Tssetsarkin *et al.*, *Nucl. Acids Res.* 44:3330-3350 (2016)). Three pups from each litter were sacrificed at 4, 7, and 10 dpi, brains were dissected and used to prepare 10% homogenate in L-15/ 1x SPG solution. Virus titers in the brain suspensions were assayed by titration in Vero cells.

[0280] 2- Survival study of newborn SW mice after IC infection

[0281] Three 3-day-old SW mice were inoculated IC with 10^4 pfu of virus diluted in 10 μ L of L-15/ 1x SPG solution. Mice were returned to cages to their mothers and monitored daily for onset of neurologic symptoms (paralysis) during 21 days, at which point survived animals were humanely euthanized.

[0282] 3-Neuroinvasiveness study in adult immunodeficient SCID mice

[0283] rLGTVs were diluted in L-15/1x SPG solution to a concentration of 10^6 pfu/mL. Three-week-old male SCID mice (Taconic) in groups of five animals were injected IP with 0.1mL of each virus (dose of 10^5 pfu/mouse). Mice were monitored for onset of neurologic disease (paralysis) for 52 dpi. Mice were bled on 7, 23, 35 and 52 dpi to assess virus titer in the serum. Brains from mice that survived the experiment (52 dpi) and those who died (or developed) paralysis after viral infection (23-35 dpi) were extracted, homogenized in L-15/1x SPG solution, and viral titer in each homogenate was assessed in Vero cells.

[0284] 4-Safety and protective efficacy of bicistronic LGTVs in adult B6 IFN $\text{RI}^{-/-}$ mice

[0285] rLGTVs (**FIG. 9**) were diluted in L-15/1x SPG solution to a concentration of 10^6 pfu/mL. Three-week-old male B6 IFN $\text{RI}^{-/-}$ mice (Taconic) in groups of 5 animals were immunized IP with 0.1mL of each virus (dose of 10^5 pfu per mouse) or with 0.1mL of L-15/1x SPG (mock). Mice were monitored for onset of morbidity/mortality for 28 dpi, at which point survived animals were bled to determine neutralizing antibody titer. At 31 dpi, mice were infected IP with 10^2 pfu of wt LGTV. Mice were bled on 1 and 4 days post challenge (dpc) to determine titer of wt LGTV in the serum. At 25 dpc, mice that survived the challenge were bled to determine neutralizing antibody titer using the 50% plaque reduction neutralization assay (PRNT₅₀) against LGTV TP-21 strain as described previously (Pletnev *et al.*, *J. Virol.* 75:8259-8267 (2001)).

[0286] 5-Safety and protective efficacy of bicistronic LGTVs in adult C3H mice

[0287] Three-week-old C3H female mice (Taconic) were infected intraperitoneally with 10^5 pfu of IRES-124(3m) or mock inoculated with L-15 medium supplemented with 1x SPG and monitored daily for signs of neurotropic disease until 28 dpi. At 29 dpi, mice were challenged with 10^4 pfu of wt LGTV and

monitored for morbidity for an additional 28 days. Mice were bled on 1 and 30 dpi (30 dpi corresponds to 1 dpc with wt LGTV) for the detection of virus in serum and on 28 or 56 dpi for measurement of neutralizing antibody against LGTV TP-21 strain.

[0288] Example 2. Synergistic IRES/micro-RNA based approach for development of an attenuated, bicistronic live-virus vaccine against Zika virus

[0289] In addition to Langkat virus (LGTV), we developed recombinant bicistronic Zika virus clones. ZIKV cDNA construct, which utilizes genetic configuration similar to those described for bicistronic LGTV, does not yield an infectious virus in Vero cells (FIG. 22A). An approach was developed where only prM, E and NS1 genes were expressed in cap-dependent manner (ORF1 - see FIG. 22B). The ORF2 was expressed from translation control of IRES. In ORF2 the codon-optimized copy of C gene (Copt) was fused with truncated prM gene encoding 99 N-terminal amino acids (AA) of prM protein and preserving furin protease cleavage site. This sequence was fused with the 5' end-truncated NS1 gene encoding 120 C-terminal AA of NS1 (which also was codon-optimized) followed by NS2A-NSS genes and 3'NCR. In addition, two novel Vero cells adaptive mutations (pr-F132L in the prM protein and E-H158Y in the E protein) were introduced. The resulting virus (ZV-IRES version 2) replicated efficiently in Vero cells reaching titer of $6.5 \log_{10}$ (pfu/mL). The ZV-IRES version 2 contains 4 sites for miRNA target insertions (arrows). Viruses expressing combination targets for CNS-specific mir-124 and mir-9, as well as testis and placenta-specific miRNAs have been developed.

[0290] Example 3. Synergistic IRES/micro-RNA based approach for development of an attenuated, live-virus vaccine against Japanese encephalitis virus or West Nile virus.

A live-virus vaccine against Japanese encephalitis virus or West Nile virus can be constructed using the same genetic organization as described in FIG. 22B. Examples are provided in FIG. 22C.

[0291] Example 4. Additional synergistic IRES/micro-RNA based approach for development of an attenuated, live-virus vaccine against Zika virus.

[0292] Using a genetic mapping method we identified minimal sequences of genome duplication (FIG. 26A; C trn region encoding 50 AA of C protein) that are necessary and sufficient for efficient ZIKV-IRES virus replication in Vero cells. This construct is named ZIKV-IRES version 3 (SEQ ID NO: 16). Insertion of heterologous sequences (miRNA targets for mir-124) between NS1 gene and IRES substantially reduced virus fitness in Vero cells, whereas insertion of miRNA targets after truncated C gene (C trn; FIG. 26A) did not interfere with virus replication. In addition, a novel Vero adaptive mutation (748 G→A (pr-R94K)) additionally increased virus fitness in Vero cells.

[0293] The ZIKV-IRES version 3 vaccine candidate was not pathogenic (did not cause morbidity or mortality after i.p. infection with 10^5 pfu ZIKV-IRES version 3) to Tac314 (IFNRI^{-/-}) or AG129 (IFNRI^{-/-} and IFNRII^{-/-}) mice (type I, or type I and type II interferon receptor knockout mice, respectively). Adult AG129 (IFNRI^{-/-} and IFNRII^{-/-}) or Tac314 (IFNRI^{-/-}) mice were mock infected or immunized with 10^5 pfu

of bicistronic ZIKV. At 29 dpi mice were challenged with 10^5 pfu of ZIKV-wt (FIG. 27A). Moreover, ZIKV-IRES version 3 caused only brief viremia (detectable for 2 days of post infection), but it was not detected in the brain, spleen, testis or epididymis of Tac314 or AG129 mice. In addition, ZIKV-IRES version 3 did not replicate in placental and fetus tissues of pregnant AG129 mice infected with a 10^5 pfu dose on day 7 of pregnancy.

[0294] A single immunization of Tac314 or AG129 mice with ZIKV-IRES version 3 led to development of robust ZIKV-specific neutralizing antibody response in 100% of vaccinated animals at 28 days post-inoculation with ZIKV-IRES version 3 and at 27 days post-challenge with ZIKV-wt (FIG. 27B). The developed immunity was sufficient to prevent mortality and viremia in mice that were challenged i.p. with 10^5 pfu of wild type ZIKV (strain Paraiba 2015) on day 28 post-immunization. Moreover, no replication of wild-type ZIKV was detected 2 days post-challenge in brain, spleen, testis or epididymis of Tac314 or AG129 mice immunized with ZIKV-IRES version 3 (FIG. 27C).

[0295] *Aedes albopictus*-derived C6/36 cells were infected with ZIKV-IRES version 3 or with original ZIKV isolate (ZIKV-wt), which was used for construction of the infection clone ZIKV-NS3mut, or with recombinant ZIKV-NS3mut virus at MOI of 0.01 in duplicates. Cell culture supernatants were collected daily followed by titration in Vero cells. ZIKV-IRES version 3 was unable to replicate in mosquito C6/36 cells, indicating that a developed vaccine will be safe for the environment (FIG. 28).

[0296] While the present disclosure has described embodiments in some detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of the disclosure.

CLAIMS

1. A nucleic acid molecule comprising an attenuated flavivirus or chimeric flavivirus genome comprising one or more target nucleotide sequences complementary to a host-specific micro-RNA (miRNA) and a nucleotide sequence encoding one or more flavivirus proteins that are under translational control of an internal ribosome entry site sequence (IRES).
2. The nucleic acid molecule of claim 1, wherein the one or more miRNA target nucleotide sequences are effective in restricting replication of the flavivirus in the presence of the complementary host-specific miRNA.
3. A nucleic acid molecule comprising an attenuated flavivirus or chimeric flavivirus genome comprising:

a first open reading frame comprising a nucleic acid encoding at least a first portion of a C protein, a nucleic acid encoding a prM protein, a nucleic acid encoding an E protein, and a nucleic acid encoding an NS1 protein; and

a second open reading frame under the translation control of an internal ribosome entry site sequence (IRES) comprising a nucleic acid encoding at least a second portion of a C protein, and a nucleic acid encoding an NS2 protein, and NS3 protein, an NS4 protein, and an NS5 protein.
4. The nucleic acid molecule of claim 3, further comprising one or more target nucleotide sequences complementary to a host-specific micro-RNA (miRNA).
5. The nucleic acid molecule of any one of claims 1 to 4, wherein the IRES is effective in converting the flavivirus genome into a bicistronic flavivirus genome, thereby causing attenuation of the flavivirus.
6. The nucleic acid molecule of any one of claims 1 to 5, wherein the flavivirus genome is selected from the group consisting of a Zika virus (ZIKV) genome, a West Nile virus (WNV) genome, a tick-borne encephalitis virus (TBEV) genome, a yellow fever virus (YFV) genome, Langat virus (LGTV), a Japanese encephalitis virus (JEV) genome, dengue virus (DENV) genomes, a Powassan virus (POWV) genome and an Usutu virus (USUV) genome.
7. The nucleic acid molecule of claim 6, wherein the flavivirus genome is a Zika virus (ZIKV) genome.
8. The nucleic acid molecule of claim 7, wherein the Zika virus (ZIKV) genome is GenBank Accession No. AY632535, LC002520, KU055503, KU055505, KU055502, KU955591, KU955594, or KX280026.

9. The nucleic acid molecule of claim 6, wherein the West Nile virus (WNV) genome is NY99-35262 prototypic strain (GenBank Accession No. AR196835) or recent isolates of WN02 genotype virus.
10. The nucleic acid molecule of claim 1 or claim 4, wherein the attenuated flavivirus or chimeric flavivirus comprises at least two target nucleotide sequences complementary to a host-specific micro-RNA (miRNA).
11. The nucleic acid molecule of claim 10, wherein the attenuated flavivirus or chimeric flavivirus comprises at least three target nucleotide sequences complementary to a host-specific micro-RNA (miRNA).
12. The nucleic acid molecule of claim 10 or claim 11, wherein the target nucleotide sequences are the same or different.
13. The nucleic acid molecule of claim 1 or claim 4, wherein the host-specific micro-RNA (miRNA) is broadly expressed in human tissues.
14. The nucleic acid molecule of claim 13, wherein the miRNA is selectively expressed in an epithelial tissue, connective tissue, nervous system tissue, reproductive organ tissues, or placenta.
15. The nucleic acid molecule of claim 13, wherein the miRNA is selectively expressed in the cardiovascular system, digestive system, endocrine system, excretory system, lymphatic system, integumentary system, muscular system, nervous system, reproductive system, respiratory system, skeletal system, immune system or hematopoietic system..
16. The nucleic acid molecule of claim 13, wherein the miRNA is a human miRNA.
17. The nucleic acid molecule of claim 1 or claim 4, wherein the host-specific micro-RNA (miRNA) is expressed in an arthropod vector.
18. The nucleic acid molecule of claim 17 wherein the arthropod vector is a mosquito or tick.
19. The nucleic acid molecule of claim 18, wherein the miRNA is a mosquito-specific miRNA selected from the group consisting of mir-184, mir-275, and mir-1.
20. The nucleic acid molecule of claim 1 or claim 4, wherein the one or more target nucleotide sequences complementary to a host-specific micro-RNA (miRNA) are complementary to at least one of SEQ ID NOs: 1, 2, 6-12, or 17.
21. The nucleic acid molecule comprising an attenuated flavivirus or chimeric flavivirus genome of any one of claims 1 to 20, wherein said nucleic acid molecule is a single stranded RNA molecule.

22. The nucleic acid molecule comprising an attenuated flavivirus or chimeric flavivirus genome of any one of claims 1 to 20, wherein said nucleic acid molecule is a complementary DNA (cDNA) molecule.
23. The nucleic acid molecule of claim 1 or claim 4, wherein the nucleic acid comprises a nucleic acid sequence at least 95% identical to any one of SEQ ID NOs: 13-17.
24. The nucleic acid molecule of any one of claims 1 to 22, comprising a chimeric flavivirus genome, wherein said chimeric genome comprises a first nucleic acid sequence encoding at least one structural protein from a first flavivirus and a second nucleic acid sequence encoding the remaining structural and nonstructural proteins from a second flavivirus.
25. The nucleic acid molecule of claim 24, wherein the first nucleic acid sequence is from a Zika virus genome.
26. The nucleic acid molecule of claim 24, wherein the second nucleic acid sequence is from a Zika virus genome.
27. The nucleic acid molecule of claim 24, wherein the first or second nucleic acid sequence is from a Zika virus genome and the remaining nucleic acid sequence is from a dengue virus genome.
28. A live attenuated flavivirus or chimeric flavivirus comprising the nucleic acid molecule of any one of claims 1 to 27.
29. The live attenuated flavivirus of claim 28, wherein said flavivirus is a Zika virus (ZIKV), a West Nile virus (WNV), a tick-borne encephalitis virus (TBEV), a yellow fever virus (YFV), Langat virus (LGTV), a Japanese encephalitis virus (JEV), a dengue virus (DENV), a Powassan virus (POWV) or an Usutu virus (USUV), or a chimeric flavivirus thereof.
30. An immunogenic composition comprising the live attenuated virus of claim 28 or claim 29.
31. The immunogenic composition of claim 30, wherein the live attenuated virus is immunogenic against a Zika virus.
32. A multivalent immunogenic composition comprising at least two different live attenuated flaviviruses of claim 28 or claim 29, wherein at least one of the live attenuated flaviviruses is immunogenic against a Zika virus.
33. The live attenuated flavivirus or chimeric flavivirus of claim 28 or claim 29, further comprising at least one additional attenuating mutation selected from the group consisting of: (a) an attenuating deletion in the 3'UTR or (b) an attenuating point mutation.

34. The live attenuated flavivirus or chimeric flavivirus of claim 33, wherein the attenuating deletion in the 3'UTR is a deletion in the 3'UTR that corresponds with a $\Delta 30$, $\Delta 31$, $\Delta 30/31$, or $\Delta 86$ mutation in the DENV serotype 4 background.
35. The live attenuated flavivirus or chimeric flavivirus of claim 33, wherein the attenuating point mutation corresponds with a mutation at nucleotide 4861 or 4995 of the NS3 gene in the DENV serotype 4 background.
36. The nucleic acid molecule of any one of claims 1 to 27, wherein the IRES is from a poliovirus (PV) or an encephalomyocarditis virus (EMCV).
37. An attenuated flavivirus or chimeric flavivirus comprising a recombinant flavivirus genome that encodes (a) a first open reading frame (ORF1) that is under 5'-cap-dependent translational control and which codes for at least one flavivirus protein and (b) a second open reading frame (ORF2) that is under IRES-dependent translational control and which codes for the remaining flavivirus proteins not encoded by ORF1, and wherein said genome further comprises at least one miRNA-target sequence that is complementary to a host-specific miRNA.
38. An attenuated flavivirus or chimeric flavivirus comprising a recombinant flavivirus genome that encodes:
- a first open reading frame comprising a nucleic acid encoding at least a first portion of a C protein, a nucleic acid encoding a prM protein, a nucleic acid encoding an E protein, and a nucleic acid encoding an NS1 protein; and
- a second open reading frame under the translation control of an internal ribosome entry site sequence (IRES) comprising a nucleic acid encoding at least a second portion of a C protein, and a nucleic acid encoding an NS2 protein, and NS3 protein, an NS4 protein, and an NS5 protein.
39. The attenuated flavivirus or chimeric flavivirus of claim 38, further comprising one or more target nucleotide sequences complementary to a host-specific micro-RNA (miRNA).
40. The attenuated flavivirus or chimeric flavivirus of claim 37 or claim 39, wherein the at least one miRNA-target sequence is effective in restricting replication of the flavivirus in the presence of the complementary host-specific miRNA.
41. The attenuated flavivirus or chimeric flavivirus of any one of claims 37 to 40, wherein the recombinant flavivirus genome comprises a Zika virus (ZIKV) genome, a West Nile virus (WNV) genome, a tick-borne encephalitis virus (TBEV) genome, a yellow fever virus (YFV) genome, Langat virus (LGTV), a Japanese encephalitis virus (JEV) genome, a dengue virus (DENV) genome, a Powassan virus (POWV)

genome, or an Usutu virus (USUV) genome, which further comprises the IRES sequence and the least one miRNA-target sequence inserted therein.

42. The attenuated flavivirus or chimeric flavivirus of any one of claims 37 to 41, wherein the flavivirus genome is a Zika virus (ZIKV) genome.

43. The attenuated flavivirus or chimeric flavivirus of claim 42, wherein the Zika virus (ZIKV) genome is GenBank Accession No. AY632535, LC002520, KU055503, KU055505, KU055502, KU955591, KU955594, or KX280026.

44. The attenuated flavivirus or chimeric flavivirus of claim 41, wherein the West Nile virus (WNV) genome is GenBank Accession No. AR196835.

45. The attenuated flavivirus or chimeric flavivirus of claim 40, wherein the host-specific miRNA is expressed in a human.

46. The attenuated flavivirus or chimeric flavivirus of claim 40, wherein the host-specific miRNA is expressed in an arthropod vector.

47. The attenuated flavivirus or chimeric flavivirus of claim 46, wherein the arthropod vector is a mosquito or tick.

48. The attenuated flavivirus or chimeric flavivirus of claim 47, wherein the miRNA is a mosquito-specific miRNA selected from the group consisting of mir-184, mir-275, and mir-1.

49. The attenuated flavivirus or chimeric flavivirus of claim 37 or claim 39, wherein the at least one miRNA target sequence is complementary to at least one of SEQ ID NOs: 1, 2, 6-12, or 17.

50. A bicistronic attenuated flavivirus comprising a recombinant flavivirus genome comprising a first nucleotide sequence encoding a first open reading frame (ORF1) coding for at least one flavivirus protein or functional variant thereof and a second nucleotide sequence encoding a second open reading frame (ORF2) coding for the remaining flavivirus proteins not coded for by ORF1, wherein the first open reading frame is under IRES-dependent translational control, and where said genome further comprises at least one miRNA-target sequence.

51. A bicistronic attenuated flavivirus comprising a recombinant flavivirus genome comprising:

a first nucleic acid encoding first open reading frame comprising a nucleic acid encoding at least first portion of a C protein, a nucleic acid encoding a prM protein, a nucleic acid encoding an E protein, and a nucleic acid encoding an NS1 protein; and

a second nucleic acid encoding a second open reading frame under the translation control of an internal ribosome entry site sequence (IRES) comprising a nucleic acid encoding at least a second portion of a C protein, and a nucleic acid encoding an NS2 protein, and NS3 protein, an NS4 protein, and an NS5 protein.

52. The bicistronic attenuated flavivirus of claim 51, further comprising one or more target nucleotide sequences complementary to a host-specific micro-RNA (miRNA).

53. The bicistronic attenuated flavivirus of claim 50 or claim 52, wherein the at least one miRNA-target sequence is effective in restricting replication of the flavivirus in the presence of a complementary host-specific miRNA.

54. The bicistronic attenuated flavivirus of any one of claims 50 to 53, wherein the flavivirus is a Zika virus (ZIKV), a West Nile virus (WNV), a tick-borne encephalitis virus (TBEV), a yellow fever virus (YFV), Langat virus (LGTV), a Japanese encephalitis virus (JEV), a dengue virus (DENV), a Powassan virus (POWV), or an Usutu virus (USUV).

55. The bicistronic attenuated flavivirus of claim 54, wherein the flavivirus is a Zika virus (ZIKV).

56. The bicistronic attenuated flavivirus of claim 55, wherein the Zika virus (ZIKV) is GenBank Accession No. AY632535, LC002520, KU055503, KU055505, KU055502, KU955591, KU955594, or KX280026.

57. The bicistronic attenuated flavivirus of claim 54, wherein the West Nile virus (WNV) is GenBank Accession No. AR196835.

58. The bicistronic attenuated flavivirus of claim 50 or claim 52, wherein the host-specific miRNA is expressed in a human.

59. The bicistronic attenuated flavivirus of claim 50 or claim 52, wherein the host-specific miRNA is expressed in an arthropod vector.

60. The bicistronic attenuated flavivirus of claim 59, wherein the arthropod vector is a mosquito or tick.

61. The bicistronic attenuated flavivirus of claim 60, wherein the miRNA is a mosquito-specific miRNA selected from the group consisting of mir-184, mir-275, and mir-1.

62. The bicistronic attenuated flavivirus of claim 50, wherein the first open reading frame (ORF1) encodes the capsid protein or a functional variant thereof.

63. An immunogenic composition comprising the bicistronic attenuated flavivirus of any one of claims 50 to 62.

64. The immunogenic composition of claim 63, wherein the bicistronic attenuated flavivirus is immunogenic against a Zika virus.

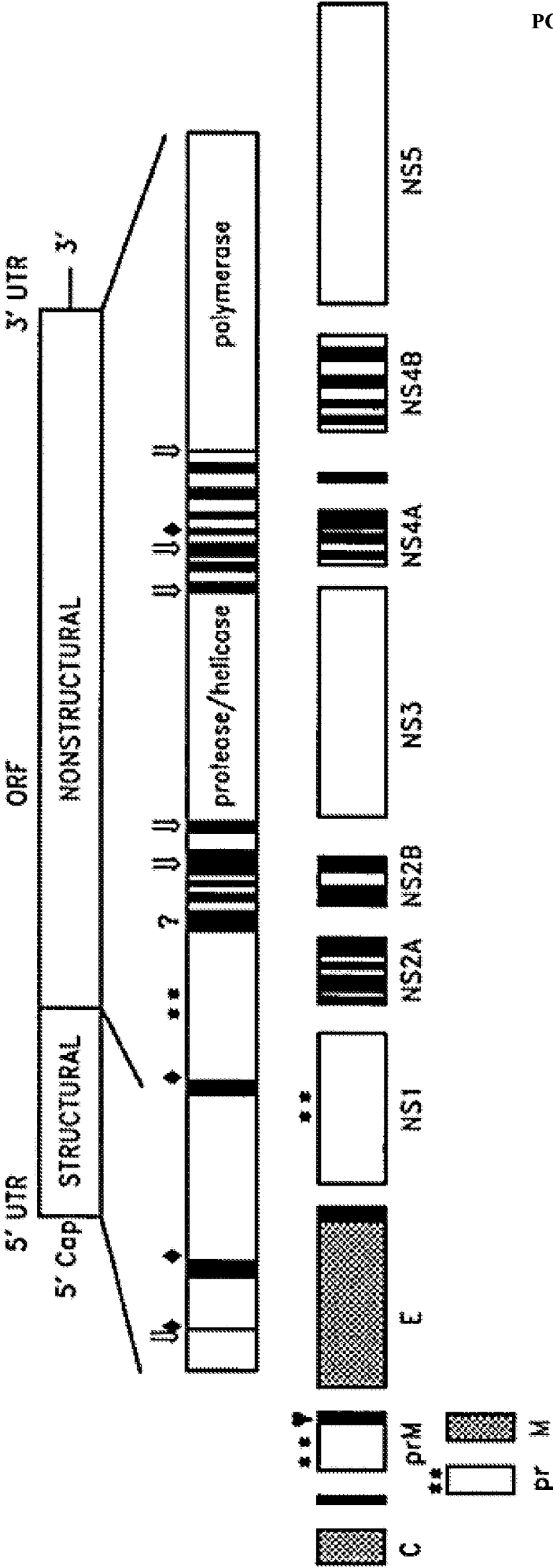
65. A multivalent immunogenic composition comprising at least two different bicistronic attenuated flaviviruses of any one of claims 50 to 62, wherein at least one of the bicistronic attenuated flaviviruses is immunogenic against a Zika virus.

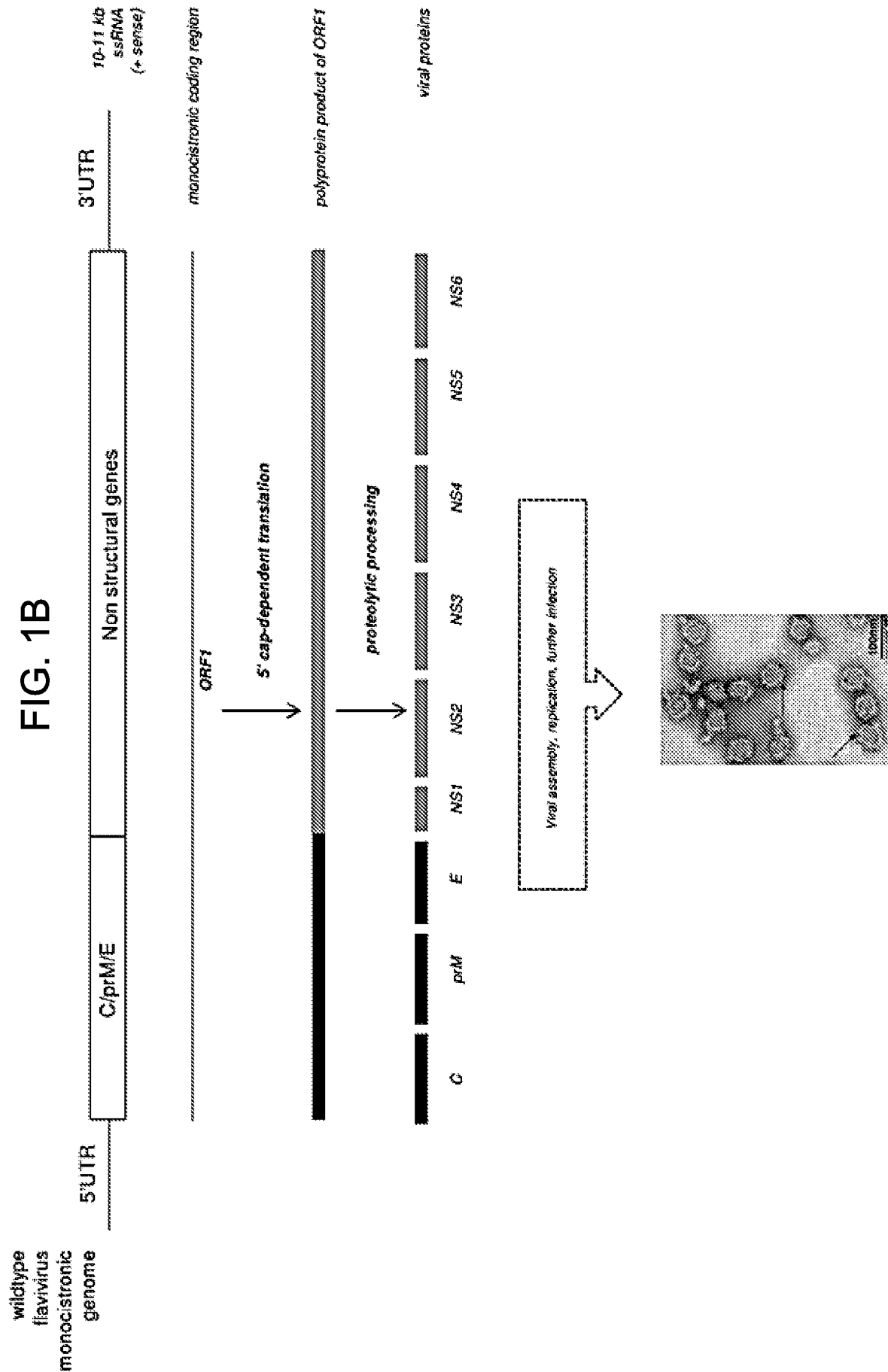
66. A method of inducing an immune response in a subject infected with a flavivirus comprising administering an effective amount of the composition of any one of claims 30 to 32 or 63 to 65.

67. The method of claim 66, wherein the flavivirus is a Zika virus.

68. A method of preventing or treating a Zika virus infection in a subject, wherein the method comprises administering to the subject an effective amount of a flavivirus comprising administering an effective amount of the composition of any one of claims 63 to 65.

FIG. 1A





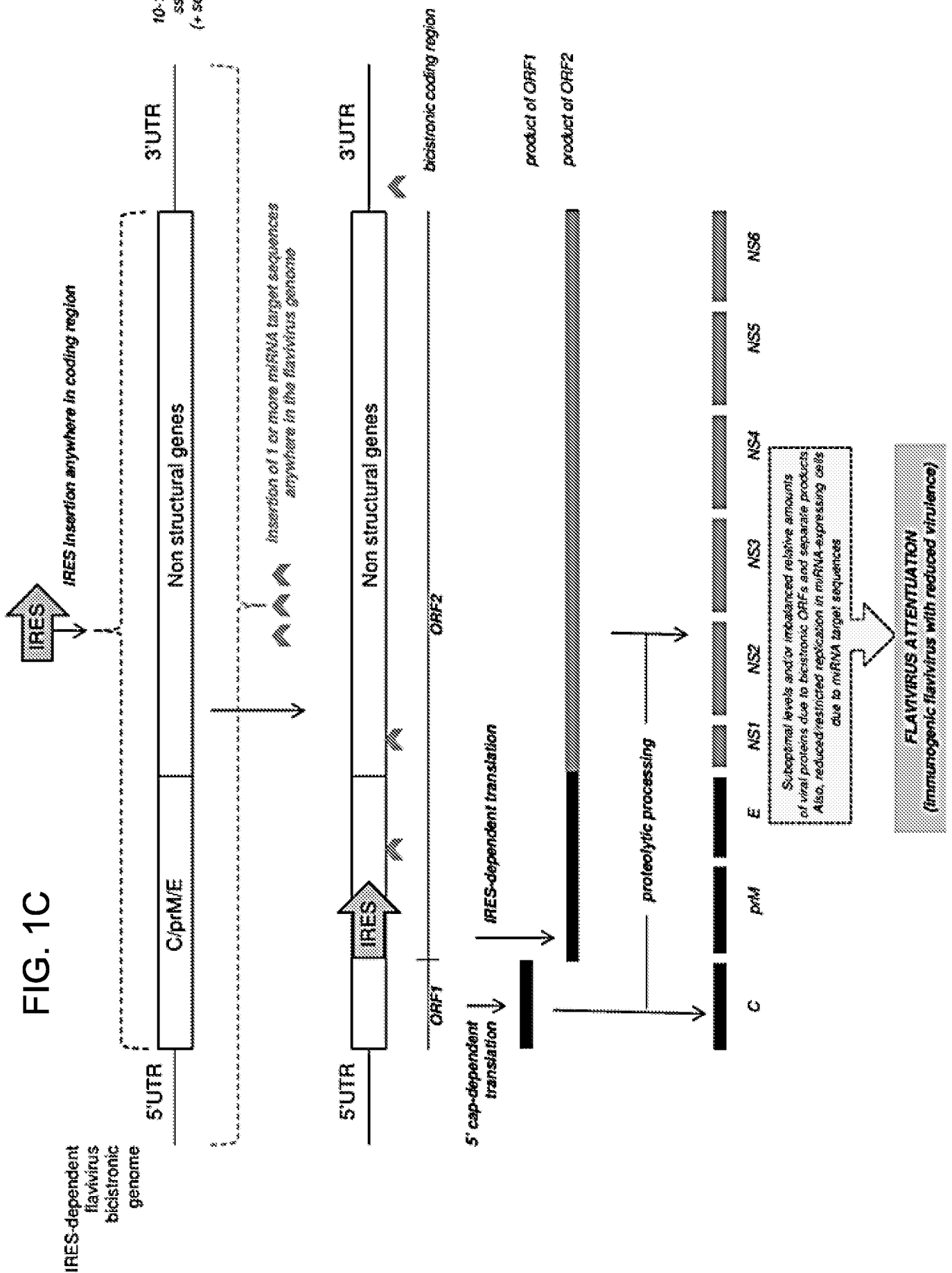


FIG. 1D

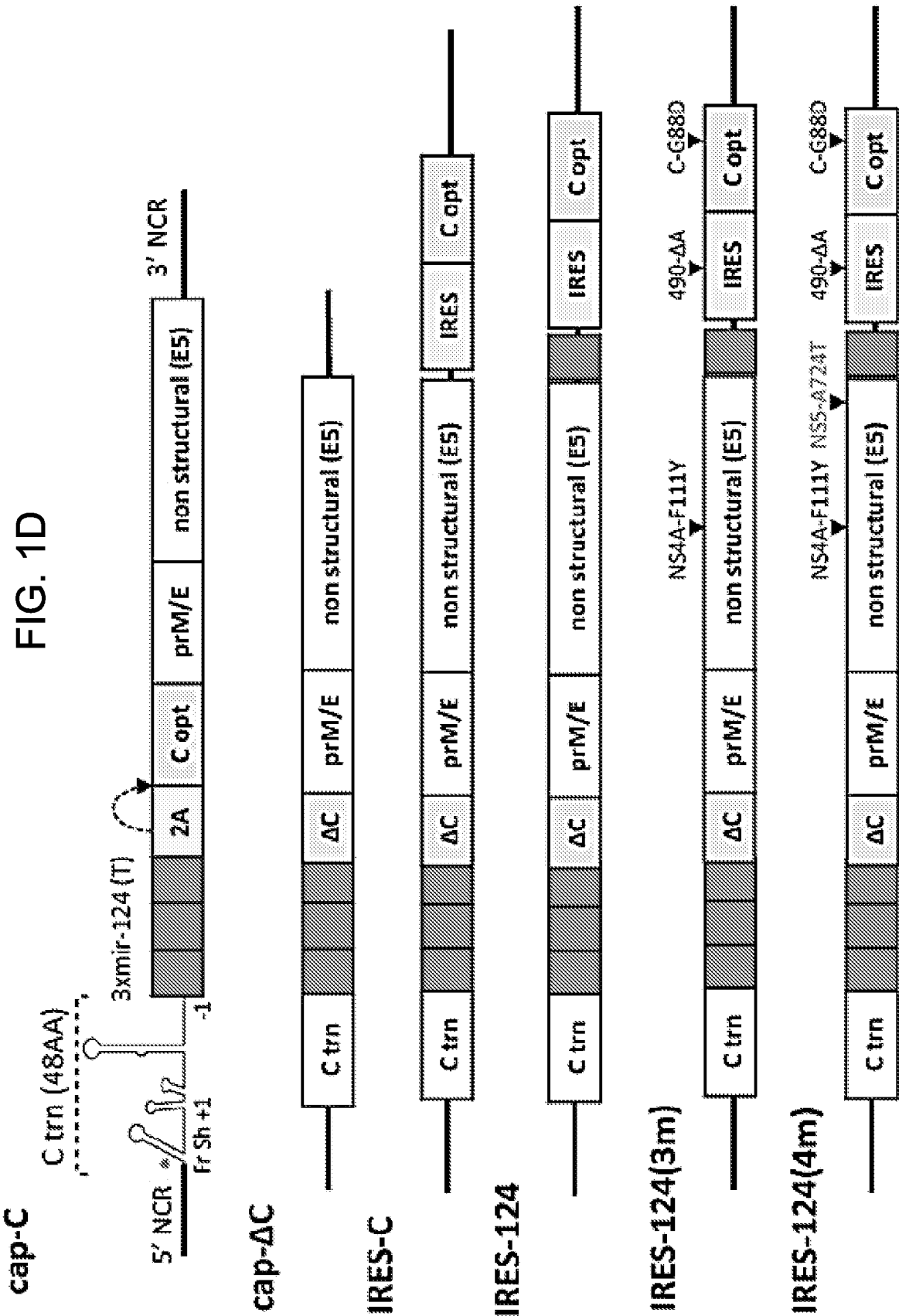


FIG. 2

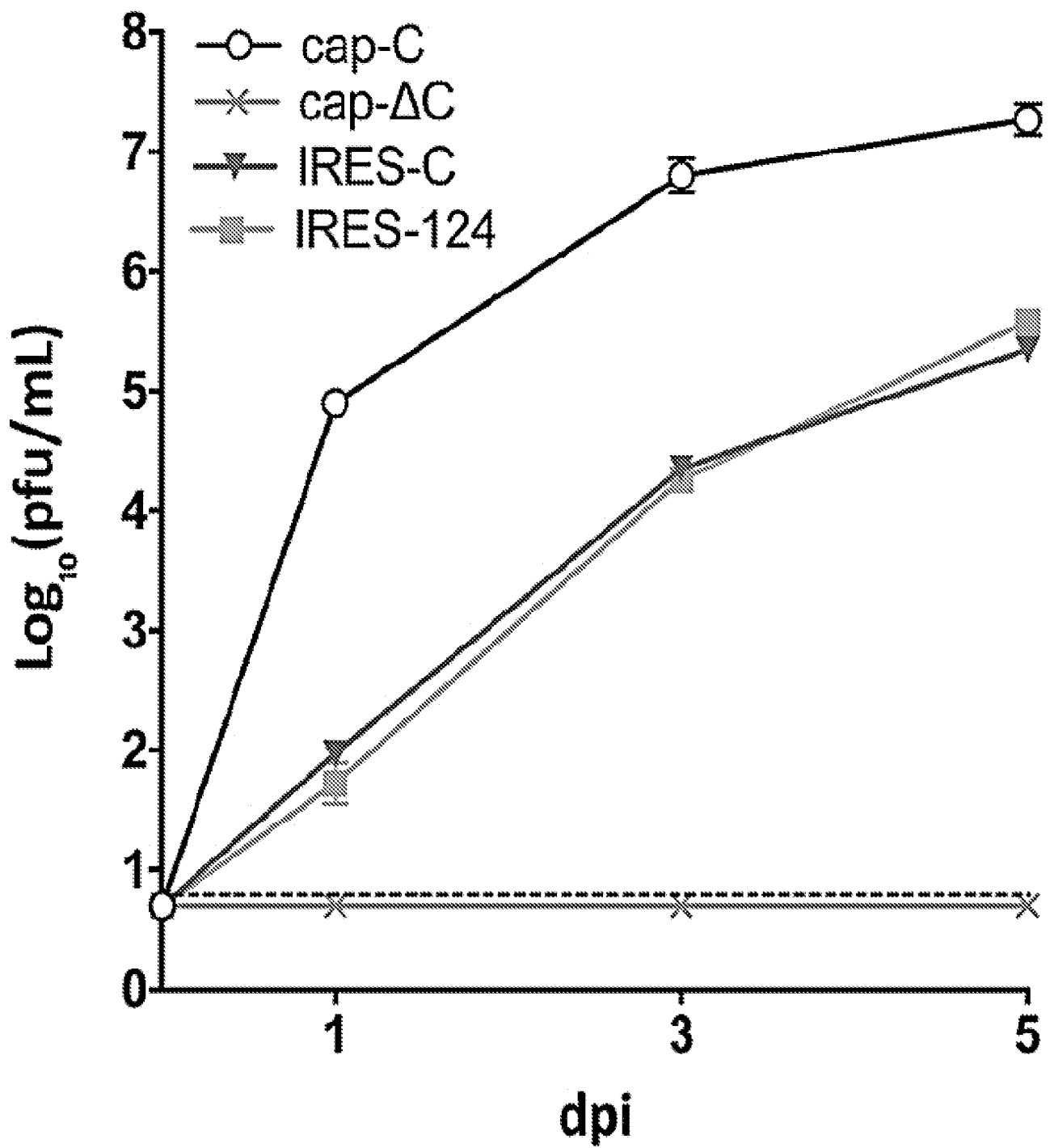


FIG. 3A

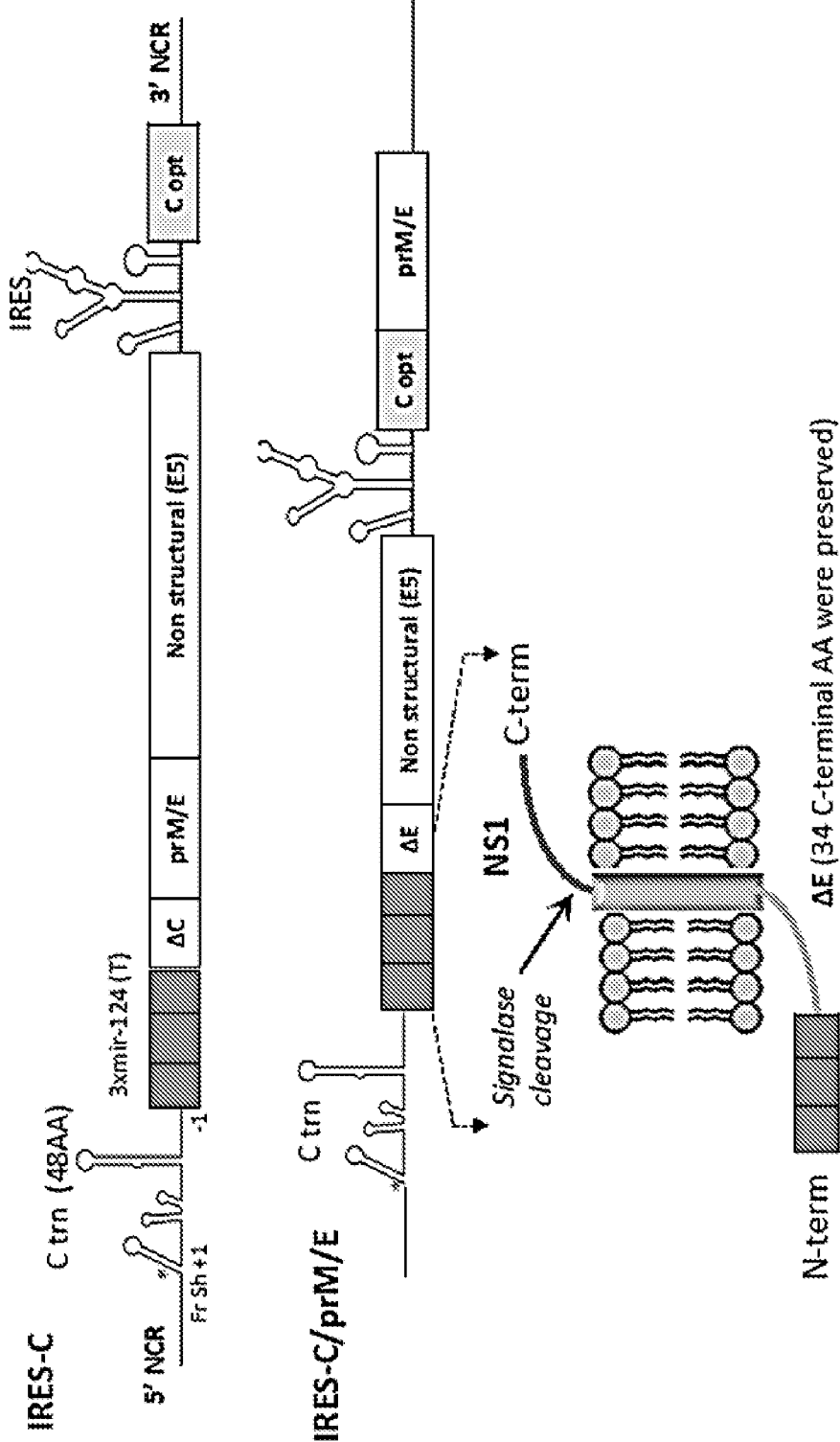


FIG. 3B

FIG. 4A

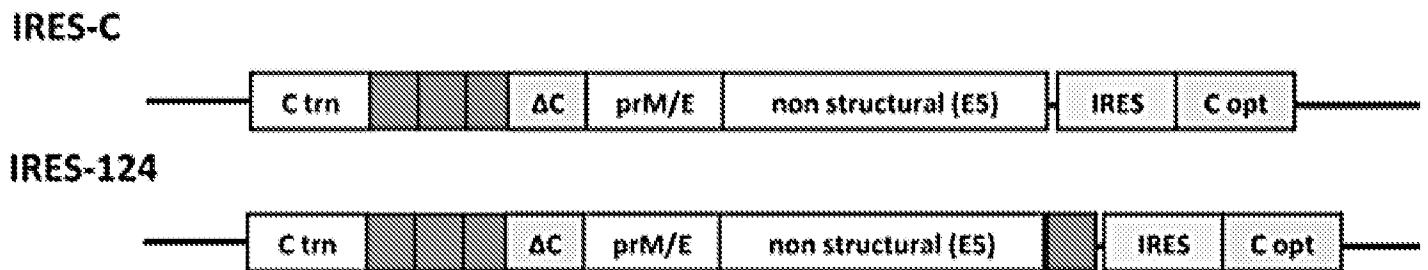


FIG. 4B

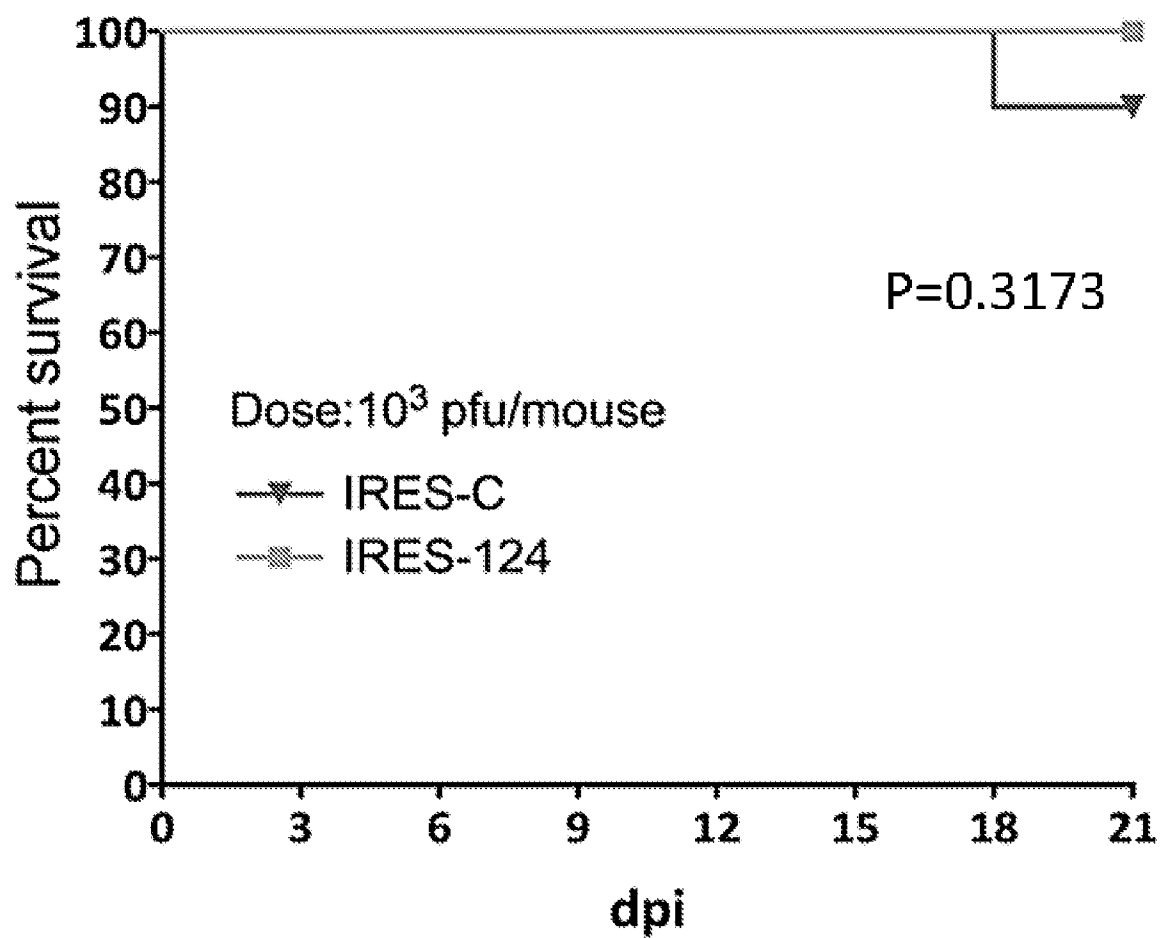


FIG. 5

Gene	Nucleotide	Amino acid
M	G ₂₁₇ →A	A ₇₃ →T
NS4A	U ₃₃₂ →A	F ₁₁₁ →Y
IRES	ΔA ₄₉₀ (deletion)	N/A
C optimized	G ₂₆₃ →A	G ₈₈ →D

Numbers indicate position of the mutation within the gene or protein.

N/A - not applicable

FIG. 6

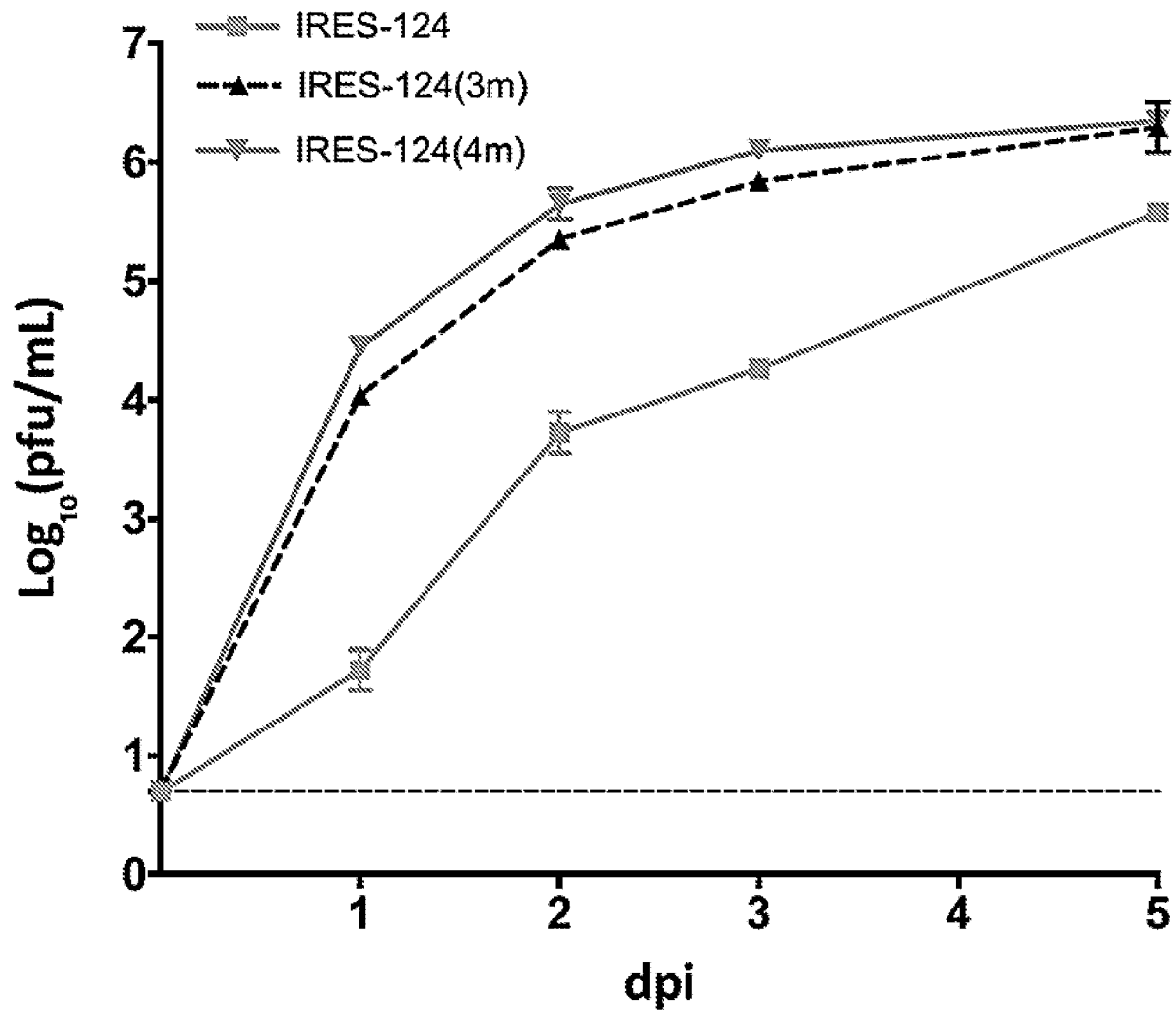


FIG. 7

Virus	No. of mice	Viremia ^a at 1 dpi	PRNT _{50%} against LGTV at 28 dpi	Viremia ^a at 30 dpi (1 day post challenge)	Survival at 56 dpi	PRNT _{50%} against LGTV at 56 dpi
mock	5	-	-	2.7±0.4	5/5 (100%)	28.7 ±8.5
IRES-124(3m)	5	2.58±0.19	204±161	<1.7	5/5 (100%)	173±144

^a – Virus load in the serum was determined by titration in Vero cells. Limit of virus detection was 1.7 Log₁₀(pfu/mL).

FIG. 8

Gene	Nucleotide ^a	Amino acid
E	G ₆₀₇ → A	D ₂₀₃ → N
NS5	G ₂₁₇₀ → A	A ₇₂₄ → T

^a - numbers indicate position of the mutation within the gene or protein...

FIG. 9

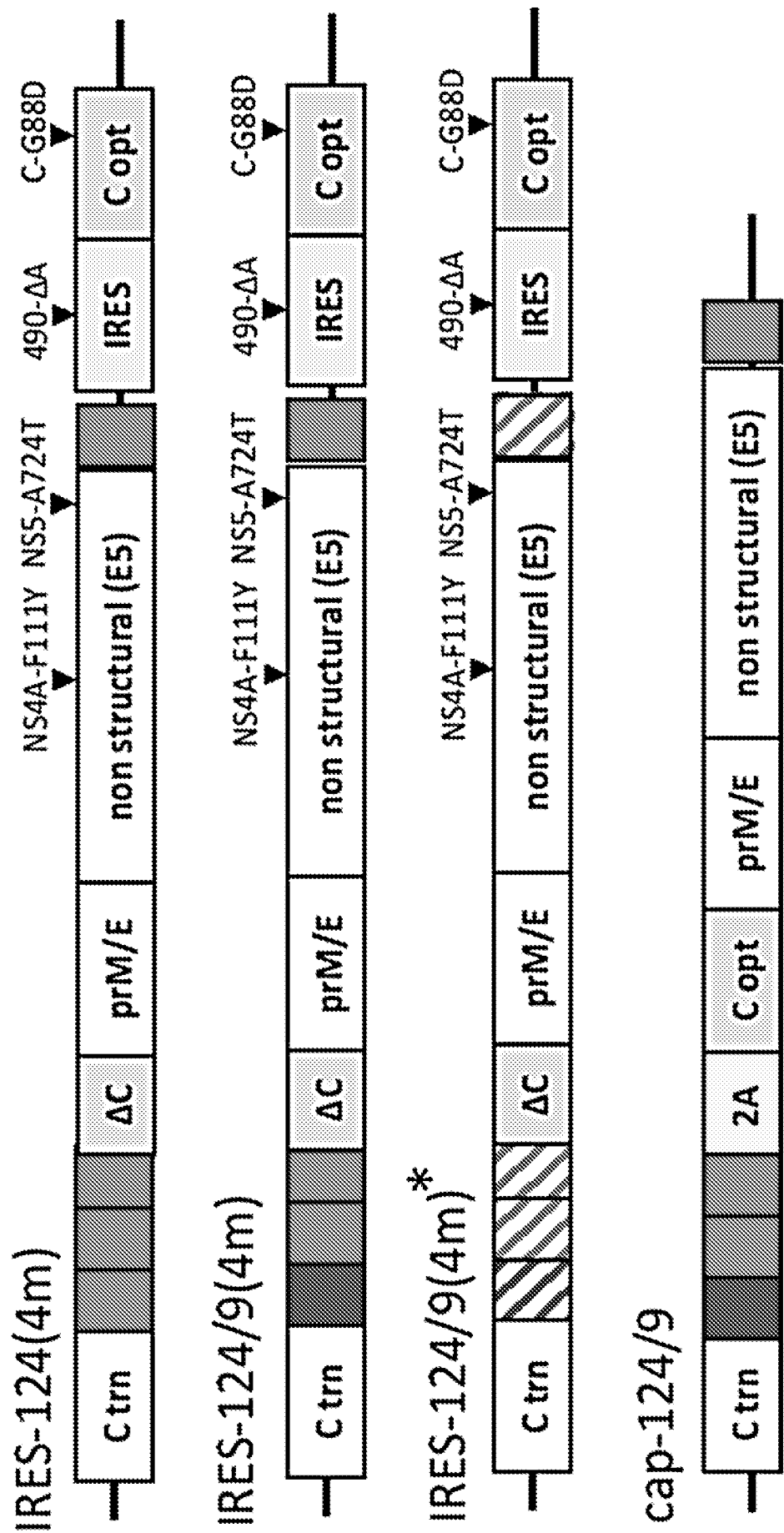


FIG. 10

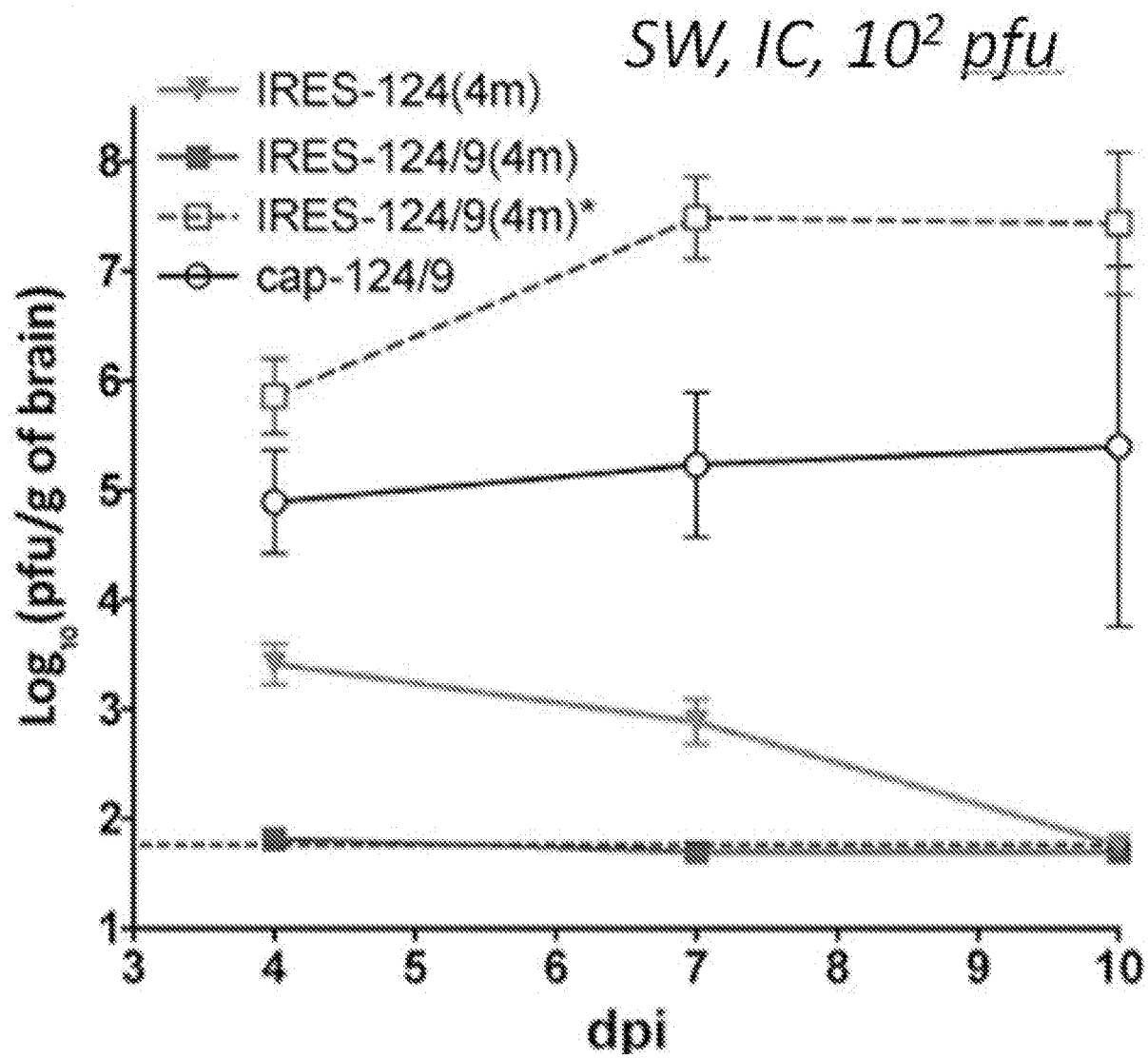


FIG. 11

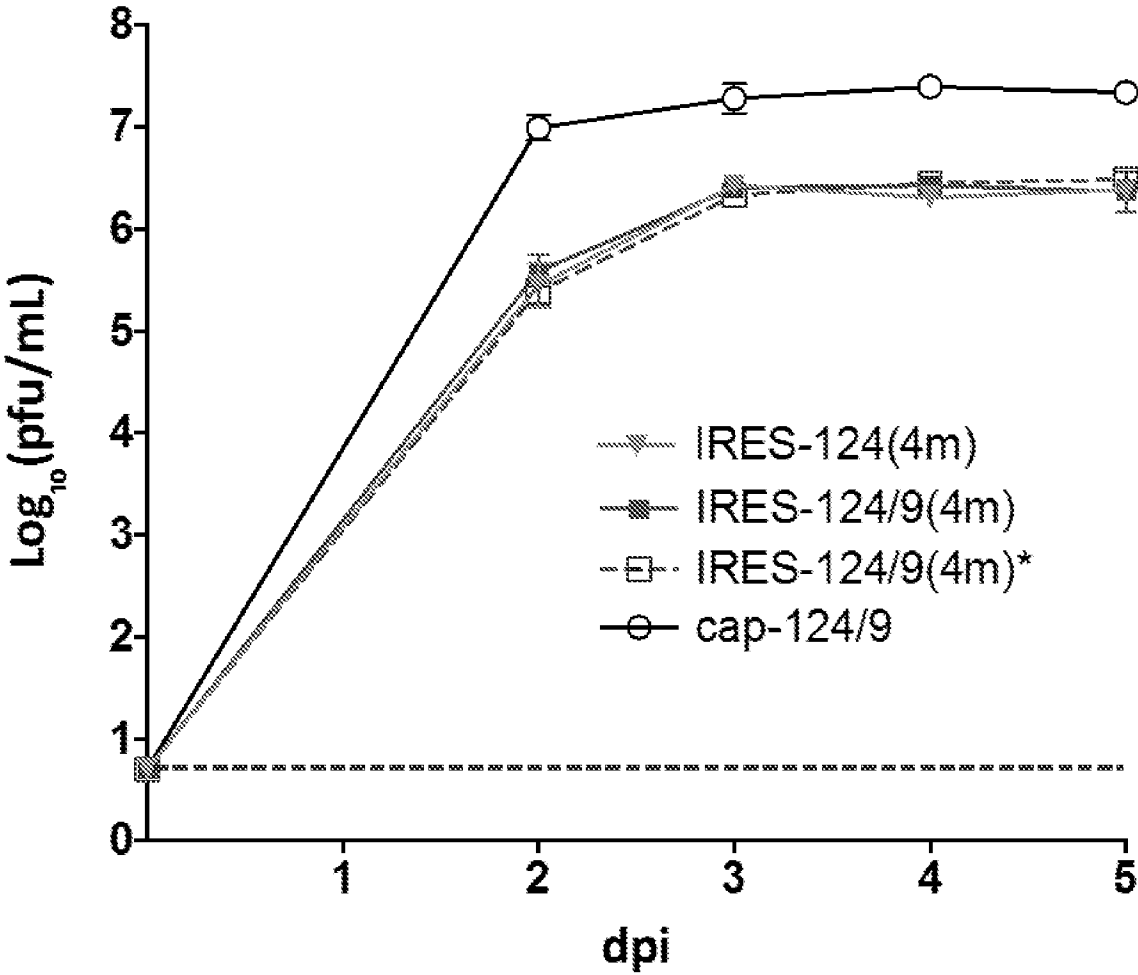


FIG. 12

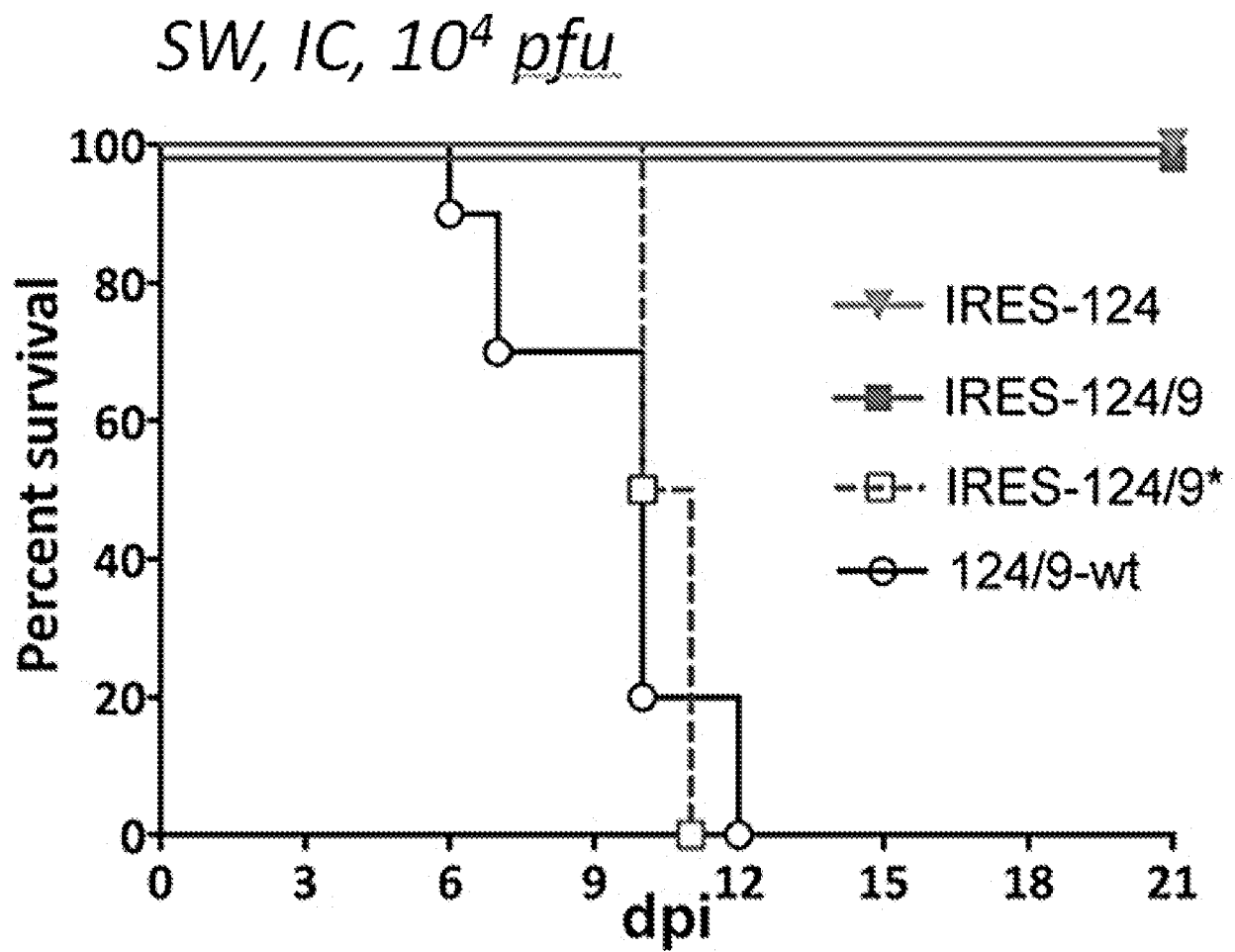


FIG. 13

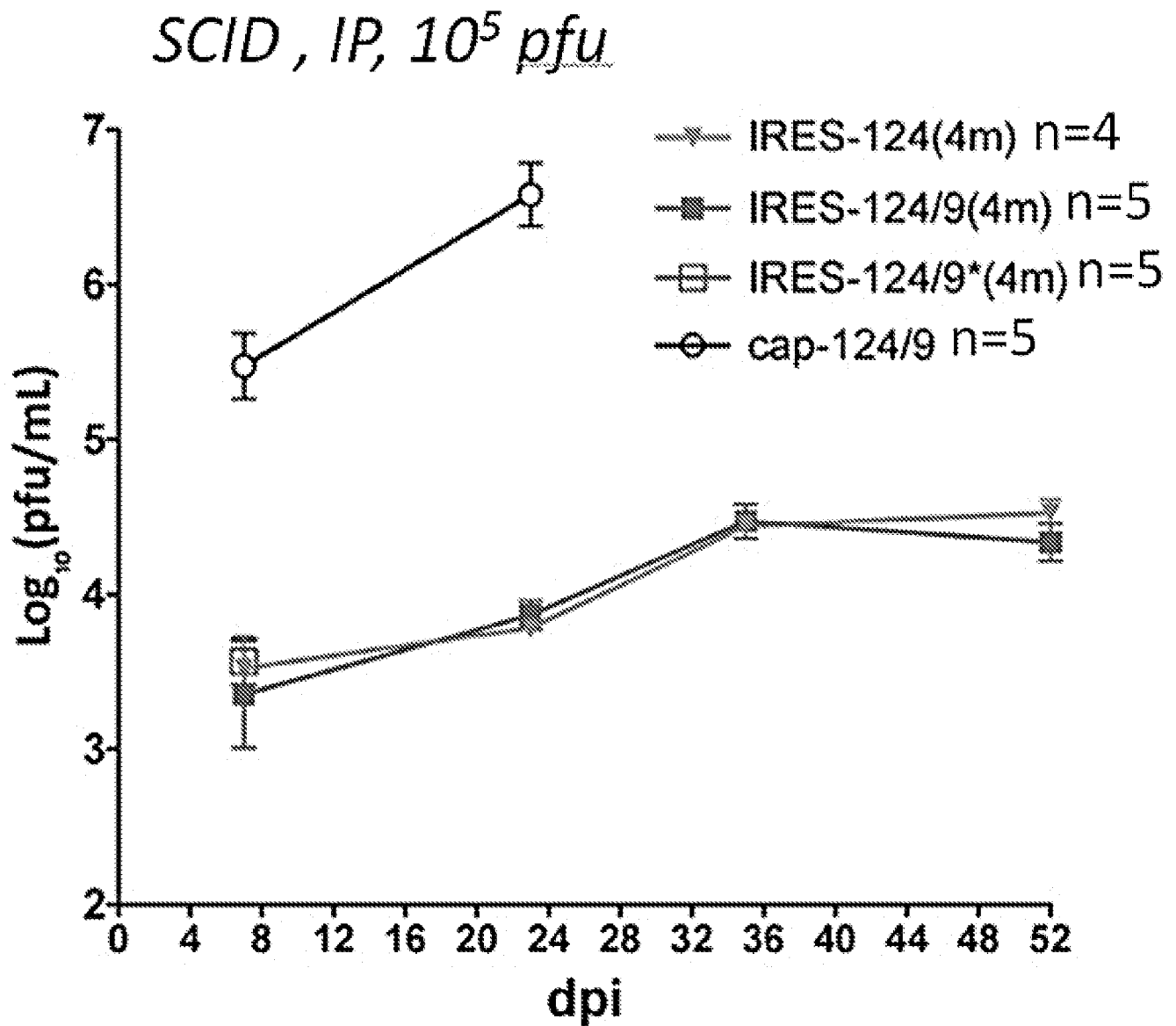


FIG. 14

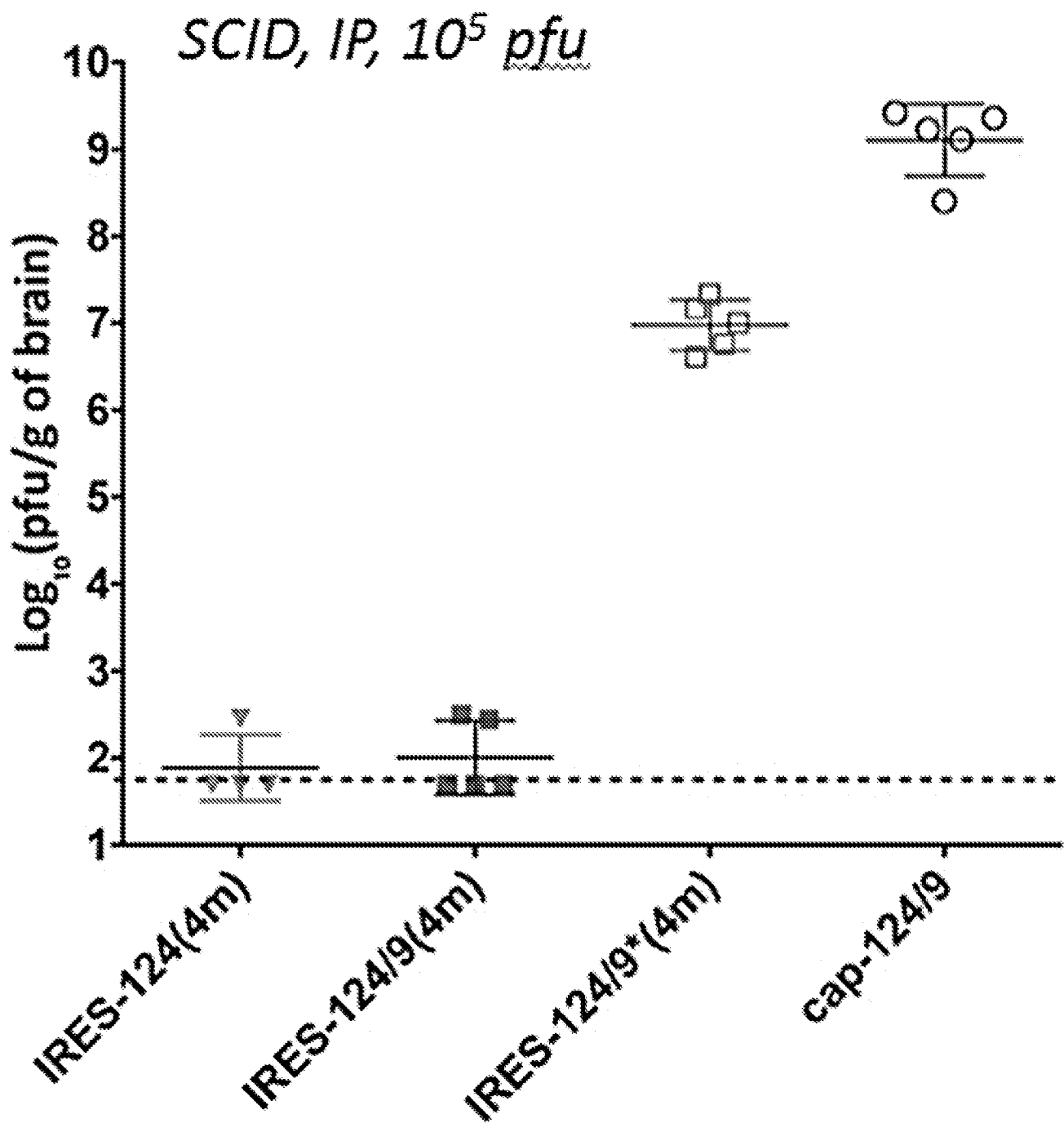


FIG. 15

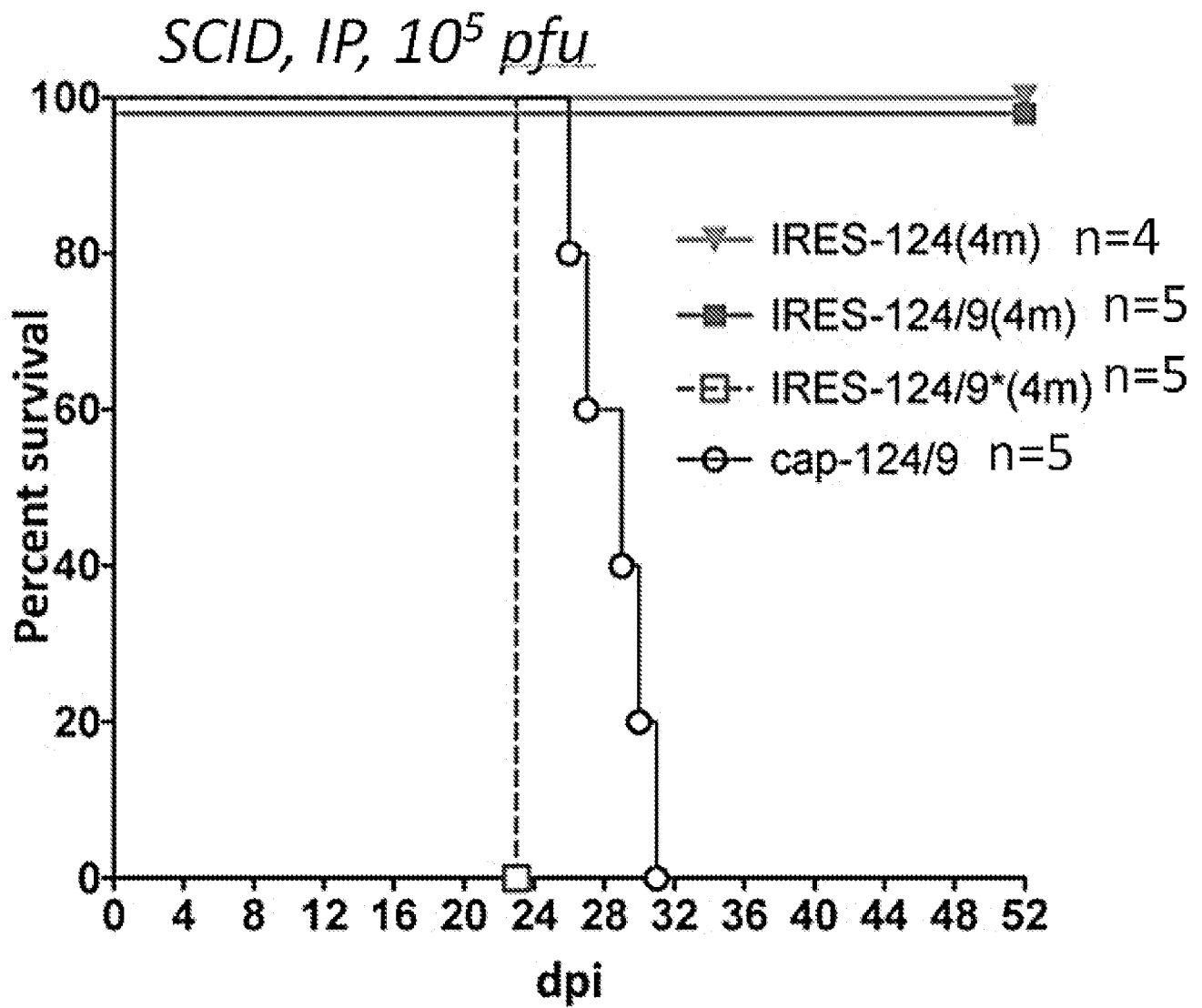


FIG. 16

cap-124/9

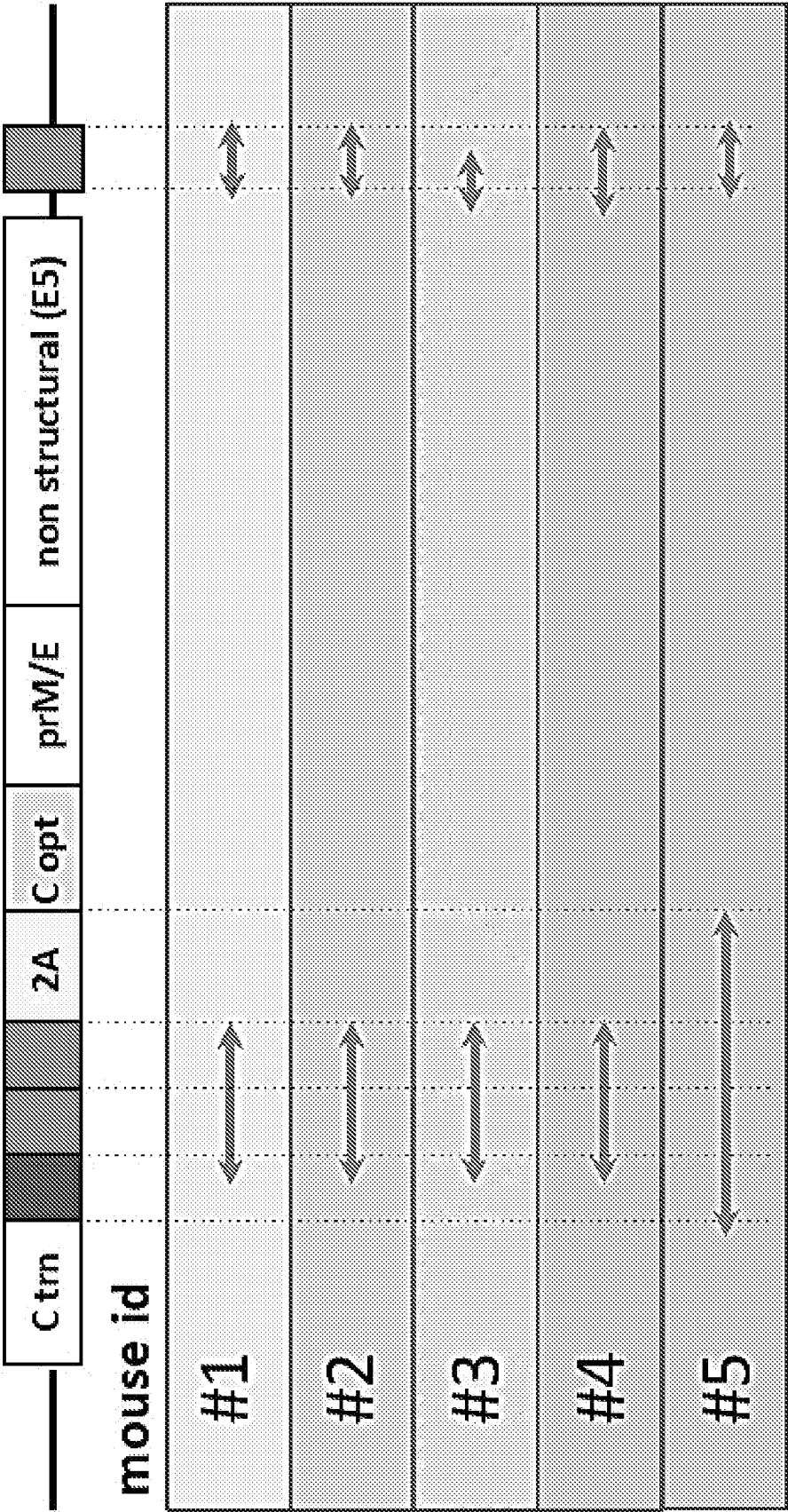


FIG. 17

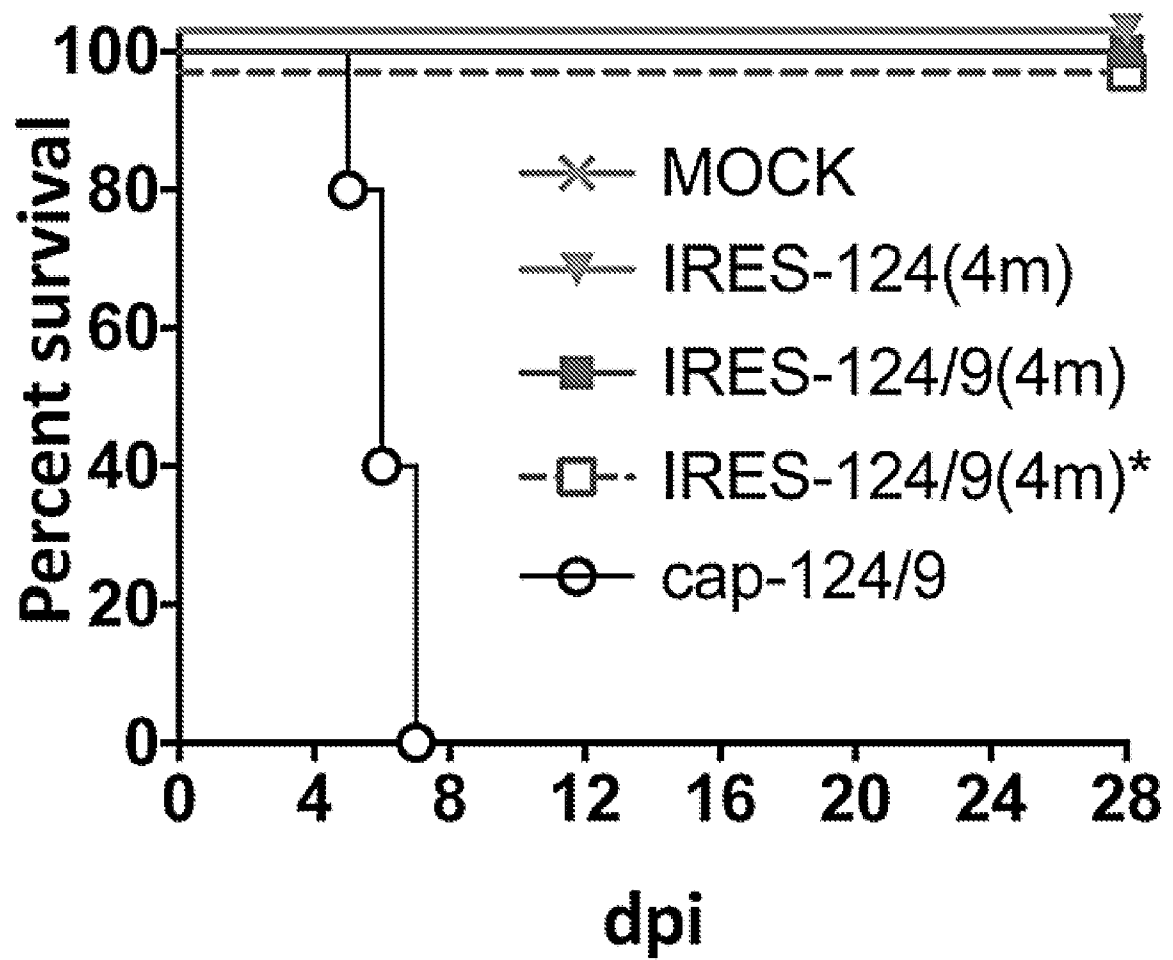


FIG. 18

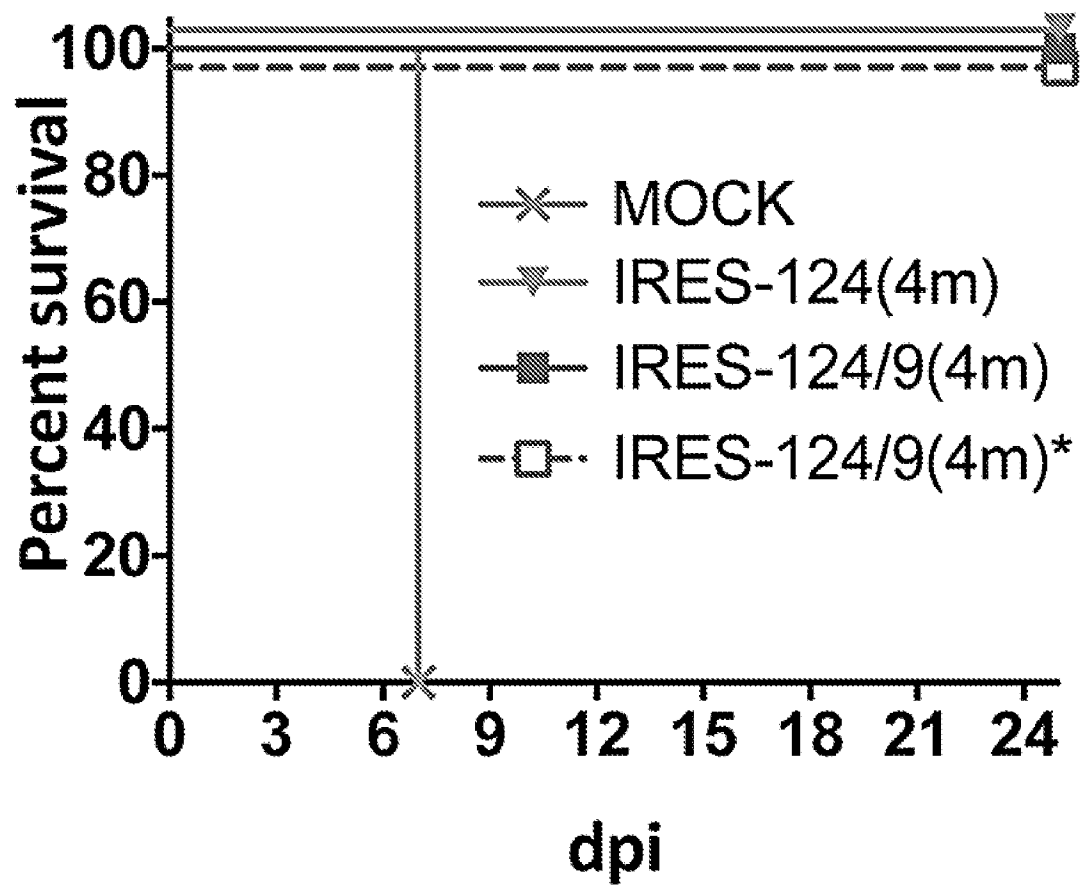


FIG. 19

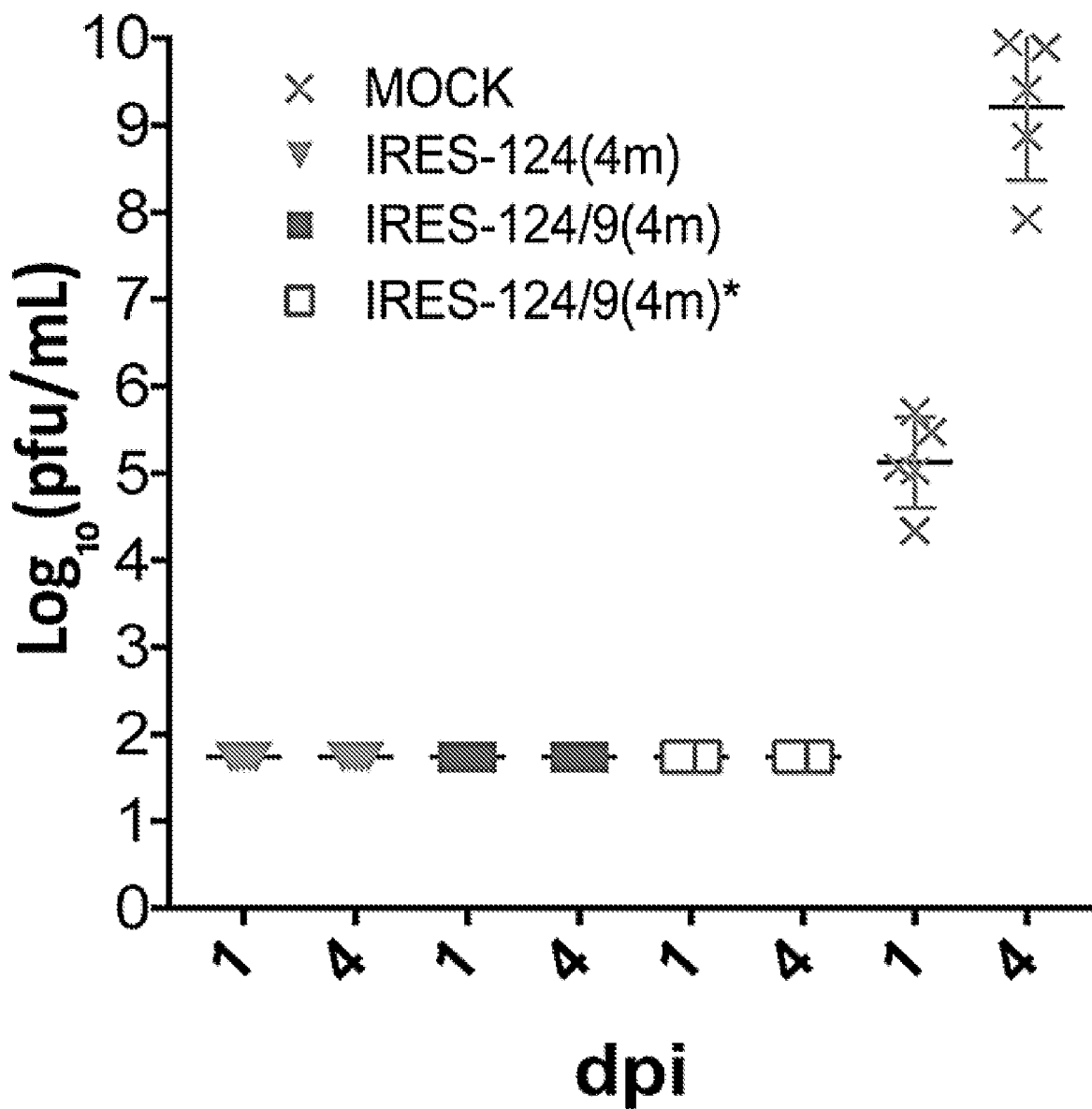


FIG. 20

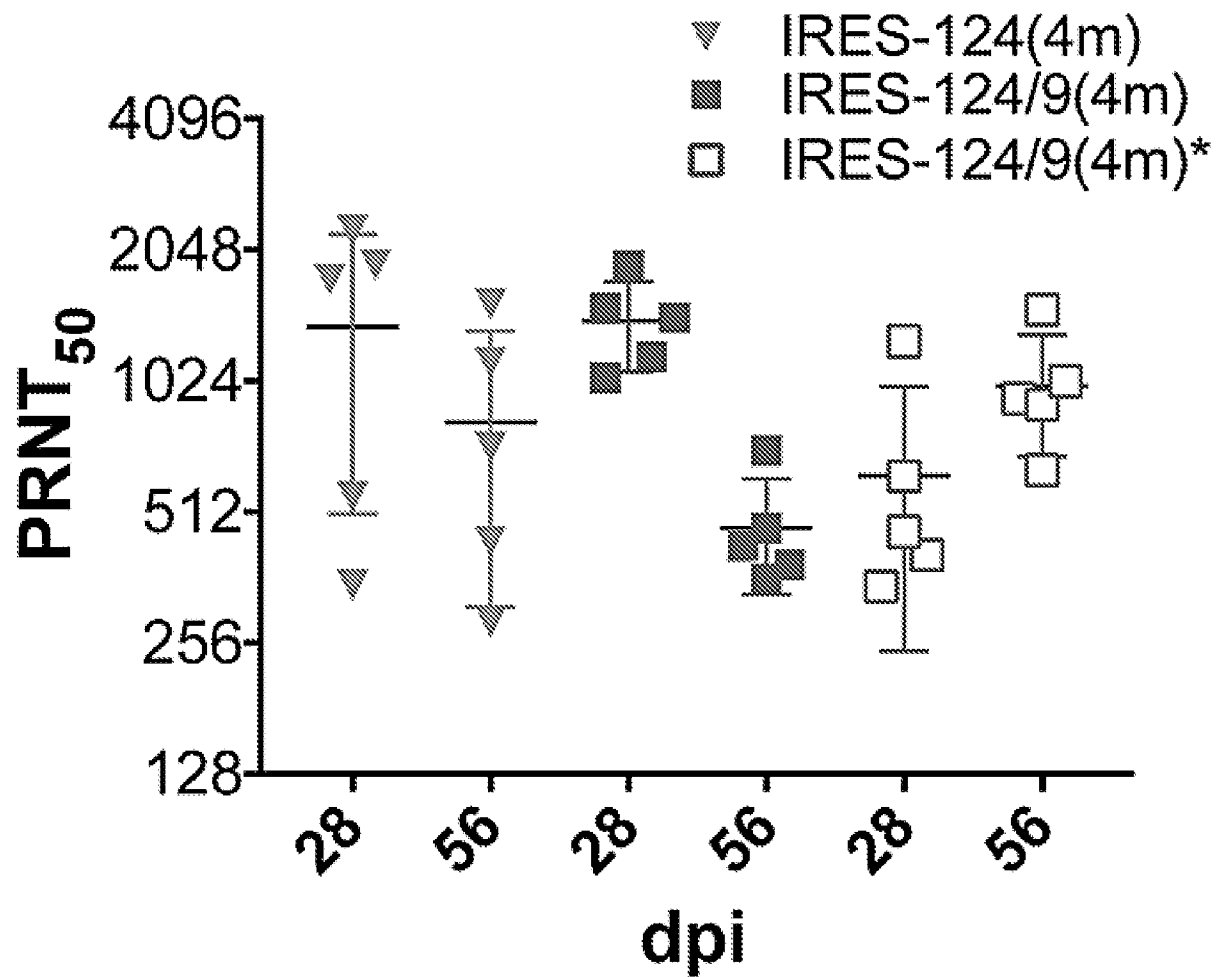


FIG. 21A

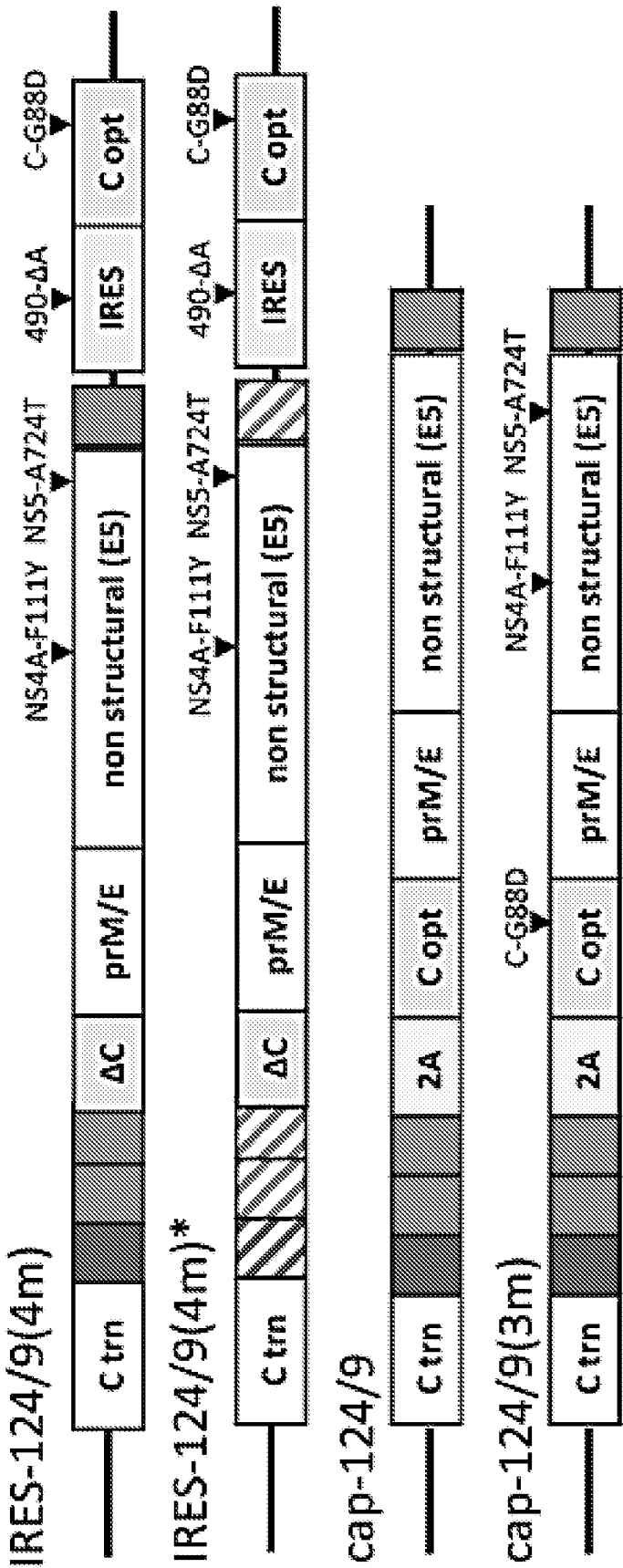


FIG. 21B

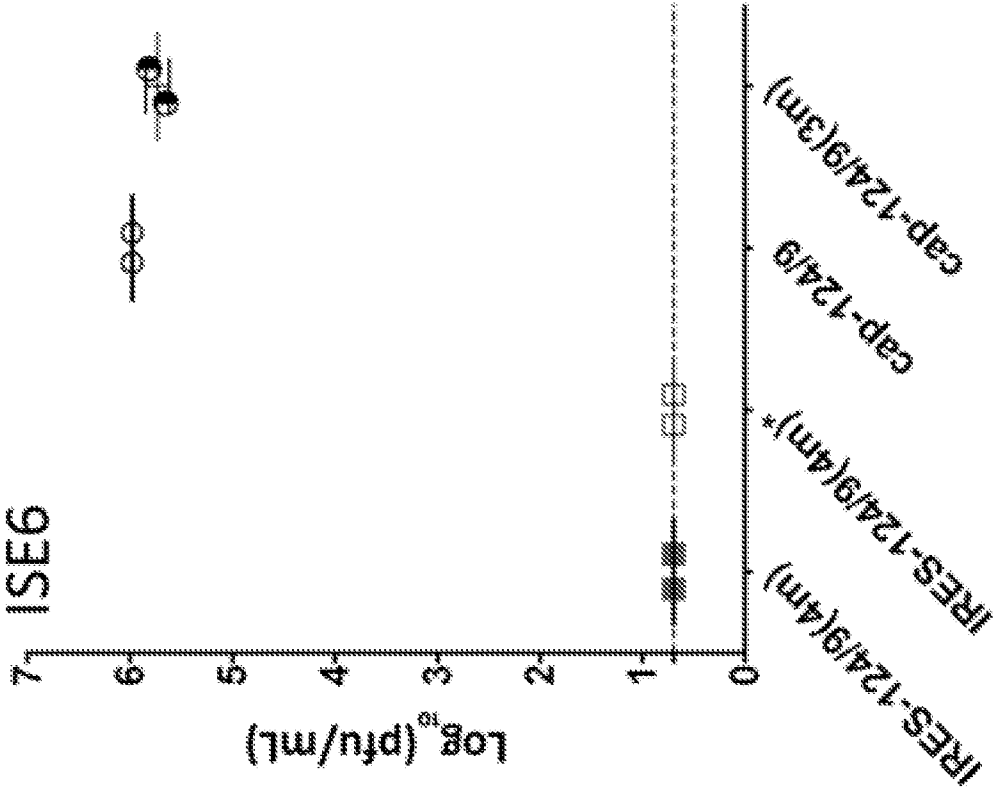


FIG. 21C

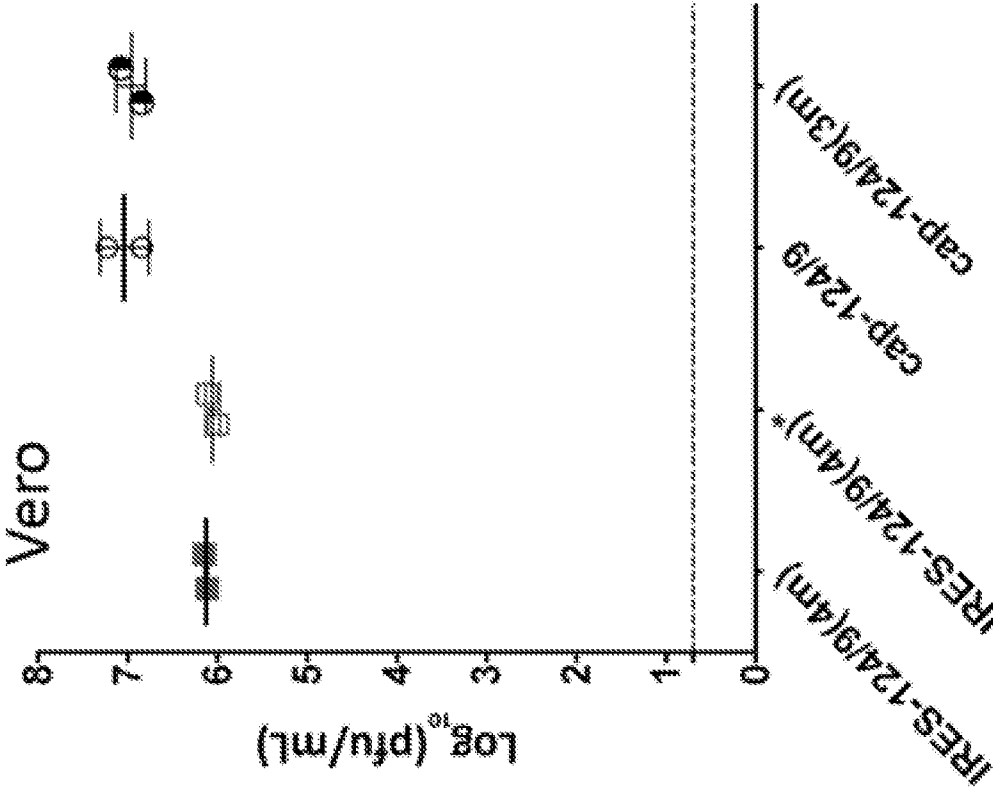


FIG. 22A

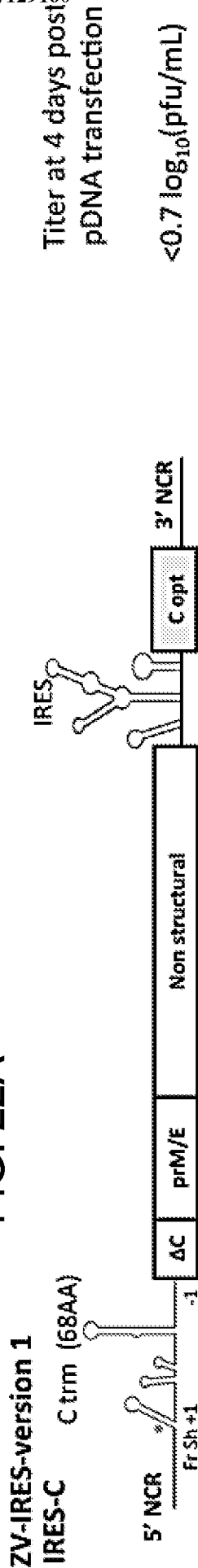
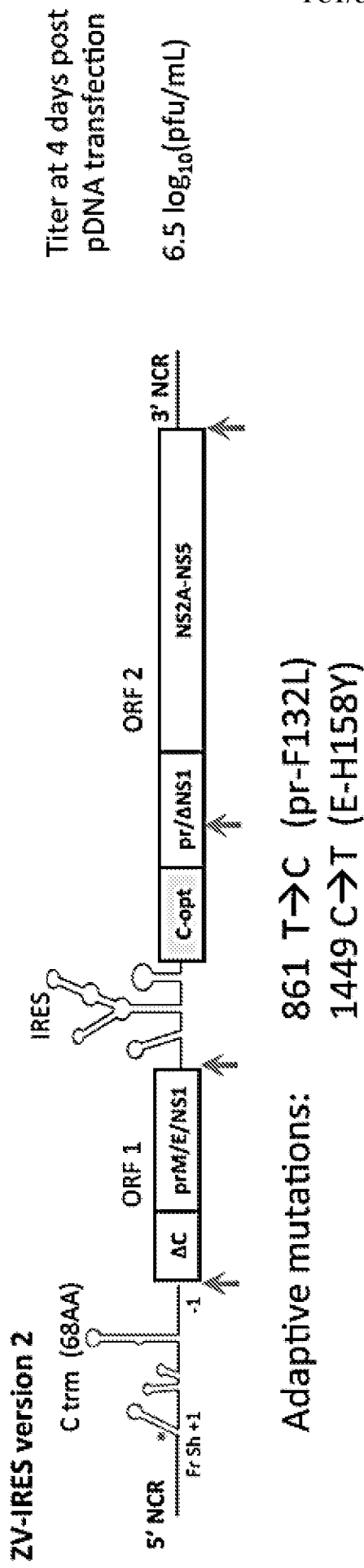


FIG. 22B



JV-IRES-miR or WNV-IRES-miR

FIG. 22C

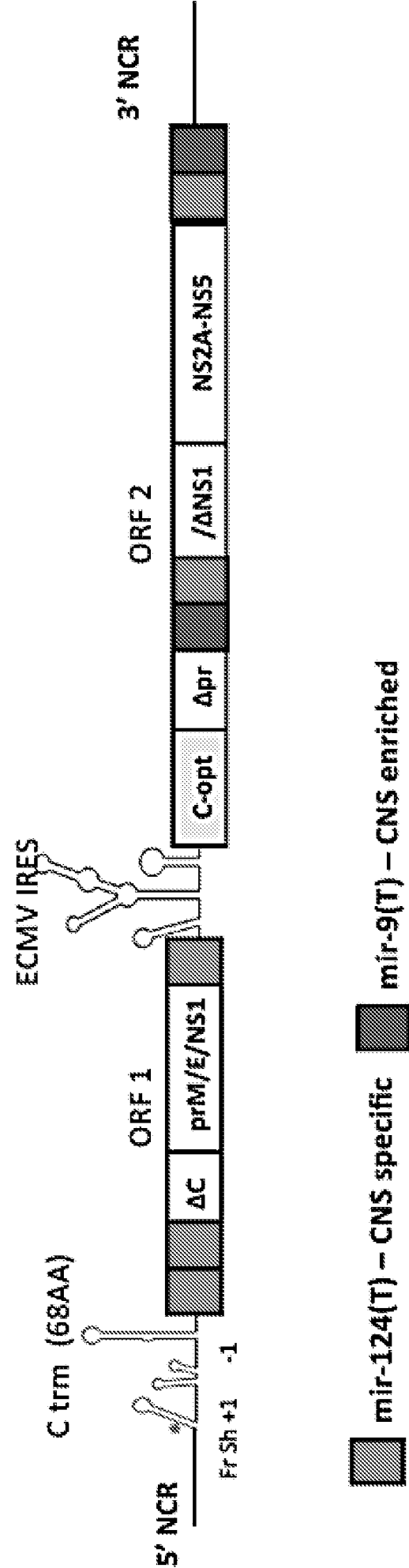


FIG. 23A

COMPLETE NUCLEOTIDE SEQUENCE OF IRES-124/9

AGATTTTCTTGCGCGTGCATGCGTGTGCTTCAGACAGCCCAGGCAGCGACTGTGAT
 TGTGGATATTCTTTCTGCAAGTTTTGTGCTGAACGTGTTGAGAAAAAGACAGCTTA
 GGAGAACAAGAGCTGGGA**ATG**GCCCGGGAAGGCCGTTCTA**AAA**AGGAAAGGGGGGGG
 GTCCCCCTCGACGAGCGTCGAAAGTGGCCCCAAAGAAGACGCGTCAGTTGCGGGTC
 CAAATGCCAAATGGACTTGTACTGATGCGCATGCTGGGAGTTCTGTGGCag**TCATA**
CAGCTAGATAACCAAAGAgaaCGTTCCCTTTGGGCATT**CACCGCGTGCCTTAGAATC**
CACTGAGgGGCATTACCGCGTGCCTTACACCGCCGAGGAAGTAGAAGGACGACCA
 TTGACTGGATGACTCCCTTGTTGATCACTGTCATGCTTGGAAATGTGCCTCACGGCA
 ACTGTCCGTAGAGAGAGAGATGGGTCAATGGTGATTCTGTGCTGAAGGGCGAGATGC
 TGCAACCCAGGTTAGAGTGGAGAATGGCACTTGTGTCAATTCTTGCTACAGACATGG
 GATCATGGTGTGATGACTCGTTGGCTTACGAATGCGTGACCATTGACCAGGGAGAG
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 CACACGCACAGCGTGACCTGACCGGGCGGGGACACCAATGGTTAGAGGGAGAGGCT
 GTGAAGGCACACCTGACCCGCGTTGAGGGTTGGGTGTGGAAGAACAAATTGTTTCAC
 CCTTTCTCTTGTGATGGTGGCCTGGTTGATGGTGGATGGATTGTTGCCCAGGATTC
 TCATTGTGGTTGTGGCTCTCGCATTTGGCACCGGCCTACGCGTCGAGATGCACCCAC
 CTGGAAAACAGAGACTTCGTCACTGGGGTGCAGGGGACGACCCGCCTCACATTGGT
 TCTCGAGCTGGGGGGCTGCGTGACGGTCACGGCGGATGGGAAGCCATCCCTCGACG
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 AGCATCGGCAGGGTCCCTCCAGAAGACCAGGAAGGGGATTGAGAGACTCACCGTCT
 TGGAGAACATGCTTGGGACTTTGGCTCAGTGGGAGGTGTGATGACGTCCATTGGGA
 GAGCCATGCACACCGTCCCTGGGAGGAGCGTTTAAACACCCTCCTAGGGGGGGTGGGC
 TTCCTACCCAAGATACTACTAGGCGTAGCTATGGCCTGGCTGGGCTTGAACATGAG
 GAACCCAACCTTGTCCATGGGCTTCCTACTCTCAGGGGGCTTGGTGCTGGCCATGA

start codon
"A" insertion
mir-9
mir-124
mir-124

FIG. 23B

CTCTGGGGGTTGGAGCCGATGTGGGCTGCGCAGTGGACACAGAGCGAATGGAGGTA
 AGTATCAAGGTTACAAGGCAGGTTTAAGGAGACCAATAGAACTGGGCTTGTGCGAG
 ACAGAGAAGACTCTTGGCGTTTCTGATAGGCACCTATTGGTCTTACTGACATCCACT
 TTGCCTTTCTCTCCACAGCTCCGATGTGGAGAAGGACTGGTGGPATGGAGAGAGGT
 GTCAGAAATGGTACGACAACTACGTGTTCCACCCAGAGACTCCGGCGGTCTCTGGCAT
 CGGCAGTTCAGAGGGCCCTATGAAGAGGAAATCTGTGGCATTGTCCCTCAAAATAGA
 CTGGAGATGGCCATGTGGAGAAGCTCTCTAGTGGAGCTCAACCTGGCTCTAGCGGA
 GGGTGAGGCAAACCTGACTGTTGTGGTCGACAAGGCAGACCCAGTGACTACAGGG
 GGGGCGTTCCCGGGCTCCTTAACAAGGGAAAAGACATTAAAGTATCTTGGAGGAGT
 TGGGGGCGATCCATGCTCTGGAGTGTTCGCGAGGCCCCCTCGCAGGTTTCATGATTGG
 AGTGGAAGGAGGTAGAGAGTGCCCCCTTTGCCAGAAGGAAGACAGGTGTGATGACGG
 TGGCAGAGTTTGGGATTGGTCTTCGCACGAAAGTCTTCATGGACTTGCGGCAAGAA
 TTGACAACAGAGTGTGACACAGGGGTCATGGGGGCTGCGGTCAAGAATGGAATGGC
 CGTCCACACTGATCAAAGTCTGTGGATGAAATCCATCAAGAATGATACAACTGTAA
 CAATAGTTGAGCTGATTGTGACAGACCTGAGAACTGCACTTGCCAGCTAGCCAC
 ACCATTGACAATGCCGGAGTAGTGAAGTCAAAGCTGTTTCTACCGGCCAGTCTGGC
 TGGACCTAGGTCAACCTACAATGTGATACCTGGCTATGCAGAGCAAGTGAGGGGAC
 CGTGGGCGCACACCCCGGTGAGAATAAAAAGAGAAGAATGCCCTGGAACCCGAGTG
 ACCATTGACAAGGCATGTGACAAGAGAGGGGCGTCGGTGAGAAGCACTACAGAGAG
 TGGGAAAGTCATACCCGAATGGTGCTGTGCGACCTGTGAGCTGCCCCCGGTACAT
 ATCGCACTGGGACTGATTGTTGGTATGCCATGGAGATTAGGCCGGTACACACTCAG
 GGTGGCCTGGTCAGATCCATGGTAGTGGCTGACAACGGGGCTCTACTCAGTGAAGG
 TGGAGTTCGGGGGGTAGTAGCGCTCTTTGTGGTTTTTGGAGCTGGTGATTAGAAGGC
 GACCAGCCACGGGGGGCACGGTGATCTGGGGAGGCATTGCCATTCTGGCTCTTTTG
 GTGACGGGACTAGTCTCTGTGGAGAGTCTGTTTCGTTACCTTGTAAGCAGTCGGTCT
 GGTGTTTCAGCTGGAAGTGGGACCAGAGGCTGTGGCTATGGTGCTTCTACAGGCAG
 TTTTGTGAGATGAGAACGTGTCTACTGAGTGGGTTTTGTGCTTCGTAGAAGCATAACC
 ACGAGAGAAATTGTGACAGTGTACTTCCTCCTCCTGGTGTGGAGATGGGCATTCC
 AGTGAAGGGTCTGGAGCATCTCTGGAGATGGACTGATGCGCTGGCGATGGGGGCCA
 TCATCTTCAGGGCATGCACTGCAGAGGGGAAGACAGGCATTTGGTTTGTCTTTTGGCT
 GCATTTCATGACCCAGAGTGACATGAACATCATCCATGATGGCTTGACCGCTTTTCT
 TTGTGTGGCCACTACCATGGCCATATGGAGATACATCAGGGGCCAGGGAGAGAGAA
 AAGGACTGACATGGATCGTCCCGCTTGCTGGCATCCTTGGAGGAGAGGGCTCAGGA
 GTACGACTGCTGGCCTTCTGGGAGTTGGCGGCCTCCAGGGGGAGACGTTCTTTAA
 TGAGCCTATGACTGTCATAGGAGTGATGCTGACTTTGGCCAGTGGAATGATGCGCC
 ACACGTCTCAGGAGGCGGTGTGTGCCATGGCGCTGGCGGCTTCTCTTCTACTCATG
 CTCACGCTTGGAAGTAGGAAAATGCAGTTGTTGGCTGAGTGGAGTGGGAACATTGA
 GTGGAATCCAGAACTGACCAGTGAGGGCGGTGAAGTGAGCCTGCGCGTCAGACAAG
 ATGCCCTTGGAAGTTGCACCTGACTGAACTGGAGAAGGAGGAACGCATGATGGCT
 TTTTGGCTGGTGGTAGGCTTGATTGCCTCGGCCTTCCACTGGTTCGGGATCCTTAT
 CGTCATGGGACTGTGGACCATTTCGGAGATGCTTGGCTCACCAGGAGAACGGATC
 TTGTGTTTTTCAGGATGCTCCGAAGGGCGAAGTGACAGCCGTCGCTCGATGTAAAA
 AGTGGAGTGTAACGCATATATACTCCAGGCCTGCTCTGGGGACAAAGACAGATTGG
 AGTGGGCTACGGGGCAAAGGGTGTGCTACACACCATGTGGCATGTCACTAGGGGGG
 CTGCCCTGCTGGTTGATGGAGTTGCAGTGGGCCCCCTACTGGGCTGATGTGCGGGAG
 GATGTGGTTTTGTTATGGAGGCGCTGGAGTCTGGAAAGCAGGTGGAGGGGTGAGAC
 TGTGCAGGTGCATGCCTTCCCACCCGGCAGGGCACATGAAACACACCAATGCCAAC
 CTGGAGAGTTGATATTGGAGAATGGGAGGAAGATGGGAGCCATTCCCATTTGATCTC

introne

4528T>C

FIG. 23C

GCCAAAGGAACATCAGGCAGTCCAAATTATGAACTCACAGGGAGAGGTTCGTTGGCCT
 GTATGGAAACGGCCTCAAGACTAATGACACGTATGTCAGCAGCATAGCACAGGGTG
 AAGTGGAAAAAAGTAGACCAAATCTCCCACAGTCAGTAGTTGGCACGGGCTGGACA
 GCCAAGGGACAGATCACGGTGTGGATATGCACCCTGGCTCGGGGAAGACCCATCG
 GGTCCCTCCCTGAGCTCATCCGTCAATGTGTGGAGCGGCGATTGCGAACCTTGGTCT
 TGGCCCCCACACGAGTGGTGCTGAGGGAGATGGAACGCGCCCTTAGTGGGAAGAAC
 GTGCGGTACCATTTCGCCTGCAGTCACAGAGCAGCACGCCAATGGTGCAATCGTTGA
 TGTCAATGTGCCATGCTACATATGTCAACAGAAGACTCCTCCCACAGGGGAGACAAA
 ACTGGGAGGTTGGCTATCATGGATGAGGCTCATTTGGACTGACCCCCACAGTATAGCG
 GCCAGAGGACACCTCTACTCCCTTGCCAAAGGAAAACAGATGTGCTCTCGTTCTCAT
 GACGGCAACACCACCGGGGAAGAGTGAACCCCTTTCTGAGTCCAACGGAGCCATAG
 CCAGTGAGGAGAGACAGATCCCTGATGGGGAGTGGCGGGACGGGTTTGAAGTGGATC
 ACAGAGTATGAGGGTAGGACAGCCTGGTTTCGTTCCGTCAATTGCCAGAGGAGGAGC
 CATAGCAAGGGCTTTGAGGCAGCGAGGGGAAGAGTGTCAATTTGCTTAAACAGCAAGA
 CCTTTGACAAGGAGTACTCAAGAGTGAAGGATGAAAAACCCGACTTTGTGGTGACC
 ACGGACATATCAGAGATGGGGGCGAATCTTGATGTACCCCGCGTCATTGATGGCCG
 GACAAACATCAAACCGGAGGAGGTGGACGGCAGGATCGAACTCACAGGGACGAGAC
 GTGTGACAACCGCGTCTGCTGCACAGCGCAGGGGAAGAGTGGGCAGGCAGGGGGGT
 CGCACAGATGAATACATATACTCAGGCCAGTGTGATGATGATGACAGTGGTCTTGT
 GCAGTGGAAAGAGGGCCAGATACTCTTGACAAACATCACAACAGCAAGAGGGCCGG
 TTGCCACATTCTATGGCCCTGAGCAGGAGAGGATGACAGAGACGCCAGGCCACTAC
 CGCCTGCCAGAGGAGAAGAGAAAACACTTCAGACATCTGTTGGCTCAGTGTGATTT
 CACACCATGGCTGGCCTGGCATGTTGCGGCCAATGTTGCTTCAGTGAAGTATAGGA
 GCTGGACATGGGAAGGACCAGAGGAAAAATGCTGTGGATGAGAACAATGGAGAAGT
 GTGACATTGAGAAGCCCAAATGGAGCAGAAAGAACTCAGGCCCGTATGGAGAGA
 CGCCCGTATGTTTAGAGAGGGCCGTGACATAAGGGAGTTTGTGTCTATGCTTCTG
 GAAGACGCAGTGTGGGAGATGTGCTCATGGGAATGCTGCGGGTGGCAAGCGCTCCTA
 CGGCAAAGATGCACCAGTGCAATGGATGTGTTTTACACGCTCATGCATGAAGAGCC
 TGGGAGCAGAGCCATGCGAATGGCTGAACGAGATGCCCTGAGGCCTTCTGACGG
 CAGTGGAGATGCTTGTCTGGGGCTTGCAACCCTGGGAGTTGTCTGGTGTGTTTGT
 GTGCGCACCTCGGTGAGTAGGATGGTGTGTTGGGGACCTAGTTCTCGCCACCTCACT
 GATCTTCTGTGGGCGCGAGGTGTTGGCTACGGGAACATGGCTGGGGTGGCACTGG
 TGTATTACACCTACTGACAGTCTTGCACCCGGAGACCGGAAAGCAGCGGAGTAGT
 GATGACAACAACTGGCATACTTTCTGCTAACATTGTGCGGCCCTGGCCGGGATGGT
 GGCTGCCAATGAAATGGGCCTCTTGAGAGAAACTAAAGCAGATCTCGCAGCACTCT
 TCGCTAGAGACCAAGGGGAGACTGTCCGATCGGGGGAGTGGACCAATTTGGACATC
 CAGCCGGCAAGATCATGGGGCACATATGTTCTGGTGGTATCTTTGTTACCCCCCTA
 TATGCTGCACCAATTGCAGACAAGAAATCCAGCAGTTGGTGAACAGTGCAGTGGCTT
 CAGGAGCCCAGGCAATGAGAGATCTAGGGGGAGGGACTCCTTTCTTTGGTGTGGCG
 GGACATGTCTTGGCACTCGGGATCGCGTCTCTCGTTGGAGCGACCCCGACATCTTT
 AATCCTGGGAGTGGGGCTGGCGGCTTTCCACCTGGCCATAGTGGTGTGCGGACTGG
 AGGCAGAGTTAACACAAAGGGCTCACAAAGTGTTCTTCTCGGCCATGGTCCGGAAC
 CCAATGGTGGACGGGGATGTCATCAACCCATTTGGAGATGGTGAGGCAAAGCCTGC
 GCTCTATGAGAGGAAGCTCAGCCTTATCCTGGCTTTGGTGTGCTTGGCCTCGG
 TGGTCATGAATCGGACATTTGTGGCACTTACTGAGGCAGGAGCCGTGGGAGTGGCT
 GCAGCAATGCAGTTGCTGAGACCTGAGATGGACGTTTGTGGACTATGCCTGTGGC
 CTGTGGCATGAGTGGAGTGGTCAGGGGAAGCCTGTGGGGCCTTCTGCCACTCGGCC
 ACAGACTGTGGCTCCGGACCACTGGGACACGGCGTGGTGGATCCGAGGGAGACACC

Vero
 adaptation
 NS4A U332>A
 (F111>Y)

FIG. 23D

CTGGGTGACATGTGGAAGGCCCGGTTGAACAGCTGCACAAAAGAGGAGTTCTTCGC
 CTACCGCAGAGCTGGAGTGATGGAGACTGACCGTGAAAAGGCCAGAGAGCTTTTAA
 AAAGAGGGGAACTAACATGGGCTTGGCGGTTTCCAGGGGAACTTCCAAGCTGGCA
 TGGATGGAAGAGAGGGGCTACGTCAACCCTGAAAGGGGAAGTGGTCCGACCTGGGCTG
 CGGCAGAGGCGGCTGGTCATACTATGCAGCATCACGACCAGCGGTTATGAGTGTGA
 GAGCATACACCATCGGTGGAAAGGGACATGAATCACACCGCATGGTAACCAGCCTC
 GGGTGGAAACCTGATAAAATTCAGAGCAGGCATGGATGTGTTCTCAATGGAGCCTCA
 TCGAGCAGATGCAATCCTATGTGACATAGGGGAAAGCAATCCAGACGCAGTGGTGG
 AAGGAGAGAGATCACGGCGTGTGATTCTTCTTATGGAGCAATGGAAAAATCGCAAT
 CCAACAGCGACATGTGTGTTCAAGGTCCTGGCTCCTTATCGTCCCGAGGTAATAGA
 GGCACCTGCACAGGTTCCAACCTCAATGGGGAGGGGGTCTTGTGAGGACACCTTTTT
 CCCGAAATTCCACCCATGAAATGTACTTCTCAACTGCCATCACGGGGAACATTGTA
 AATTCAAGTGAACATCCAGTCTCGAAAGCTGCTGGCTAGGTTTGGAGACCAAAGAGG
 GCCAACCCGAGTGGCCGAGATCGATCTGGGTGTGGGCACTAGGTGCGTAGTGCTGG
 CAGAGGACAAGGTCAAAGAAAAAGATGTGATGGAAAGGATCCAGGCCCTTGAAGGAC
 CAGTACTGCGACACCTGGCAGGAGGATCATGAACATCCATACAGAACATGGCAGTA
 CTGGGGGAGCTACAAGACACCGGCCACAGGCTCATCAGCTTCGCTGCTGAATGGGG
 TCGTGAAGCTTCTCAGTTGCCCATGGAATGCTAGGGAAGATGTGGTGCGGATGGCA
 ATGACTGACACAACCTGCATTGGGGCAGCAGAGGGTCTTTAAGGACAAAGTCGACAC
 CAAGGCACAGGAGCCGCAACCCGGGACCAAAATCATCATGAGAGCGGTGAATGACT
 GGCTGCTTGAGCGACTGGTCAAGAAGAGTGGGCCACGCATGTGCTCCAGGGAGGAG
 TTCATAGCGAAAGTCCGGTCAATGCGGCCCTTGGCGCTTGGTCAGATGAGCAAAA
 CAAATGGAAGAGTGGGAGAGAAGCAGTGGAAAGATCCTGAGTTCTGGAGTCTTGTG
 AGGCTGAGAGAGAGCGCCACTTGCAGGGGAGATGCGCCCATTTGTGTGTACAACATG
 ATGGGGAAAAGAGAGAAAAAGCTTGGTGAATTTGGAGTAGCCAAGGGCAGCAGGGC
 CATCTGGTACATGTGGCTGGGCAGTCGGTTCTTGGAGTTTGAGGCCCTCGGTTTCC
 TCAATGAGGATCACTGGGCGTCCAGGGCTTCTAGTGGAGGTAAGTATCAAGGTTAC
 AAGGCAGGTTTAAGGAGACCAATAGAAACTGGGCTTGTGCGAGACAGAGAAGACTCT
 TGGCTTTCTGATAGGCACCTATTGGTCTTACTGACATCCACTTTGCCTTTCTCTCC
 ACAGCTGGAGTGGAAAGGAATCAGCCTCAACTACCTGGGGTGGCATTGTGAAAAA
 GGCTTCCTTGAGTGGTGGTCTGTTTTATGCCGACGACACTGCTGGCTGGGACACGA
 AGATCACCAATGCAGATCTGGATGATGAGGAGCAGATCCTTCGTTACATGGATGGA
 GACCACAAGAACTGGCGGCCACAGTGTAAAGGAAGGCCTATCATGCCAAGGTGGT
 GAGAGTGGCTAGGCCAGCGTGAGGGGGGCTGTGTCATGGACATCATAACGCGTA
 GAGACCAGAGAGGCTCAGGACAGGTTGTGACTTATGCTCTAAACACAATCACAAAC
 ATCAAGGTGCAGCTGGTGAGGATGATGGAAGGTGAGGGTGTGATTGAGGTGGCGGA
 CTCCCACAACCCAAGACTGCTACGTGTGGAATAATGGCTCGAAGAGCATGGAGAGG
 AAAGGCTGAGCAGAATGCTGGTGAGCGGTGACGACTGTGTGGTCAGGCCAGTGGAT
 GACAGGTTCAAGCAAGGCCCTTACTTCCTCAATGACATGGCAAAAACCCGGAAGA
 CACCGGTGAGTGGGAGCCCTCAACCGGATTCGCTAGCTGGGAAGAGGTCCCTTTT
 GCTCACACCACTTTTCATGAACTTGTGATGAAGGACGGACCGCACTCTTGTGGTGGCG
 TGCCGTGACCAGGATGAGTTGGTGGGCAGGGCACGTGTCTCACCTGGCTGCGGCTG
 GAGTGTGCGGGAGACGGCCTGTCTATCGAAGGCTTACGGACAAATGTGGCTGTTGA
 GCTACTTCCACAGGAGGACTTGAGGACTCTGGGGTTCGCCATATGTTCCGGCAGTG
 CCCGTGGACTGGGTCCCAACAGGAAGAACAACCTGGAGCATAATGCAAGTGGTGC
 CTGGATGACAACCGAAGACATGCTGGAGGTGTGGAACCGGGTGTGGATCTATGACA
 ACCCATTCATGGAAGACAAGACAAGAGTTGATGAGTGGAGAGACACTCCATACCTG
 CCAAAATCACAGGACATCCTGTGTTTCATCATTTGGTGGGAAGGGGTGAAAGAGCAGA

No EcoRIintrone

Vero
adaptation:
NS5 G2170>A
(A724>T)

FIG. 23E

GTGGGCAAAGAACATCTGGGGAGCCGCTGGAGAAGGTGAGAAGGATGATCGGACCAG	
AACACTACAGGGACTACTTGTCAAGCATGGACAGACACGACCTACACTGGGAGCTC	
AAACTGGAGAGCTCAATATTTTAAAGCCAGGGCATTTCACCGCGTGCCTTAACGCC	<u>stop codon</u>
CTCTCCCTCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGGAATAAGGCCGGT	<u>mir-124</u>
GTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGCTTTTTGGCAATGTGAGG	
GCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCTAGGGGTCTTTCCCTCT	
CGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAG	
CTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCA	<u>IRES</u>
CCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAA	
GGCGGCACAACCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAA	
GGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCAGAAGGTACCCCAT	
TGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAG	<u>Vero</u>
GTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCTTTGAAAAAC	<u>adaptation:</u>
ACGATGATAAGCTTGCCACAACCCACAAGGAGACGACCTTCCATGGCTGGCAAAGC	<u>IRES AA490</u>
TGTGCTGAAGGGCAAAGGCGGCGCCCTCCCCGCCGCGCCAGCAAGGTCCGCCCCA	
AAAAGACCCGCCAGCTGCGCGTGCAGATGCCCAACGGCCTGGTGTAAATGAGGATG	<u>C-opt</u>
TTAGGCGTGCTGTGGCAGCCCTGACCGGCACCGCCCGCAGCCCCGTGCTGAAGGC	
CTTCTGGAAGGTGGTGGCCCTGAAGCAGGCCACCCTGGCCCTGCGCAAGATCAAGC	<u>Vero</u>
GCACCGTGAGCACCTGATGGTGGACCTGCACCGCCGAGGAAGTAGAAGGTAAAGC	<u>adaptation:</u>
CAGACACAAGSAGTCCAACCTGGAGGGCTCTTGAAAACTCGTCCAGAAACCAAAAC	<u>C-opt G263>A</u>
AGATGAGCAASTCAACAGGAGATGATAACTCGTACGAGCTGATCTCCAACACACAA	<u>(G88>D)</u>
GAAAAATGGTGGGATGCGGCAACGCACGAGGCTCGTGACGGGGAAATGATCGCTCC	
CGACGCACCCCTCCATTGGAGACAACCTTCGTGAGATCCCCCAGGTGTTTAGGGGCA	
CACGCCTGAGSTAAGCAAGCCCCAGGGCGCATTCGGGCAGCACACCAGTGAGAGTG	
GTGACGGGAAACTGGTCACTCCCGACGGAGCTGCGCCTTGTGAAACTTTGTGAGAC	
CCCTTGCGTCCAGAGAAGGSCGAACCTGGGCGTTATAAGGAGGCCCCCAGGGGGAAA	
CCCCCTGGGAGSAGGGAAGAGAGAAATTGGCAACTCTCTTCAGGATATTTCCCTCCTC	
CTATACCAAATTCCCCCTCGTCAGAGGGGGGCGGTTCTTGTCTCCCTGAGCCAC	
CATCACCTAGACACAGATAGTCTGAAAAGGAGGTGATGCGTGTCTCGGAAAAACAC	
CCGCTGGGTGCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCGAAG	
GAGGACGCACGTCCACTCGGATGGCTAAGGGAGAGCCACGAGCTCCTCGACAGATC	
ATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACA	<u>RBZ</u>
CCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTA	
TTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTACAAATAAA	
GCATTTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAACTCATCAAGATAATAAA	<u>pA signal</u>
GGAAATTTATTTTCAATTGCAATAGTGTGTTGGAATTTTTTGTGTCTCTCACTCGGA	<u>translation</u>
AGGACATATGGGAGGGCAAATCATTTAAACATCAGAATGAGTATTTGGTTTAGAG	<u>terminator</u>
TTTGGCAACATATGCCCATATGTAAGATATCAGGCTTCGAGCAAGACGTTTCCCGT	<u>from</u>
TGAATATGGCTCATAACACCCCTTGTAATACTGTTTATGTAAGCAGACAGTTTTAT	<u>pTriEx1.1</u>
TGTTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGACA	
CAACGTGGCTTTGTTGAATAAAATCGAACTTTTGCTGAGTTGAAGGATCAGATCACG	
CATCTTCCCGACAACGCAGACCGTTCCGTGGCAAAGSCAAAAGTTCAAAATCACCAA	
CTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTCCCTCACTTTCTGGCTGG	
ATGATGGGGCGATTCAGGCCTGGTATGAGTCAGCAACACCTTCTTCACGAGGCAGA	
CCTCAGCGCTAGCGGAGTGTATACTGGCTTACTATGTTGGCACTGATGAGGGTGTG	
AGTGAAGTGCTTCATGTGGCAGGAGAAAAAGGCTGCACCGGTGCGTCAGCAGAAT	<u>p15A origin</u>
ATGTGATACAGGATATATTCCGCTTCCTCGCTCACTGACTCGCTACGCTCGGTGCT	<u>of</u>
TCGACTGCGCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCTTGGAAGATG	<u>replication</u>

FIG. 23F

CCAGGAAGATACTTAACAGGGAAGTGAGAGGGCCGCGGCAAAGCCGTTTTTCCATA
 GGCTCCGCCCCCTGACAAGCATCACGAAATCTGACGCTCAAATCAGTGGTGGCGA
 AACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCTGGCGGCTCCCTCGTGCGC
 TCTCCTGTTCTGCCTTTTCGGTTTACCGGTGTCATTCCGCTGTTATGGCCGCGTTT
 GTCTCATTCCACGCCTGACACTCAGTTCGGGGTAGGCAGTTCGCTCCAAGCTGGAC
 TGTATGCACGAACCCCCCGTTTCAGTCCGACCGCTGCGCCTTATCCGGTAACTATCG
 TCTTGAGTCCAACCCGGAAGACATGCAAAAGCACCCTGGCAGCAGCCACTGGTA
 ATTGATTAGAGGAGTTAGTCTTGAAGTCATGCGCCGGTTAAGGCTAAACTGAAAG
 GACAAGTTTTTGGTGACTGCGCTCCTCCAAGCCAGTTACCTCGGTTCAAAGAGTTGG
 TAGCTCAGAGAACCCTTCGAAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTTCAGAG
 CAAGAGATTACCGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAAGGGGTC
 TGACGCTCAGTGGAAACGAAAACCTACGTTAAGGGATTTTGGTCATGAGATTATCAA
 AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAA
 AGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACC
 TATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTTG
 AGATAACTACGATACGGGAGGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCG
 CGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCCAGCCAGCCGGAAG
 GGCCGAGCGCAGAAGTGGTCTGCACTTTATCCGCCCTCCATCCAGTCTATTAATT
 GTTGCCGGGAAGCTAGAGTAAGTAGTTTCGCCAGTTAATAGTTTTCGCAACGTTGTT
 GCCATTGCTGCAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTAG
 CTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG
 CGGTTAGCTCCTTCGGTCCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCGAGTGTTA
 TCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAG
 ATGCTTTTCTGTGACTGGTGGTACTCAACCAAGTCATTCTGAGATAGTGTATGC
 GGCGACCGAGTTGCTCTTGGCCCGCGTCAACACGGGATAATACCGCGCCACATAGC
 AGAACPTTAAAAGTGTCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCAAG
 GATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGACCCCACTGAT
 CTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAA
 AATGCCGCAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTT
 CCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACA
 TATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCGA
 AAAGTGCCACCTGACGTGTGACGCGGCGCATGTGCTTACATAACTTACGGTAAA
 TGGCCCCGCTGGCTGACCGGCCAACGACCCCCCGCCCAATTGACGTCAATAATGACGT
 ATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTAT
 TTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCC
 CCCTATTGACGTCAATGACGGTAAATGGCCCCGCTGGCATTATGCCCAGTACATGA
 CCTTATGGGACTTTCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACC
 ATGGTGATGCGGTTTTTGGCAGTACATCAATGGGCGTGCATAGCGGTTTGAATCAGC
 GGGATTTCCAAGTCTCCACCCCATTCAGCTCAATGGGAGTTTTGTTTTGGCACCAAA
 ATCAACGGGACTTTCCAANATGTCTAACAACCTCCGCCCATTCAGCGCAATGGGC
 GSTAGCGGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGT

AmpR

CMV promoter
 from pCmv
 sport6
 (Invitrogen)

FIG. 24A

COMPLETE NUCLEOTIDE SEQUENCE OF IRES-124

AGATTTTCTTGCGCGTGCATGCGTGTGCTTCAGACAGCCCAGGCAGCGACTGTGAT
 TGTGGATATTCTTTCTGCAAGTTTGTGCTGAACTGTTGAGAAAAAGACAGCTTA
 GGAGAACAAGAGCTGGGAATGGCCGGGAAGGCCGTTCTAAAAAGGAAAGGGGGGGG
 GTCCCCCTCGACGAGCGTCGAAAGTGGCCCCAAAGAAGACGCGTCAGTTGCGGGTC
 CAAATGCCAAATGGACTTGTACTGATGCGCATGCTGGGAGTTCTGTGGCAgGGCAT
TCACCGCGTGCCTTAgaaCGTTCCTTTGGGCATTTCACCGCGTGCCTTAGAAATCCAC
 TGAGgGGCATTTCACCGCGTGCCTTACACCGCCGAGGAAGTAGAAGGACGACCATTG
 ACTGGATGACTCCCTTGTTGATCACTGTGCTGCTTGGAAATGTGCCTCACGGCAACT
 GTCCGTAGAGAGAGAGATGGGTCAATGGTGATTCTGTGCTGAAGGGCGAGATGCTGC
 AACCCAGGTTAGAGTGGAGAATGGCACTTGTGTGCTTCTTGCTACAGACATGGGAT
 CATGGTGTGATGACTCGTTGGCTTACGAATGCGTGACCATTGACCAGGGAGAGGAA
 CCCGTTGATGTAGACTGCTTTTGCAGAGGTGTTGAGAAGGTGACACTGGAGTATGG
 ACGTTGTGGAAGGCGTGAAGGTTTACGTTCCAGGCGCTCCGTGCTGATTCCGTCAC
 ACGCACAGCGTGACCTGACCGGGCGGGGACACCAATGGTTAGAGGGAGAGGCTGTG
 AAGGCACACCTGACCCGCGTTGAGGGTTGGGTGTGGAAGAACAATTTGTTACCCCT
 TTCTCTTGATGGTGGCTGGTTGATGGTGGATGGATTGTTGCCAGGATTCTCA
 TTGTGGTTGTGGCTCTCGCATTGGCACCGGCCCTACCGCTCGAGATGCACCCACCTG
 GAAAACAGAGACTTCGTCCTGAGGGTGCAGGGGACGACCCGCTCACATTGGTTCT
 CGAGCTGGGGGGCTGCGTGACGGTCACGGCGGATGGGAAGCCATCCCTCGACGTCT
 GGCTCGACTCCATCTACCAAGAGAGCCCTGCACAGACAAGGGAGTATTGCCATCAT
 GCAAAGTTGACTGGGACAAAGGTGGCTGCCAGATGCCCGACAATGGGGCCAGCCAC
 GCTTCCAGAGGAGCACCAGGTGGAACAGTGTGCAAGAGAGATCAGAGCGACAGGG
 GATGGGGTAACCATTTGTGGACTATTTGGCAAAGGGAGCATCGTAACCTGCGTCAAG
 TTCCTTGTGAGGACAAGAAGAAGGCCACAGGACATGTGTATGATGTGAACAAAAT
 CACCTATACCATAAAGGTGGAGCCACACACTGGAGAGTTTGTGGCAGCCAATGAGA
 CTCCTCAGGGAGGAAGTCCAGCTCGTTTACAGTGTGATCAGAAAAACAATCTTG
 ACTCTTGGAGACTATGGTGATGTATCACTCTTGTGTAGAGTGGCCAGTGGGGTGGGA
 CCTTGCTCAGACTGTGGTTTGGCTTTGGACAAGACACATGAGCATCTGCCACAG
 CATGGCAGGTGCATCGTGACTGGTTTTAATGATCTGGCCCTCCCTTGAAGCATGAT
 GGAGCTGAGGCTTGAACGAAGCAGGACGGCTCGTGGAGTTTGGGACGCCCTCATGC
 AGTGAAAATGGATGTGTTCAATCTGGGAGACCAGACGGGAGTGTGTTGAAGTCAC
 TGGCTGGGGTCCCTGTAGCTAGCATAGAGGGAACCTAAGTATCATCTAAAGAGTGA
 CATGTTACTTGTGAGGTTGGCTTGGAAAACTGAAGATGAAAGGATTGACATACAC
 AGTCTGTGACAAGACAAAGTTCACTTGGAAAAGAGCACCCACAGACAGCGGTCATG
 ACACCGTGGTCATGGAGGTTGGCTTCTCGGGAACCTAGGCCTTGACAGATTCCAGTA
 CGTGCGGTTGCACACGGAGTTCCTGAGGTTAACGTGGCCATGCTCATAACACCTAA
 CCCGACAATGGAGAACAACGGAGGTGGATTCAATTGAGATGCAGCTCCCTCCTGGAG
 ATAACATAATCTATGTTGGTGACCTGAACCATCACTGGTTTCAGAAAGGGGAGCAGC
 ATCGGCAGGGTCCCTCCAGAAGACCAGGAAGGGGATTGAGAGACTCACCGTCCTTGG
 AGAACATGCTTGGGACTTTGGCTCAGTGGGAGGTGTCATGACGTCCATTGGGAGAG
 CCATGCACACCGTCCCTGGGAGGAGCGTTTAAACACCCTCCTAGGGGGGGTGGGCTTC
 CTACCCAAGATACTACTAGGCGTAGCTATGGCCTGGCTGGGCTTGAACATGAGGAA

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FIG. 24B

CCCAACCTTGTCCATGEGGCTTCCTACTCTCAGGGGGCTTGGTSGCTGGCCATGACTC
 TGGGGGTTGGAGCCGATGTGEGCTGCGCAGTGGACACAGAGCGAATGGAGCTCCGA
 TGTGGAGAAGGACTGGTGGTATGGAGAGAGGTGTGAGAATGGTACGACAACTACGT
 GTTCCACCCAGAGACTCCGGCGGTTCTGGCATCGGCAGTTCAGAGGGCCATATGAAG
 AGGAAATCTGTGGCATTGTCCCTCAAAATAGACTGGAGATGGCCATGTGGAGAAGC
 TCTCTAGTGGAGCTCAACCTGGCTCTAGCGGAGGGTGAGGCAAACCTGACTGTTGT
 GGTCGACAAGGCAGACCCCAGTGACTACAGGGGGGGCGTTCCCGGGCTCCTTAACA
 AGGSAAGACATTAAAGTATCTTGGAGGAGTTGGGGGCGATCCATGCTCTGGAGT
 GTTCCCGAGGCCCCCTCGCAGGTTTCATGATTGGAGTGGAAAGGAGGTAGAGAGTGGCC
 CTTTGGCCAGAAGGAAGACAGCTGTGATGACGGTGGCAGAGTTTGGGATTGGTCTTC
 GCACGAAAGTCTTCATGGACTTGCGGCAAGAATTGACAACAGAGTGTGACACAGGG
 GTCATGGGGGCTGCGGTCAAGAATGGAATGGCCGTCCACACTGATCAAAGTCTGTG
 GATGAAATCCATCAAGAATGATACAACTGTAACAATAGTTGAGCTGATTGTGACAG
 ACCTGAGAACTGCACTTGGCCAGCTAGCCACACCATTGACAATGCCGGAGTAGTG
 AACTCAAAGCTGTTTCTACCGGCCASTCTGGCTGGACCTAGGTCAAACCTACAATGT
 GATACCTGGCTATGCAGAGCAAGTGAGGGGACCGTGGGCGCACACCCCGGTCAGAA
 TAAAAAGAGAAGAATGCCCTGGAACCCGAGTGACCATTGACAAGGCATGTGACAAG
 AGAGGGGCGCTCGGTGAGAAGCACTACAGAGAGTGGGAAAGTCATACCCGAATGGTG
 CTGTCCACCTGTGAGCTGCCCCCGGTCACATATCGCACTGGGACTGATTGTTGGT
 ATGCCATGGAGATTAGGCCGGTACACACTCAGGGTGGCCTGGTCAGATCCATGGTA
 GTGGCTGACAACGGGGCTCTACTCAGTGAAGGTGGAGTTCCGGGGGTAGTAGCGCT
 CTTTGTGTTTTTGGAGCTGGTGATTAGAAGGGCACCAGCCACGGGGGGCACGGTGA
 TCTGGGAGGCAATTGCCATTCTGGCTCTTTTGGTGACGGGACTAGTCTCTGTGGAG
 AGTCTGTTTCGTTACCTTGTAGCAGTCGGTCTGGTGTTCAGCTGGAAGTGGGACC
 AGAGGCTGTGGCTATGGTGCTTCTACAGGCAGTTTTTGGAGATGAGAACGTGTCTAC
 TGAGTGGGTTTTGTGCTTCGTAGAAGCATAACCACGAGAGAAATTTGTGACAGTGTAC
 TTCCTCCTCCTGGTGTGGAGATGGGCATTCCAGTGAAGGGTCTGGAGCATCTCTG
 GAGATGGACTGATGCGCTGGCGATGGGGGCCATCATCTTCAGGGCATGCACTGCAG
 AGGGGAAGACAGGCATTGGTTTGCTTTTGGCTGCATTTCATGACCCAGAGTGACATG
 AACATCATCCATGATGGCTTGACCGCTTTTCTTTGTGTGGCCACTACCATGGCCAT
 ATGGAGATACATCAGGGGCCAGGGAGAGAGAAAAGGACTGACATGGATCGTCCCGC
 TTGCTGGCATCCTTGGAGGAGAGGGCTCAGGAGTACGACTGCTGGCCTTCTGGGAG
 TTGGCGGCCTCCAGGGGGAGACGTTTCTTTAATGAGCCTATGACTGTATAGGAGT
 GATGCTGACTTTGGCCAGTGGAAATGATGCGCCACACGTCTCAGGAGGCGGTGTGTG
 CCATGGCGCTGGCGGCTTTCTTCTACTCATGCTCACGCTTGGAAGTAGGAAAATG
 CAGTTGTTGGCTGAGTGGAGTGGGAACATTGAGTGGAAATCCAGAAGTACCAGTGA
 GGGCGGTGAAGTGAGCCTGCGCGTCAGACAAGATGCCCTTGGAAGTTCACCTGA
 CTGAACTGGAGAAGGAGGAACGCATGATGGCTTTTGGCTGGTGGTAGGCTTGATT
 GCCTCGGCCTTCCACTGGTCGGGGATCCTTATCGTCATGGGACTGTGGACCATTTC
 GGAGATGCTTGGCTCAGCCAGGAGAACGGATCTTGTGTTTTTTCAGGATGCTCCGAAG
 GGCGAAGTGACAGCCGTCGCTCGATGTAAAAAGTGGAGTGTACCGCATATATACT
 CCAGGCCCTGCTCTGGGGACAAAGACAGATTGGAGTGGGCTACGGGGCAAAGGGTGT
 GCTACACACCATGTGGCATGTCACTAGGGGGGCTGCCCTGCTGGTTGATGGAGTTG
 CAGTGGGCCCCCTACTGGGCTGATGTGCGGGAGGATGTGGTTTGTATGGAGGCGCC
 TGGAGTCTGGAAAGCAGGTGGAGGGGTGAGACTGTGCAGGTGCATGCCTTCCACC
 CGGCAGGGCACATGAAACACACCAATGCCAACCTGGAGAGTTGATATTGGAGAATG
 GGAGGAAGATGGGAGCCATTCCCATTGATCTCGCCAAAGGAACATCAGGCAGTCCA
 ATTATGAACTCACAGGGAGAGGTGCTTGGCCTGTATGGAAACGGCCTCAAGACTAA

Spe-Nsi from
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4528 T>C

FIG. 24C

TGACACGTATGTCAGCAGCATAGCACAGGGTGAAGTGGAAAAAAGTAGACCAAATC
 TCCCACAGTCAGTAGTTGGCACGGGCTGGACAGCCAAGGGACAGATCACGGTGTG
 GATATGCACCCTGGCTCGGGGAAGACCCATCGGGTCCCTCCCTGAGCTCATCCGTCA
 ATGTGTGGAGCGGGCATTGCGAACCTTGGTCTTGGCCCCACACGAGTGGTGCTGA
 GGGAGATGGAACGGCGCCCTTAGTGGGAAGAACGTGCGGTACCATTGCGCTGCAGTC
 ACAGAGCAGCAGCCCAATGGTGCAATCGTTGATGTCATGTGCCATGCTACATATGT
 CAACAGAAGACTCCTCCACAGGGGAGACAAAACCTGGGAGGTGGCTATCATGGATG
 AGGCTCATTGGACTGACCCCCACAGTATAGCGGCCAGAGGACACCTCTACTCCCTT
 GCCAAGGAAAACAGATGTGCTCTCGTTCTCATGACGGCAACACCACCGGGGAAGAG
 TGAACCCTTTCCCTGAGTCCAACGGAGCCATAGCCAGTGAGGAGAGACAGATCCCTG
 ATGGGGAGTGGCGGGACGGGTTTGAAGTGGATCACAGAGTATGAGGGTAGGACAGCC
 TGGTTCGTTCCGTCAATTGCCAGAGGAGGAGCCATAGCAAGGGCTTTGAGGCAGCG
 AGGGAAGAGTGTCAATTTGCTTAAACAGCAAGACCTTTGACAAGGAGTACTCAAGAG
 TGAAGGATGAAAAACCCGACTTTGTGGTGACCACGGACATATCAGAGATGGGGGGC
 AATCTTGATGTCAACCCGCGTCATTGATGGCCGGACAAACATCAAACCGGAGGAGGT
 GGACGGCAGGATCGAACTCACAGGGACGAGACGTGTGACAACCGCGTCTGCTGCAC
 AGCGCAGGGGAAGAGTGGGCAGGCAGGGGGGTGCGACAGATGAATACATATACTCA
 GGCCAGTGTGATGATGATGACAGTGGTCTTGTGCAAGTGGAAAGAGGCCAGATACT
 CTTGGACAACATCACAACAGCAAGAGGGCCGGTTGCCACATTCTATGGCCCTGAGC
 AGGAGAGGATGACAGAGACGGCAGGCCACTACCGCCTGCCAGAGGAGAAGAGAAAA
 CACTTCAGACATCTGTTGGCTCAGTGTGATTTACACCATGGCTGGCCTGGCATGT
 TGCGGCCAATGTTGCTTCAGTGAAGTGAAGAGCTGGACATGGGAAGGACCAGAGG
 AAAATGCTGTGGATGAGAACAATGGAGAAGTGGTGACATTCAGAAGCCCAAATGGA
 GCAGAAAGAACAACACTCAGGCCCGTATGGAGAGACGCCCGTATGTTTAGAGAGGGCCG
 TGACATAAGGGAGTTTGTGTCTATGCTTCTGGAAGACGCAGTGTGGGAGATGTGC
 TCATGGGAATGTCTGGGGTGCCAGCGCTCCTACGGCAAAGATGCACCAGTGCAATG
 GATGTGTTTTACACGCTCATGTCATGAAGAGCCTGGGAGCAGAGCCATGCGAATGGC
 TGAACGAGATGCCCTGAGGCCTTCCTGACGGCAGTGGAGATGCTTGTCTCTGGGGC
 TTGCAACCCCTGGGAGTTGTCTGGTGTGTTTGTGTTGTGCGCACCTCGGTGAGTAGGATG
 GTGTTGGGGACCCCTAGTTCTCGCCACCTCACTGATCTTCTGTGGGCCGGAGGTGT
 TGGCTACGGGAACATGGCTGGGGTGGCACTGGTGTATTACACCTACTGACAGTCC
 TGCAACCGGAGACCGGAAAGCAGCGGAGTAGTGATGACAACAACTGGCATACTTT
 CTGCTAACATTGTGCGGCCCTGGCCGGGATGGTGGCTGCCAATGAAATGGGCCCTCTT
 GGAGAAAACATAAGCAGATCTCGCAGCACTCTTCGCTAGAGACCAAGGGGAGACTG
 TGCGATGGGGGGAGTGGACCAATTTGGACATCCAGCCGGCAAGATCATGGGGCACA
 TATGTTCTGGTGGTATCTTTGTTTACCCCCCTATATGCTGCACCAATTGCAGACAAG
 AATCCAGCAGTTGGTGAACAGTGCAGTGGCTTCAGGAGCCAGGCAATGAGAGATC
 TAGGGGGAGGGACTCCTTTCTTTGGTGTGGCGGGACATGTCTTGGCACTCGGGATC
 GCGTCTCTCGTTGGAGCGACCCCGACATCTTTAATCCTGGGAGTGGGGCTGGCGGC
 TTTCCACCTGGCCATAGTGGTGTGCGGACTGGAGGCAGAGTTAACACAAAGGGCTC
 ACAAAGTGTCTTCTCGGCCATGGTCCGGAACCCAATGGTGGACGGGGATGTCATC
 AACCCATTTGGAGATGGTGAGGCAAGCCTGCGCTCTATGAGAGGAAGCTCAGCCT
 TATCCTGGCTTTGGTGTGCTTGGCCTCGGTGGTGCATGAATCGGACATTTGTGG
 CAGTTACTGAGGCAGGAGCCGTGGGAGTGGCTGCAGCAATGCAGTTGCTGAGACCT
 GAGATGGACGTTTTGTGGACTATGCCTGTGGCCTGTGGCATGAGTGGAGTGGTCAG
 GGAAGCCTGTGGGGCCTTCTGCCACTCGGCCACAGACTGTGGCTCCGGACCACTG
 GGACACGGCGTGGTGGATCCGAGGGAGACACCCCTGGGTGACATGTGGAAGGCCCGG
 TTGAACAGCTGCACAAAAGAGGAGTTCTTCGCCCTACCGCAGAGCTGGAGTGATGGA

Vero P10 mut
 6792 T>A

FIG. 24D

GACTGACCGTGAAAAGGCCAGAGAGCTTTTAAAAAGAGGGGAACTAACATGGGGCT
 TGGCGGTTTCCAGGGGAACCTTCCAAGCTGGCATGGATGGAAGAGAGGGGCTACGTC
 ACCCTGAAAGGGGAAGTGGTCGACCTGGGCTGCGGCAGAGGCGGCTGGTCATACTA
 TGCAGCATCACGACCAGCGGTATGAGTGTGAGAGCATACACCATCGGTGGAAAGG
 GACATGAATCACACGCATGGTAACCAGCCTCGGGTGGAACCTGATAAAATTGAGA
 GCAGGCATGGATGTGTTCTCAATGGAGCCTCATCGAGCAGATGCAATCCTATGTGA
 CATAGGGGAAAGCAATCCAGACGCAGTGGTGGAAAGGAGAGAGATCACGGCGTGTGA
 TTCTTCTTATGGAGCAATGGAAAAATCGCAATCCAACAGCGACATGTGTGTTCAAG
 GTCTTGGCTCCTTATCGTCCCGAGGTAATAGAGGCACTGCACAGGTTCCAACTTCA
 ATGGGGAGGGGGTCTTGTGAGGACACCTTTTTTCCCGAAATTCACCCCATGAAATGT
 ACTTCTCAACTGCCATCACGGGGGAACATTGTAAATTTCAGTGAACATCCAGTCTCGA
 AAGCTGCTGGCTAGGTTTGGAGACCAAAGAGGGGCCAACCCGAGTGCCCGAGATCGA
 TCTGGGTGTGGGCACTAGGTGCGTAGTGTGCTGGCAGAGGACAAGGTCAAAGAAAAAG
 ATGTGATGGAAGGATCCAGGCCCTGAAGGACCAGTACTGCGACACCTGGCAGCAG
 GATCATGAACATCCATACAGAACATGGCAGTACTGGGGGAGCTACAAGACAGCGGC
 CACAGGGTCATCAGCTTCGCTGCTGAATGGGGTCTGTGAAGCTTCTCAGTTGGCCAT
 GGAATGCTAGGGAAGATGTGGTGGGATGGCAATGACTGACACAACCTGCATTCCGG
 CAGCAGAGGGTCTTTAAGGACAAAGTCGACACCAAGGCACAGGAGCCGCAACCCGG
 GACCAAAATCATCATGAGAGCGGTGAATGACTGGCTGCTTGAGCGACTGGTCAAGA
 AGAGTCGGCCACGCATGTGCTCCAGGGAGGAGTTCATAGCGAAAGTCCGGTCTGAAT
 GCGGCCCTTGGCGCTTGGTCAGATGAGCAAAACAAATGGAAGAGTCCGAGAGAAGC
 AGTGAAGATCCTGAGTTCTGGAGTCTTGGTGAGGCTGAGAGAGAGCGCCACTTGC
 AGGGGAGATGCGCCCATTTGTGTGTACAACATGATGGGGAAAAGAGAGAAAAAGCTT
 GGTGAATTTGGAGTAGCCAAGGGCAGCAGGGCCATCTGGTACATGTGGCTGGGCAG
 TCGGTTCTTGGAGTTTGGAGGCCCTCGGTTTCTCAATGAGGATCACTGGGCGTCCA
 GGGCTTCTAGTGGAGGTAAGTATCAAGGTTACAAGGCAGGTTTAAGGAGACCAATA
 GAAACTGGGCTTGTGCGAGACAGAGAAGACTCTTGCGTTTTCTGATAGGCACCTATTG
 GTCTTACTGACATCCACTTTGCGCTTCTCTCCACAGCTGGAGTGGAAAGGAATCAGC
 CTCAACTACCTGGGGTGGCATTTGAAAAAACTGGCTTCCTTGAGTGGTGGTCTGTT
 TTATGCCGACGACACTGCTGGCTGGGACACGAAGATCACCAATGCAGATCTGGATG
 ATGAGGAGCAGATCCTTCGTTACATGGATGGAGACCACAAGAACTGGCGGCCACA
 GTGCTAAGGAAGGCCTATCATGCCAAGGTGGTGAGAGTGGCTAGGCCCAGCCGTGA
 GGGGGCTGTGTATGACATCATAACGCGTAGAGACCAGAGAGGCTCAGGACAGG
 TTGTGACTTATGCTCTAAACACAATCACAAACATCAAGGTGCAGCTGGTGAGGATG
 ATGGAAGGTGAGGGTGTGCTTGGAGGTGGCGGACTCCCACAACCCAAGACTGCTACG
 TGTGGAAAAATGGCTCGAAGAGCATGGAGAGGAAAGGCTGAGCAGAATGCTGGTGA
 GCGGTGACGACTGTGTGGTCAGGCCAGTGGATGACAGGTTTACGCAAGGCCCTTTAC
 TTCCTCAATGACATGGCAAAAACCCGGAAAGACACCGGTGAGTGGGAGCCCTCAAC
 CGGATTCGCTAGCTGGGAAGAGGTCCCTTTTGTCTCACACCCTTTTCATGAACTTG
 TGATGAAGGACGGACGCACTCTTGTGGTGGCGTGCCGTGACCAGGATGAGTTGGTG
 GGCAGGGCACGTGTCTCACCTGGCTGCGGCTGGAGTGTGCGGGAGACGGCCTGTCT
 ATCGAAGGCTTACGGACAAATGTGGCTGTTGAGCTACTTCCACAGGAGGGACTTGA
 GGACTCTGGGGTTCCGCCATATGTTCCGGCAGTGCCCGTGGACTGGGTCCCAACAGGA
 AGAACAACCTGGAGCATAACATGCAAGTGGTGCCTGGATGACAACCGAAGACATGCT
 GGAGGTGTGAACCGGCTGTGGATCTATGACAACCCATTTCATGGAAGACAAGACAA
 GAGTTGATGAGTGGAGAGACACTCCATACCTGCCAAAATCACAGGACATCCTGTGT
 TCATCATTGGTGGGAAGGGGTGAAAGAGCAGAGTGGGGCAAAGAACATCTGGGGAGC
 CGTGGAGAAGGTGAGAAGGATGATCGGACCAGAACACTACAGGGACTACTTGTCAA

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FIG. 24E

GCATGGACAGACACGACCTACACTGGGAGCTCAAACCTGGAGAGCTCAATATTTTAA
 AGCCAGGGCATTACACCGCTGCCTTAACGCCCTCTCCCTCCCCCCCCCTAACGT
 TACTGGCCGAAGCCGCTTGGAAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTT
 CCACCATATTGCGCTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTC
 TTGACGAGCATTCTTAGGGGTCTTCCCCCTCTCGCCAAAGGAATGCAAGGTCTGTT
 GAATGTCGTGAAGGAAGCAGTTCCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTG
 TAGCGAACCCTTTGCAGGCAGCGGAACCCCCCACTGGCGACAGGTGCCTCTGCGGC
 CAAAAGCCACGTGTATAAGATAACCTGCAAAGGCCGACAAACCCAGTGCCACGT
 TGTGAGTTGGATAGTTGTGGAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACA
 AGGGGCTGAAGGATGCCAGAAAGGTACCCUATTGTATGGGATCTGATCTGGGSCCT
 CGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCCCG
 AACCACGGGGACGTGGTTTTCTTTGAAAAACACGATGATAAGCTTGCCACAACCC
 ACAAGGAGACGACCTTCCATGGCTGGCAAAGCTGTGCTGAAGGGCAAAGGCGGCGG
 CCTCCCCGCGCGCCAGCAAGGTGCGGCCCAAAGACCCGCCAGCTGCGCGTGC
 AGATGCCCAACGGCCTGGTGTAAATGAGGATGTTAGGCGTGCTGTGGCACGCCCTG
 ACCGGCACCGCCCGCAGCCCCGTGCTGAAGGCCTTCTGGAAGGTGGTGCCTCTGAA
 GCAGGCCACCCTGGCCCTGCGCAAGATCAAGCGCACCGTGAGCACCCCTGATGGTGG
 ACCTGCACCGCCGAGGAAGTAGAAGGTAAAGCCAGACACAAGGAGTCCAACCTGGA
 GGGCTCTTGAAAAACTCGTCCAGAAACCAACAGATGAGCAAGTCAACAGGAGATG
 ATAACCTCGTACGAGCTGATCTCCAACACACAAGAAAAATGGTGGGATGCGGCAACG
 CACGAGGCTCGTGACGGGGAAATGATCGCTCCCGACGCACCCCTCCATTGGAGACA
 ACTTCGTGAGATCCCCCAGGTGTTTAGGGGCACACGCCTGAGSTAAGCAAGCCCCA
 GGGCGCATTCCGGCAGCACACCAGTGAGAGTGGTGACGGGAAACTGGTCACTCCCCG
 ACCGAGCTGCGCCTTGTGAACTTTGTGAGACCCCTTGCGTCCAGAGAAGGCCGAA
 CTGGGCGTTATAAGGAGGCCCCAGGGGGAAACCCCTGGGAGGAGGGAAGAGAGAA
 ATTGGCAACTCTCTTCAGGATATTTCTCTCCTATACCAAATTTCCCCCTCGTCAG
 AGGGGGGGCGGTTCTTGTCTCCCTGAGCCACCATCACCTAGACACAGATAGTCTG
 AAAAGGAGGTGATGCGTGTCTCGGAAAAACACCCGCTGGGTCCGCATGGCATCTCC
 ACCTCCTCGCGGTCCGACCTGGGCATCCGAAGGAGGACGCACGTCCACTCGGATGG
 CTAAGGGAGAGCCACGAGCTCCTCGACAGATCATAATCAGCCATACCACATTTGTA
 GAGGTTTTACTTGCTTTAAAAAACCTCCACACCTCCCCCTGAACCTGAAACATAA
 AATGAATGCAATTGTTGTTGTTAACTTGTTTATTGACGCTTATAATGGTTACAAAT
 AAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTTCACTGCATTCTAGT
 TGTGGTTTGTCCAAACTCATCAAGATAATAAAGGAAATTTATTTTCATTGCAATAG
 TGTGTTGGAATTTTTTTGTGTCTCTCACTCGGAAGGACATATGGGAGGGCAAATCAT
 TTAAAACATCAGAATGAGTATTTGGTTTAGAGTTTGGCAACATATGCCCATATGTA
 AGATATCAGGCTTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTT
 GTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTTCATGATGATATATTTTTATC
 TTGTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTGTTGAATAAATC
 GAACTTTTGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGT
 TCCGTGGCAAAGCAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCT
 CATCAACCGTGGCTCCCTCACTTTCTGGCTGGATGATGGGGCGATTCAAGGCCTGGT
 ATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCGCTAGCGGAGTGATAC
 TGGCTTACTATGTTGGCACTGATGAGGGTGTGAGTGAAGTGCTTCATGTGGCAGGA
 GAAAAAAGGCTGCACCGGTGCGTCAGCAGAAATATGTGATACAGGATATATTCCGCT
 TCCTCGCTCACTGACTCGCTACGCTCGGTGCTTCGACTGCGGCGAGCGGAAATGGC
 TTACGAACGGGGCGGAGATTTCTGGAAGATGCCAGGAAGATACTTAACAGGGGAAG
 TGAGAGGGCCGCGCAAAGCCGTTTTTCCATAGGCTCCGCCCCCTGACAAGCATC

Vero P 10> A delVero P10 C-
G88DRBZpA signaltranslation
terminator
from
pTriEx1.1p15A origin
of

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ACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATAC CAGGCGTTTCCCTGGCGGCTCCCTCGTGCGCTCTCCTGTTCCCTGCCTTTCCGGTTT ACCGGTGTCAATCCGCTGTTATGGCCGCGTTTGTCTCATTCACCGCCTGACACTCA GTTCCGGGTAGGCAGTTCCSCTCCAAGCTGGACTGTATGCACGAACCCCCCGTTCAG TCCGACCGCTGCGCCTTATCCGGTAACCTATCGTCTTGAGTCCAACCCGGAAAGACA TGCAAAAGCACCACCTGGCAGCAGCCACTGGTAATTGATTTAGAGGAGTTAGTCTTG AAGTCATGCGCCCGTTAAGGCTAAACTGAAAGGACAAGTTTTGGTGA CTGCGCTCC TCCAAGCCAGTTACCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAAC CGCCCTGCAAGGCGGTTTTTTTCGTTTTTCAGAGCAAGAGATTACGCGCAGACCAAAA CGATCTCAAGAAGATCATCTTATTAAGGGGTCTGACGCTCAGTGGAACGAAACTC ACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTT TAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCT GACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCTG TTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCT TACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACC GGCTCCA GATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGC AACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGAAGCTAGAGTAAGTA GTTCCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGTG TCACGCTCGTCGTTTGGTATGGCTTCATT CAGCTCCGGTTCCCAACGATCAAGGCG AGTTACATGATCCCCCATGTTGTGCAAAAAGCGGTTAGCTCCTTCGGTCTCCGA TCGTTGTG CAGAAGTAAGTTGGCCG CAGTGTTATCACTCATGGTTATGGCAGCACTG CATAATTCTCTTACTGT CATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTA CTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGG CGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATT GGAAAACGTTCTTCGGGGCGAAAAC TCTCAAGGATCTTACCGCTGTTGAGATCCAG TTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTT CACCA GCGTTTCTGGGTGAGCAAAAACAGGAAGGCCAAAATGCCGCAAAAAGGGGAATAAGG GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCAT TTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATA AACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTGTGACG CGGCCGCAATGTCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAA CGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAG GGACTTTCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCA GTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAA ATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGC AGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTTGGCAGTAC ATCAATGGGCGTGGATAGCGGTTT GACTCACGGGGATTTCCAAGTCTCCACCCCAT TGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAATGTC GTAACAACCTCCGCCCCATTGACGCAAAATGGGCGGTAGGCGTG TACGGTGGGAGGTC TATATAAGCAGAGCTCGTTTAGTGAACCGT	replication
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FIG. 25A

COMPLETE NUCLEOTIDE SEQUENCE OF ZY-IRES Version 2

AGTTGTTGATCTGTGTGAATCAGACTGCGACAGTTTCGAGTTTGAAGCGAAAGCTAG
 CAACAGTATCAACAGGTTTTATTTTGGATTTGGAAACGAGAGTTTCTGGTCA**ATGAA**
 AAACCCAAA**AAA**AGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACGCGGA
 GTAGCCCGTGTGAGCCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCT
 GGGTCATGGGCCCATCAGGATGGTCTTGGCGATTCTAGCCTTTTGGAGATTCACGG
 CAATCAAGCCATCACTGGGTCTCATCAAT**CT**AGGAAGGAGAAGAAGAGACGAGGCG
 CAGATACTAGTGTGCGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAG
 GTCAGTACAGTGGGAGTGCATACTATATGTACTTGGACAGAAACGATGCTGGGGA
 GGCCATATCTTTTCCAACCACATTGGGGATGAATAAGTGTATATACAGATCATGG
 ATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCTGGATGAG
 GGGGTGGAACAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTGT
 GTACGGAACTGCCATCACA**AAAAA**AGGTGAAGCACGGAGATCTAGAA**GAGCTGTGA**
CGCTCCCCCTCCCACTTAGGAAGCTGCAAACGCGTCCGAAACCTGGTTGGAA
 TCAAGAGAATACACAAAGC**at**TTGATTAGAGTCGAAAATTGGATA**CT**CAGGAACCC
 TGGCTTCGCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCTCAAC**a**AGCC
 AAAAAAGTCATATACTTGGTCATGATACTGCTGATTGCCCGGCATACAGCATCAGG
 TGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGGGT
 TGATGTTGTCTTGGAAACATGGAGGTTGTGTACCGTAATGGCACAGGACAAACCGA
 CTGTGACATAGAGCTGGTTACAACAACAGTCAGCAACATGGCGGAGGTAAGATCC
 TACTGCTATGAGGCATCAATATCAGACATGGCTTCCGGACAGCCGCTGCCCAACACA
 AGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGT
 TAGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGCCTGGTG
 ACATGCGCTAAGTTT**GCATGCT**CCAAGAAAATGACCGGAAGAGCATCCAGCCAGA
 GAATCTGGAGTACCGGATAATGCTGTCAGTTTCATGGCTCCAGCACAGTGGGATGA
 TCGTTAATGACACAGGA**TAT**GAAACTGATCAGAATAGAGCCAAGGTTGAGATAACG
 CCCAATTCACCAAGAGCCGAAGCCACCCCTGGGGGGTTTGGAAAGCCTAGGACTTGA
 TTGTGAACCGAGGACAGGCCCTTGACTTTTCAGATTTGTATTACTTGACTATGAATA
 ACAAGCACTGGTTGGT**t**CACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCAC
 GCTGGGGCAGACACCGGAACTCCACACTGGAACAACAAAGAAGCACTGGTAGAGTT
 CAAGGACGCACATGCCAAAAGGCCAACTGTCTGGTCTTAGGGAGTCAAGAAGGAG
 CAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGA
 AGGCTGTCTCTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAA
 GGGCGTGTCACTACTCCTTGTGTACCGCAGCGTTCACATTCACCAAGATCCCGGCTG
 AAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCT
 TGCAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAACTCTGACCCCACTTGGGAG
 GTTGATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAACTCTAAGATGATGC
 TGGAACCTTGATCCACCATTTGGGGACTCTTACATTGTTCATAGGAGTCGGGGAGAAG
 AAGATCACCCACCACTGGCACAGGAGTGGCAGCACCATTTGGAAAAGCATTGTAAGC
 CACTGTGAGAGGTGCCAAGAGAATGGCAGTCTTGGGAGACACAGCCTGGGACTTTG
 GATCAGTTGGAGGCGCTCTCAACTCATTTGGGCAAGGGCATCCATCAAATTTTGGGA
 GCAGCTTTCAAATCATTTGTTGGAGGAATGTCCTGGTTCTCACAAT**t**CTCATTGG
 AACGTTGCTGATGTGGTTGGGTCTGAACACAAAGAATGGATCTATTTCCCTTATGT
 GCTTGGCCTTAGGGGGAGTGTGATCTTCTTATCCACAGCCGTCTCTGCT**GATGTG**

+1A frame
shift

C deletion

861 t>C (pr-
F132L)

SphI not
single but
can be used
for cloning
with Mlu and

XmaI
1449 C>T
E-H158Y

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[illegible]

FIG. 25C

GTCCATCATTTGAGGTCTACTACTGCCAGTGGACGAGTAATAGAAGAqTGGTGTTCG	NS1 135
AGAGAaTGTACTATGCCTCCGCTTTCATTCCGAGCCAAgGAoGGtTGCTGGTACGG	<u>optimised</u>
cATGGAGATCCGACCcCGCAAGGAgCCGGAGtcgAAtTTGGTCAGATCCGTCGTAA	
CGGCAGGATCAACTGATCACATGGATCACTTCTCCCTTGGAGTGCCTTGTGATTCTG	<u>NS2A</u>
CTCATGGTSCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCATAAGCAC	
ATCAATGGCAGTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGG	
CTAAGCTTGCAATTTTGATGGGtGCCACCTTCGCGGAAATGAACACTGGAGGAGAT	
GTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGACCAGCGTTGCTGGTATC	
TTTCATCTTCAGAGCTAATTGGACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCT	
CGTGTCTTTTGCAAACCTGCGATCTCCGCCTTGGAAGGCGACCTGATGGTTCTCATC	
AATGGTTTTTGCTTTGGCCTGGTTGGCAATACGAGCGATGGTTGTTCCACGCACTGA	
TAACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGC	<u>XmaI</u>
TTGTGGCGTGGAGAGCAGGCCTTGCTACTTGCGGGGGGTTTATGCTCCTCTCTCTG	
AAGGGAAAAGGCAGTGTGAAGAAGAACTTACCATTGTGTCATGGCCCTGGGACTAAC	
CGCTGTGAGGCTGGTCGACCCCATCAAtGTGGTGGGACTGCTGTTGCTCACAAGGA	
GTGGGAAGCGGAGCTGGCCCCCTAGCGAAGTACTCACAGCTGTTGGCCTGATATGC	
GCATTGGCTGGAGGGTTCCGCCAAGGCAGATATAGAGATGGCTGGGCCCATGGCCGC	
GGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGAGTGTGGACATGTACA	
TTGAAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGT	
CCCCGGCTCGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTGGTGGAGGATGA	
CGGTCCCCCATGAGAGAGATCATACTCAAGGTGGTCTGATGACCATCTGTGGCA	
TGAACCCAATAGCCATAACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGACT	
GGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGG	
GGAGACCACAGATGGAGTGTACAGAGTAATGACTCGTAGACTGCTAGGTTCAACAC	
AAGTTGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGTGGCACGTCACA	
AAAGGATCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGT	
CAAGCAGGATCTGGTGTCTACTGTGGTCCATGGAAGCTAGATGCCGCCTGGGACG	
GGCACAGCGAGGTGCAGCTCTTGCCCGTGCCCCCGGAGAGAGAGCGAGGAACATC	
CAGACTCTGCCCGGAATATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCT	
GGATTACCCAGCAGGAACCTCAGGATCTCCAATCCTAGACAAGTGTGGGAGAGTGA	
TAGGACTTTATGGCAATGGGGTCGTGATCAAAAATGGGAGTTATGTTAGTGCCATC	
ACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGCCTTCGATGCT	
GAAGAAGAAGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTGGGAAAACCAGGA	
GAGTCTTCCCTGAAATAGTCCGTGAAGCCATAAAAACAAGACTCCGTAAGTGTGATC	
TTAGCTCCAACCAGGGTTGTGCGTGTGAAATGGAGGAAGCCCTTAGAGGGGCTTCC	
AGTGCGTTATATGACAACAGCAGTCAATGTCACCCACTCTGGAACAGAAATCGTCG	
ACTTAATGTGCCATGCCACCTTCACTTCACGTCTACTACAGCCAATCAGgGTCCCC	
AACTATAATCTGTATATTATGGATGAGGCCCACTTCACAGATCCCTCAAGTATAGC	
AGCAAGAGGATACATTTCAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCA	
TGACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGACTCCAACCTACCAATT	
ATGGACACCGAAGTGGAAGTCCCAGAGAGAGCCTGGAGCTCAGGCTTTGATTGGGT	
GACCGATCATTTTGGAAAAACAGTcTGGTTTGTTCGAAGCGTGAGGAACGGCAATG	
AGATCGCAGCTTGTCTGACAAAGGCTGGAAAAACGGGTCTATACAGCTCAGCAGAAAG	
ACTTTTGAGACAGAGTTCCAGAAAAACAAACATCAAGAGTGGGACTTTGTGCTGAC	
AACTGACATTTTCAGAGATGGGCGCCAACCTTTAAAGCTGACCGTGTATAGATTCCA	
GGAGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTcTGGCTGGACCC	
ATGCCTGTACACATGCCAGCGCTGCCAGAGAGGGGGCGCATAGGCAGGAATCC	
CAACAAACCTGGAGATGAGTATCTGTATGGAGGTGGGTGCGCAGAGACTGACGAAG	
	<u>5680 C>T</u>

FIG. 25D

ACCATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGAT
 GGCCTCATAGCCTCGCTCTATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGG
 AGAGTTCAAGCTTAGGACGGAGCAAAGGAAGACCTTTGTGGAACTCATGAAAAGAG
 GAGATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAATAACCTACACA
 GATAGAAGATGGTGCTTTGATGGCACGACCAACAAtACCATAATGGAAGACAGTGT
GCCGGCAGAGGTGTGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGAGGTGGA
 TGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGAAGTCATTCAAGGAGTTTGCC
 GCTGGGAAAAGAGGAGCGGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGG
 ACACATGACAGAGAGATTCCAGGAAGCCATTGACAACCTCGCTGTGCTCATGCGGG
 CAGAGACTGGAAGCAGGCCTTACAAAGCCGCGGCGGCCAATTGCCGGAGACCCTA
 GAGACCATTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTTTTTCGT
 CTTGATGAGGAACAAGGGCATAGGGAAGATGGGCTTTGGAATGGTGACTCTTGGGG
 CCAGCGCATGGCTCATGTGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGTC
 CTCATTGTTGTGTTCCATTGCTGGTGGTGCTCATACCTGAGCCAGAAAAGCAAAG
 ATCTCCCCAGGACAACCAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGG
 GCTTGATTACCGCCAATGAACTCGGATGGTTGGAGAGAACAAGAGTGACCTAAGC
 CACCTAATGGGAAGGAGAGAGGAGGGAGCAACCATAGGATTCTCAATGGACATTGA
 CCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCC
 CAGCCGTCCAACATGCAGTGACCACTTCATACAACAACACTACTCCTTAATGGCGATG
 GCCACGCAAGCTGGAGTGTTGTTGGTATGGGCAAAGGGATGCCATTCTACGCATG
 GGACTTTGGAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACCCCTGA
 CCTAATAGTGGCCATCATTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGG
 CTGCAGGCAGCAGCTGCGCGTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAA
 GAACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACACAATGACAATTGACC
 CCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGCCcGTCTCCAGC
 GCCATACTGTGCGGACCGCCTGGGGGTGGGGaGAGGCTGGGGCCCTGATCACAGC
 CGCAACTTCCACTTTGTGGGAAGGCTCTCCGAACAAGTACTGGAACCTCCTACAG
 CCACTTCACTGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATC
 TACAcAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGGGGGTGGAACAGGAGA
 GACCCTGGGAGAGAAATGGAAGGCCCGCTTGAACCAGATGTGCGCCCTGGAGTTCT
 ACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAGAGAAGAGGCCCGCCGCC
 CTCAAGGAcGGTGTGGCAACGGGAGGCCATGCTGTGTCCCGAGGAAGTGCAAAGCT
 GAGATGGTTGGTGGAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATCTTG
 GATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCACCATCCGCAAAGTTCAAGAA
 GTGAAAGGATACACAAAAGGAGGCCCTGGTCATGAAGAACCCGTGTTGGTGCAAAG
 CTATGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTCTTTCATATGGCGG
 CTGAGCCGTGTGACACGTGCTGTGTGACATAGGTGAGTCATCATCTAGTCCTGAA
 GTGGAAGAAGCACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTTGAAAA
 AAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCCATACACCAGCACTATGATGG
 AAACCCTGGAGCGACTGCAGCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTC
 TCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCGAAAAGCAACACCAT
 AAAAAGTGTGTCCACCACGAGCCAGCTCCTCTTGGGGCGCATGGACGGGCCaAGGA
 GGCCAGTGAAATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGGCTGTGGTA
 AGCTGCGCTGAAGCTCCCAACATGAAGATCATTGGTAACCGCATTGAAAGGATCCG
 CAGTGAGCACGCGGAAACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGGG
 CTTACCATGGAAGCTATGAGGCCCCACACAAGGGTCAGCGTCCTCTCTAATAAAC
 GGGGTTGTGAGGCTCCTGTCAAACCCCTGGGATGTGGTGACTGGAGTCACAGGAAT
 AGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTCAAGGAAAAAGTGG

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ACACTAGGTTGCCAGACCCCCCAAGGAAGGCACTCGTCAGGTTATGAGCATGGTCTCT	<u>introne</u>
TCCTGGTTGTGGAAAGAGCGTAAGTATCAAGGTTACAAGGCAGGTTTAAGGAGACCA	
ATAGAACTGGGCTTGTGCGAGACAGAGAAGACTCTTGGCGTTTCTGATAGGCACCTA	
TTGGTCTTACTGACATCCACTTTGCCTTTCTCTCCACAGCTAGGCCAAACACAAACG	
GCCACGAGTCTGTACCAAAGAAGAGTTtATCAACAAGGTTTCGTAGCAATGCAGCAT	
TAGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAGTGGAAGCTGTGAAC	
GATCCAAGGTTCTGGGCTCTAGTGGACAAGGAAAGAGAGCACCACCTGAGAGGAGA	
GTGCCAGAGTTGTGTGTACAACATGATGGGAAAAAGAGAAAAGAAACAAGCGGAAT	
TTGGAAAGGCCAAGGGCAGCCGCGCCATCTGGTATATGTGGCTAGGGGCTAGATTT	
CTAGAGTTCGAAGCCCTTGGAATCTTGAACGAGGATCACTGGATGGGGAGAGAGAA	
CTCAGGAGGTGGTGTGAAGGGCTGGGATTACAAAGACTCGGATATGTCTTAGAAG	
AGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGAC	
ACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGA	
GAAAGGGCAcAGGGCCCTTGGCATTGGCCATAATCAAGTACACATACCAAACAAAG	
TGGTAAAGGTCTTAGACCAGCTGAAAAAGGGAAAAACAGTTATGGACATTATTTTCG	
AGACAAGACCAAAGGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTAC	
CAACCTAGTGGTGCAACTCATTCCGAATATGGAGGCTGAGGAAGTTCTqGAGATGC	
AAGACTTGTGGCTGCTGCGGAGGTGAGAGAAAGTGACCAACTGGTTGCAGAGCAAC	
GGATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGATTGCGTTGTGAAGCC	
AATTGATGATAGGTTTGCACATGCCCTAAGGTTCTTGAATGATATGGGAAAAGTTA	
GGAAGGACACACAAGAGTGGAAACCCTCAACTGGATGGGACAACCTGGGAAGAAGTT	
CCGTTTTGCTCCCACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCCATTGT	
GGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGCGCGCTCTCTCCAGGGG	
CGGGATGGAGCATCCGGGAGACTGCTTGCCCTAGCAAAATCATATGCGCAAATGTGG	
CAGCTCCTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCAATGCCATTTGTTT	
ATCTGTGCCAGTTGACTGGGTTCCAACCTGGGAGAACTACCTGGTCAATCCATGGAA	
AGGGAGAATGGATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGATT	
GAGGAGAACGACCACATGGAAGACAAGACCCAGTTACGAAATGGACAGACATTCC	
CTATTTGGGAAAAAGGGGAAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGC	
GCACCACCTGGGCTGAGAACATTAAAAACACAGTCAACATGGTGCGCAGGATCATA	
GGTGATGAAGAAAAGTACATGGACTACCTATCCACCCAAGTTCGCTACTTGGGTGA	
AGAAGGGTCTACACCTGGAGTGCTGTAAGCACCAATCTTAATGTTGTCAGGCCTGC	
TAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGTGACCCCCCAGGAGAAGCT	
GGGAAACCAAGCCTATAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCT	
GCCTGTGAGCCCCCTCAGAGGACACTGAGTCAAAAAACCCACGCGCTTGGAGGCGC	
AGGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCCTTCAATCTGGGGCCTGAACTG	
GAGATCAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAGACCCCCCGGA	
AAACGCCAAACAGCATATTGACGCTGGGAAAGACCAGAGACTCCATGAGTTTCCAC	
CACGCTGGCCGCCAGGCACAGATCGCCGAATAGCGGCGGCGGTTGTGGGAAATCC	<u>RBZ</u>
ATGGGTCTGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCG	
AAGGAGGACGCACGTCCACTCGGATGGCTAAGGGAGAGCCACGAGCTCCTCGACAG	
ATCATAATCAGCCATACCACATTTGTAGAGGTTTACTTGCTTTAAAAAACCTCCC	
ACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGT	
TTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTACAAAT	
AAAGCATTTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAACTCATCAAGATAAT	<u>pA signal</u>
AAAGGAAATTTATTTTCATTGCAATAGTGTGTTGGAATTTTTTGTGTCTCTCACTC	
GGAAGGACATATGGGAGGGCAAATCATTTAAACATCAGAATGAGTATTTGGTTTA	<u>translation terminator</u>
GAGTTTGGCAACATATGCCCATATGTAA	

FIG. 25F

GATATCAGGCTTCSAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTG
 TATTACTGTTTATGTAAGCAGACAGTTTATTGTTTCATGATGATATATTTTATCT
 TGTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTGTTGAATAAAATCG
 AACTTTTGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGTT
 CCGTGGCAAAGCAAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCTC
 ATCAACCGTGGCTCCCTCACTTTCTGGCTGGATGATGGGGCGATTGAGGCCTGGTA
 TGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCGCTAGCGGAGTGATACT
 GGCTTACTATGTTGGCACTGATGAGGGTGTGAGTGAAGTGCTTCATGTGGCAGGAG
 AAAAAAGGCTGCACCGGTGCGTCAGCAGAATATGTGATACAGGATATATTCCGCTT
 CCTCGCTCACTGACTCGCTACGCTCGGTGCTTCGACTGCGGCGAGCGGAAATGGCT
 TACGAACGGGGCGGAGATTTCTGGAAGATGCCAGGAAGATACTTAACAGGGAAAGT
 GAGAGGGGCGCGGCAAAAGCCGTTTTTCCATAGGCTCCGCCCCCTGACAAGCATCA
 CGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATACC
 AGGCGTTTTCCCTGGCGGCTCCCTCGTGGCTCTCCTGTTCTGCTTTTCGGTTTA
 CCGGTGTCATTCCGCTGTTATGGCCGCGTTTTGTCTCATTCCACGCCTGACACTCAG
 TTCCGGGTAGGCAGTTCGCTCCAAGCTGGACTGTATGCACGAACCCCCCGTTTCAGT
 CCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGAAAGACAT
 GCAAAAGCACCCTGGCAGCAGCCACTGGTAATTGATTTAGAGGAGTTAGTCTTGA
 AGTCATGCGCCGGTTAAGGCTAAACTGAAAGGACAAGTTTTTGGTGACTGCGCTCCT
 CCAAGCCAGTTACCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCCTTCGAAAACC
 GCCCTGCAAGGCGGTTTTTTTCGTTTTTCAGAGCAAGAGATTACGCGCAGACCAAAAC
 GATCTCAAGAAGATCATCTTATTAAGGGGTCTGACGCTCAGTGGAACGAAAACCTCA
 CGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTT
 AAATTAAAAATGAAGTTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTG
 ACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGT
 TCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTT
 ACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAG
 ATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTTCA
 ACTTTATCCGCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAG
 TTCGCCAGTTAATAGTTTGGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGTGT
 CACGCTCGTCTGTTTGGTATGGCTTCATTTCAGCTCCGGTTCCCAACGATCAAGGCGA
 GTTACATGATCCCCATGTTGTGCAAAAAGCGGTTAGCTCCTTCGGTCTCCGAT
 CGTTGTCAGAAGTAAGTTGGCCGCACTGTTATCACTCATGTTATGGCAGCACTGC
 ATAATTCTCTTACTGTGATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTAC
 TCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGC
 GTCAACACGGGATAATACCGCGCCACATAGCAGAAGTTTAAAAGTGCTCATCATTG
 GAAAACGTTCTTCCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGT
 TCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAG
 CGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGG
 CGACACGGAAATGTTGAATACTCATACTCTTCCTTTTCAATATTATTGAAGCATT
 TATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAA
 ACAAATAGGGGTTCGCGGCACATTTCCCGAAAAGTGCCACCTGACGTGTCGACGC
 GGCCGCATGTCGTTACATAACTTACGGTAAATGGCCCCGCTGGCTGACCGCCCAAC
 GACCCCCGCCCATTTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGG
 GACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAG
 TACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAA
 TGGCCCGCTGGCATTATGCCAGTACATGACCTTATGGGACTTTCCTACTTGGCA
 GTACATCTACGTATTAGTCATCGCTATTACCATGGTGTATGCGGTTTTGGCAGTACA

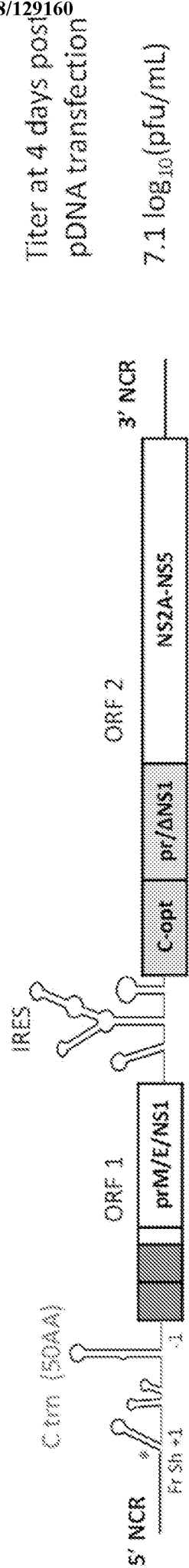
from
pTriEx1.1

CMV promoter
 from pCmv-
sport6
 (invitrogen)

FIG. 25G

<u>TCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTC</u> <u>CAAGTCTCCACCCCATT</u> <u>GACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTC</u> <u>CAAAATGTCG</u> <u>TAACRACTCCGCCCCATTGACGCAANTGGGCGGTAGGCGTG</u> <u>TACGGTGGGAGGTCT</u> <u>ATATAAGCAGAGCTCGTTTAGTGAACCGT</u>	
--	--

FIG. 26A



Titer at 4 days post
pDNA transfection

7.1 log₁₀(pfu/mL)

Vero cell adaptive mutations:

- 748 G→A (pr-R94K)
- 861 T→C (pr-F132L)
- 449 C→T (E-H158Y)

FIG. 26B

AGTTGTTGATCTGTGTGAATCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGC AACAGTATCAACAGGTTTTATTTGGATTGGAAACGAGAGTTTCTGGTCATGAAAA ACCCAAAaAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACGCGGAGT AGCCCGTGTGAGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGG GTCATGGGCCCATCAGGATGGTCTTGCGATTCTgGGCATTACCCGCGTGCCTTAGA ATCCACTGAGgGGCATTACCCGCGTGCCTTATCTAGGAAGGAGAAGAAGAGAC GAGGCGCAGATACTAGTGTGGAATTGTTGGCTCCTGCTGACCACAGCTATGGCAG CGGAGGTCACTAGACGTGGGAGTGCATACTATATGTAATTGGACAGAAACGATGCT GGGGAGGCCATATCTTTCCAACCACATTGGGGATGAATAAGTGTATATACAGATC ATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCTGGAT GAGGGGGTGAACCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGT	+1A frame shift miR-124 miR-124, C deletion
TGTGTACGGAACCTGCCATCACAAAAAGGTGAAGCACGGAGATCTAAAAGAGC TGTGACGCTCCCCTCCATTCCACTAGGAAGCTGCAAACGCGGTGCAAACCTGGTT	G→A (pr- R→K)
GGAATCAAGAGAATACACAAAGCAtTGATTAGAGTCGAAAATTGGATACTCAGGA ACCCTGGCTTCGCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCTCAACaA GCCAAAAAGTCATATACTTGGTCATGATACTGCTGATTGCCCCGGCATAACAGCATCA GGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGG GTTGATGTTGTCTTGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCG ACTGTCGACATAGAGCTGGTTACAACAACAGTCAGCAACATGGCGGAGGTAAAGATC CTACTGCTATGAGGCATCAATATCAGACATGGCTTCGGACAGCCGCTGCCCAACACA AGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTT AGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGCCTGGTG ACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCCAGA GAATCTGGAGTACCGGATAATGCTGTCAGTTTCATGGCTCCAGCACAGTGGGATGAT	861 t→C SphI
CGTTAATGACACAGGATATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACG CCaAATTCACCAAGAGCCGAAGCCACCCTGGGGGTTTTGGAAGCCTAGGACTTGAT TGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATGAATAACA AGCACTGGTTGGTtCACAAAGGAGTGGTTCCACGACATTCCATTACCTTGGCACGCTG GGGCAGACACCGGAACTCCACACTGGAACAACAAAGAAGCACTGGTAGAGTTCAAG GACGCACATGCCAAAAGGCAAACCTGTCGTGGTTCTAGGGAGTCAAGAAGGAGCAGT TCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGC TGTCCTCTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCG TGTCATACTCCTTGTTACCGCAGCGTTCACATTCACCAAGATCCCGGCTGAAACACT GCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCTTGCAAG GTTCCAGCTCAGATGGCGGTGGACATGCAAACCTTGACCCAGTTGGGAGGTTGAT AACCGCTAACCCGTAATCACTGAAAGCACTGAGAACTCTAAGATGATGCTGGAACCT TGATCCACCATTTGGGGACTCTTACATTGTCATAGGAGTCGGGGAGAAGAAGATCAC CCACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTGAAGCCACTGTGA GAGGTGCCAAGAGAATGGCAGTCTTGGGAGACACAGCCTGGGACTTTGGATCAGTT GGAGGCGCTCTCAACTCATTGGGCAAGGGCATCCATCAAATTTTGGAGCAGCTTTC AAATCATTGTTTGGAGGAATGTCCTGGTTCTACAAATtCTCATTGGAACGTTGCTGA TGTGGTTGGGTCTGAACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGG GGGAGTGTTGATCTTCTATCCACAGCCGTCTCTGCTGATGTGGGGTGCTCGGTGGA CTTCTCAAAGAAGG	1449 C→T silent to make EcoRI NS1

FIG. 26C

AGACGAGATGCGGTACAGGGGTGTTCTGCTATAACGACGTTGAAGCCTGGAGGGACAGG TACAAGTACCATCCTGACTCCCCCGTAGATTGGCAGCAGCAGTCAAGCAAGCCTGGGAA GATGGTATCTGCGGGATCTCCTCTGTTTCAAGAATGGAaAACATCATGTGGAGATCAGTAG AAGGGGAGGTAAAGTATCAAGGTTACAAGGCAGGTTTAAAGGAGACCAATAGAAACTGGGC TTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTGACATCC ACTTTGCCTTTCTCTCCACAGCTCAACGCAATCcTGAAGAGAATGGAGTTCAACTGACGG TCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGCCTG TGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAATCGTACTTCGTGAGAGCAGCAA AGACAAATAACAGCTTTGTCTGGATGGTGACACACTGAAGGAATGCCCACTCaAACATA GAGCATGGAACAGCTTTCTGTGGAGGATCATGGGTTGGGGTATTTCACTAGTGTCTG GCTCAAGGTTAGAGAAGATTATTCATTAGAGTGTGATCCAGCCGTTATTGGAACAGCTGTT AAGGGaAAGGAGGCTGTACACAGTGATCTAGGCTACTGGATTGAGAGTGAGAAGAATGA CACATGGAGGCTGAAGAGGGCCCAcTgATCGAGATGAAAACATGTGAATGGCCAAAGTC CCACACATTGTGGaCAGATGGAATAGAAGAGAGTGATCTGATCATaCCCAAGTCTTTAGCT GGGCCACTCAGCCATCACAATACCAGAGAGGGCTACAGGACCCAAATGAAAGGGCCATG GCACAGTGAAGAGCTTGAAATTCGTTTTGAGGAATGCCCaGGCACTAAGGTCCACGTGGA GGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAGGGTGA TCGAGGAATGGTGCTGCAGGGAGTGCACAATGCCCCACTGTCGTTCCGGGCTAAAGATG GCTGTTGGTATGGAATGGAGATAAGGCCCAGGAAAGAACCAGAAAGCAACTTAGTAAGG	intron end of NS1
TCAGTGGTGACTGCATAAGCCAGACGCCCCCTCTCCCTCCCCCCCCCCTAAAGTTAC TGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCA CCATATTGCGTCTTTTGCAATGPGAGGGCCCGAAACCTGGCCCTGTCTCTCTG ACGAGCATTCCTAGGGGTCTTTCCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAA TGTCGTGAAGGAAGCAGTTCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAG CGACCCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAA AAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAAGTGCCACGTTGT GAGTGGATAGTTCTGGAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGG GGCTGAAGGATGCCCAGAAGGTACCCCATTTGATGGGATCTGATCTGGGGCTCGG TGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCCCGAAC CACGGGGACGTGGTTTTCTTTGAAAAACACGATGATAAGCTGCCACAACCCACA AGGAGACGACCTTCCATGAAGAAtCCGAAGAAaAAGAGCGGTGGGTTTAGAATAGTg	MluI
AAcATGTTGAAAAGGGGCGTGGCACGCGTGTCACCATTcGGTGGTCTCAAACGC CTCCCCGCaGGTCTTCTTCTGGGACAcGGcCCgATtAGAATGGTGCTCGCTATcCT GGCgTTcCTGCGCTTtACTGCCATAAAaCCGTCCCTGGGGCTTATtAAcCGCTGGG GgTCTGTTGGTAAGaAGgAAGCcATGGAGATcATTAAaAAGTTcAAGAAgGActTa GCcGCAATGTTGCGAATcATTAAcGCcCGCAAaGAGAAGAAGCGACGAGGGCGCAGAT ACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGGTCACTAGA CGTGGGAGTGCATACTATATGTACTTGGACAGAAACGATGCTGGGGAGGCCATATCTTTT CCAACCACATTGGGGATGAATAAGTGTTATATACAGATCATGGATCTTGGACACGTAATA GAAGAgTGGTGTTCAGAGAgTGTACTATGCCTCCGCTTTCATTCCGAGCCAAgGA cGGTGTCTGGTACGGcATGGAGATCCGACCcCGCAAGGAgCCGGAGtgAATTGGTC AGATCCGTCGTAAACGGCAGGATCAACTGATCACATGGATCACTTCTCCCTGGAGTGCT TGTGATTCTGCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCAT AAGCACATCAATGGCAGTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCT GGCTAAGCTTGCAATTTTGATGGGtGCCACCTTCGCGAAATGAACACTGGAGGAGATGT AGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGACCAGCGTTGCTGGTATCTTTCATC TTCAGAGCTAATTGGACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTTGC AAACTGCGATCTCCGCCTTGAAGGCGACCTGATGGTTCTCATCAATGG	Copt NS1 135 optimized NS2A

FIG. 26D

TTTTGCTTTGGCCTGGTTGGCAATACGAGCGATGGTTGTTCCACGCACTGATAACATCACCT TGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTGTGGCGTGGAG AGCAGGCCTTGCTACTTGCGGGGGGTTTATGCTCCTCTCTGAAGGGAAAAGGCAGTGTG AAGAAGAACTTACCATTTGTCATGGCCCTGGGACTAACCGCTGTGAGGCTGGTCGACCCCA TCAAtGTGGTGGGACTGCTGTTGCTCACAAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCG AAGTACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTCGCCAAGGCAGATAT AGAGATGGCTGGGCCCATGGCCGCGGTGCTGCTAATTGTCAGTTACGTGGTCTCAGGA AAGAGTGTGGACATGTACATTGAAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAA GTCAGTGGAAACAGTCCCCGGCTCGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTGG TGGAGGATGACGGTCCCCCATGAGAGAGATCATACTCAAGGTGGTCCTGATGACCATCTG TGGCATGAACCAATAGCCATACCCTTTCAGCTGGAGCGTGGTACGTATACGTGAAGACT GGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGGGGA GACCACAGATGGAGTGTACAGAGTAATGACTCGTAGACTGCTAGGTTCAACACAAGTTGGA GTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGTGGCACGTCACAAAAGGATCCGCGC TGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATCTGGTGT CATACTGTGGTCCATGGAAGCTAGATGCCGCTGGGACGGGCACAGCGAGGTGCAGCTCTT GGCCGTGCCCCCGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAATATTTAAGAC AAAGGATGGGGACATTGGAGCGGTTGCGCTGGATTACCCAGCAGGAACCTTCAGGATCTCC AATCCTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTCGTGATCAAAAA TGGGAGTTATGTTAGTGCCATACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTG CTTCGAGCCTTCGATGCTGAAGAAGAAGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTG GGAAAAACCAGGAGAGTTCTTCCTGAAATAGTCCGTGAAGCCATAAAAAACAAGACTCCGTAC TGTGATCTTAGCTCCAACCAGGGTTGTGCTGCTGAAATGGAGGAAGCCCTTAGAGGGCTT CCAGTGCGTTATATGACAACAGCAGTCAATGTCAACCACTCTGGAACAGAAATCGTCGACTT AATGTGCCATGCCACCTTCACTTCACGTCTACTACAGCCAATCAGgGTCCCCAACTATAATCT GTATATTATGGATGAGGCCACTTCACAGATCCCTCAAGTATAGCAGCAAGAGGATACATTT CAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCATGACCGCCACGCCACCAGGAA CCCGTGACGCATTTCCGGACTCCAACCTACCAATTATGGACACCGAAGTGGAAAGTCCCAGA GAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATCATTTTGGAAAAACAGTcTGGTTT GTTCCAAGCGTGAGGAACGGCAATGAGATCGCAGCTTGTCTGACAAAGGCTGGAAAACGG GTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGTTCAGAAAACAAAACATCAAGAGT GGGACTTTGTCGTGACAACTGACATTTAGAGATGGGCGCCAACCTTTAAAGCTGACCGTGT CATAGATTCCAGGAGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTcTGGCT GGACCCATGCCTGTCACACATGCCAGCGCTGCCAGAGGAGGGGGCGCATAGGCAGGAAT CCCAACAAACCTGGAGATGAGTATCTGTATGGAGGTGGGTGCGCAGAGACTGACGAAGAC CATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGATGGCCTCAT AGCCTCGCTCTATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTT AGGACGGAGCAAAGGAAGACCTTTGTGGAACCTCATGAAAAGAGGAGATCTTCCTGTTTGG CTGGCCTATCAGGTTGCATCTGCCGGAATAACCTACACAGATAGAAGATGGTGCTTTGATG GCACGACCAACAAtACCATAATGGAAGACAGTGTGCCGGCAGAGGTGTGGACCAGACACG GAGAGAAAAGAGTGCTCAAACCGAGGTGGATGGACGCCAGAGTTTGTTTCAGATCATGCGG CCCTGAAGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGGAGCGGCTTTTGGAGTGATGG AAGCCCTGGGAACACTGCCAGGACACATGACAGAGAGATTCCAGGAAGCCATTGACAACCT CGCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAAGCCGCGGGCGGCCCAATT GCCGGAGAC	Xmal 5680 C→T (NS3-S356F); Silent
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FIG. 26E

CCTAGAGACCATTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTTTTTCGTCT
 TGATGAGGAACAAGGGCATAGGGAAGATGGGCTTTGGAATGGTGA CTCTTGGGGCCAGCG
 CATGGCTCATGTGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGTCCTCATTGTTGTG
 TTCCTATTGCTGGTGGTGCTCATACTGAGCCAGAAAAGCAAAGATCTCCCCAGGACAACCA
 AATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTACCGCCAATGAACTCG
 GATGGTTGGAGAGAACAAGAGTGACCTAAGCCaCTAATGGGAAGGAGAGAGGAGGGA
 GCAACCATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGC
 TGCCTTGACAACCTTTATTACCCCAGCCGTCCAACATGCAGTGACCACTTCATACAACAATA
 CTCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGTATGGGCAAAGGGATGCCA
 TTCTACGCATGGGACTTTGGAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACC
 CCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGC
 TGCAGGCAGCAGCTGCGCGTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAAGAACC
 CTGTTGTGGATGGAATAGTGGTGACTGACATTGACACAATGACAATTGACCCCCAAGTGGA
 GAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGCcGTCTCCAGCGCCATACTGTCGCGG
 ACCGCCTGGGGGTGGGGaGAGGCTGGGGCCCTGATCACAGCCGCAACTTCCACTTTGTGG
 GAAGGCTCTCCGAACAAGTACTGGAACCTCTACAGCCACTTCACTGTGTAAACATTTTTAG
 GGGAAAGTTACTTGGCTGGAGCTTCTCTAATCTACAcAGTAACAAGAAACGCTGGCTTGGTCA
 AGAGACGTGGGGGTGGAACAGGAGAGACCCTGGGAGAGAAATGGAAGGCCCGCTTGAAC
 CAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAGAG
 AAGAGGCCCGCCGCGCCCTCAAGGAcGGTGTGGCAACGGGAGGCCATGCTGTGTCCCGAG
 GAAGTGCAAAGCTGAGATGGTTGGTGGAGCGGGGATACCTGCAGCCCTATGGAAAGGTCA
 TTGATCTTGGATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCACCATCCGCAAAGTTCA
 AGAAGTGAAAGGATACACAAAAGGAGGCCCTGGTCATGAAGAACCCGTGTTGGTGCAAAG
 CTATGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTCTTTCATATGGCGGCTGAG
 CCGTGTGACACGTTGCTGTGTGACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAG
 CACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTTGAAAAAGACCAGGAGCCTT
 TTGTATAAAAGTGTTGTGCCATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCAG
 CGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTCCCGCAACTCTACACATGAGATGT
 ACTGGGTCTCTGGAGCGAAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGCTCCT
 CTTGGGGCGCATGGACGGGGCCaAGGAGGCCAGTGAAATATGAGGAGGATGTGAATCTCGG
 CTCTGGCACGCGGGCTGTGGTAAGCTGCGCTGAAGCTCCCAACATGAAGATCATTGGTAAC
 CGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAGAACCACCCAT
 ATAGGACATGGGCTTACCATGGAAGCTATGAGGCCCCACACAAGGGTCAGCGTCCTCTCT
 AATAAACGGGGTTGTCAGGCTCCTGTCAAACCCTGGGATGTGGTGA CTGGAGTCACAGG
 AATAGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAGGAAAAAGTGGAC
 ACTAGGGTGCCAGACCCCCAAGAAGGCACTCGTCAGGTTATGAGCATGGTCTCTTCTGGT
 TGTGGAAAG**AG**GTAAGTATCAAGGTTACAAGGCAGGTTTAAGGAGACCAATAGAACTG
 GGCTTGTGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTGACAT
 CCACCTTTGCTTTCTCTCCACAG**CT**AGGCAAACACAAACGGCCACGAGTCTGTACCAAAGA
 AGAGTTtATCAACAAGGTTCTGATGAATGCAGCATTAGGGGCAATATTTGAAGAGGAAAAA
 GAGTGGAAGACTGCAGTGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGTGGACAAG
 GAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGAGTTGTGTGTACAACATGATGGGAAAA
 AGAGAAAAGAAACAAGGGGAATTTGAAAGGCCAAGGGCAGCCGCGCCATCTGGTATATG
 TGGCTAGGGGCTAGATTTCTAGAGTTCGAAGCCCTTGATTCTTGAACGAGGATCACTGGA
 TGGG

intron

FIG. 26F

GAGAGAGAACTCAGGAGGTGGTGTGAAGGGCTGGGATTACAAAGACTCGGATATGTCCT AGAAGAGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGA CACCCGCATCAGCAGGTTtGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGAGAAA GGGCAcAGGGCCTTGGCATTGGCCATAATCAAGTACACATACCAAACAAAGTGGTAAAGG TCCTTAGACCAGCTGAAAAAGGGAAAACAGTTATGGACATTATTTGAGACAAGACCAAAG GGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGCAACTCA TTCGGAATATGGAGGCTGAGGAAGTTCTgGAGATGCAAGACTTGTGGCTGCTGCGGAGGT CAGAGAAAGTGACCAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATGGCAG TCAGTGGAGATGATTGCGTTGTGAAGCCAATTGATGATAGGTTTGCACATGCCCTaAGGTTCT TTGAATGATATGGGAAAAGTTAGGAAGGACACACAAGAGTGGAACCCCTCAACTGGATGG GACAACTGGGAAGAAGTTCCGTTTTGCTCCCACCACTTCAACAAGCTCCATCTCAAGGACGG GAGGTCCATTGTGGTTCCTGCCGCCACCAAGATGAACTGATTGGCCGGGCGCGCTCTCTC CAGGGGCGGGATGGAGCATCCGGGAGACTGCTTGCTAGCAAATCATATGCGCAAATGT GGCAGCTCCTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCAATGCCATTTGTTTCATCT GTGCCAGTTGACTGGGTTCCAACTGGGAGAACTACCTGGTCAATCCATGGAAAGGGAGAAT GGATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGATTGAGGAGAACGACC ACATGGAAGACAAGACCCCAGTTACGAAATGGACAGACATTCCCTATTTGGGAAAAAGGG AAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGCGCACCACTGGGCTGAGAACAT TAAAAACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGACTA CCTATCCACCCAAGTTGCTACTTGGGTGAAGAAGGGTCTACACCTGGAGTGCTGTAA GCACCAATCTTAATGTTGTCAGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGC CTGTGACCCCCCAGGAGAAGCTGGGAAACCAAGCCTATAGTCAGGCCGAGAACGCCATG GCACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACCCCAc GCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGG GCCTGAACTGGAGATCAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAGACCC CCCGGAAAACGCAAAACAGCATATTGACGCTGGGAAAGACCAGAGACTCCATGAGTTTCCA CCACGCTGGCCGCCAGGCACAGATCGCCGAATAGCGGCGGCCGGTGTGGGGAAATCCATG GGTCT GGGTGCGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCGAAGGAGGACGCAC GTCCACTCGGATGGCTAAGGGAGAGCCACGAGCTCCTCGACAGATCATAATCAGCCATACC ACATTTGTAGAGGTTTTACTTGGCTTTAAAAAACCTCCACACCTCCCCCTGAACCTGAAACAT AAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGC AATAGCATCACAAATTTACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCC AAACTCATCAAGATAATAAAGGAAATTTATTTTCATTGCAATAGTGTGTTGGAATTTTTTGT GTCTCTCACTCGGAAGGACATATGGGAGGGCAAATCATTTAAAACATCAGAATGAGTATTT GGTTTAGAGTTTGGCAACATATGCCCATATGTAA GATATCAGGCTTCGAG CAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTGTAATCTGTTTATGTAAGCAGAC AGTTTTATTGTTTCATGATGATATATTTTTATCTTGTCATGTAACATCAGAGATTTTGAGAC ACAACGTGGCTTTGTTGAATAAATCGAACTTTTGTGAGTTGAAGGATCAGATCACGCATCT TCCCGACAACGCAGACCGTTCCGTGGCAAAGCAAAGTTCAAATCACCAACTGGTCCACCT ACAACAAAGCTCTCATCAACCGTGGCTCCCTCACTTTCTGGCTGGATGATGGGGCGATTGAG GCCTGGTATGAGTCAGCAACACCTTCTTACGAGGCAGACCTCAGCGCTAGCGGAGTGTAT ACTGGCTTACTATGTTGGCACTGATGAGGGTGTGAGTGAAGTGCTTCATGTGGCAGGAGA AAAAAGGCTGCACCGGTGCGTCAGCAGAATATGTGAT	silent Stop RBZ pA signal translation terminator p15A origin of replication
--	---

FIG. 26G

ACAGGATATATTCCGCTTCCTCGCTCACTGACTCGCTACGCTCGGTCGTTGACTGCGGCG
 AGCGGAAATGGCTTACGAACGGGGCGGAGATTTCTGGAAGATGCCAGGAAGATACTTA
 ACAGGGAAAGTGAGAGGGCCGCGGCAAAGCCGTTTTTCCATAGGCTCCGCCCCCTGACAA
 GCATCACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGAT
 ACCAGGCGTTTTCCCTGGCGGCTCCCTCGTGCGCTCTCCTGTTCTGCTTTTCGGTTTACCG
 GTGTCATTCCGCTGTTATGGCCGCGTTTGTCTCATTCCACGCCTGACACTCAGTTCGGGTA
 GGCAGTTCGCTCCAAGCTGGACTGTATGCACGAACCCCCGTTCACTCCGACCGCTGCGCC
 TTATCCGGTAACTATCGTCTTGAGTCCAACCCGGAAAGACATGCAAAAGCACCCTGGCA
 GCAGCCACTGGTAATTGATTTAGAGGAGTTAGTCTTGAAGTCATGCGCCGGTTAAGGCTA
 AACTGAAAGGACAAGTTTTGGTGAAGTGCCTCCAAGCCAGTTACCTCGGTTCAAAGA
 GTTGGTAGCTCAGAGAACCTTCGAAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTTAGAG
 CAAGAGATTACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAAGGGGTCTGAC
 GCTCAGTGGAAACGAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCT
 TCACCTAGATCCTTTTAAATTAATAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAA
 CTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTT
 GTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCA
 TCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGC
 AATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTTATCCGCCTCC
 ATCCAGTCTATTAATTGTTGCCGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGGC
 CAACGTTGTTGCCATTGCTGCAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATT
 CAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGG
 TTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTTATCACTCATGG
 TTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTG
 GTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCC
 GCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAA
 AACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTTCGATGTAA
 CCCACTCGTGACCCCACTGATCTTCAGCATCTTTTACTTTACCCAGCGTTTCTGGGTGAGCA
 AAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAAT
 ACTCATACTCTTCTTTTTCAATATTATTGAAGCATTATATCAGGGTTATTGTCTCATGAGCGG
 ATACATATTTGAATGTATTTAGAAAAATAAACAATAGGGGTTCCGCGCACATTTCCCCGAA
 AAGTGCCACCTGACGTGTGACGCGGGGGCATGTGCTTACATAACTTACGGTAAATGGCCC
GCCTGGCTGACCGCCCAACGACCCCCGCCATTGACGTCAATAATGACGTATGTTCCCATAG
TAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTACGGTAAACTGCCAC
TTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAA
ATGGCCCGCCTGGCATTATGCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACA
TCTACGTATTAGTCATCGCTATTACCATGGTGTATGCGGTTTTTGGCAGTACATCAATGGGCGT
GGATAGCGGTTTGAATCACGGGGATTTCCAAGTCTCCACCCATTGACGTCAATGGGAGTTT
GTTTTGGCACCAAAATCAACGGGACTTTCAAAATGTCGTAACAACCTCCGCCCCATTGACGC
AAATGGGCGGTAGGCGGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACC

AmpR

CMV promoter



FIG. 27A

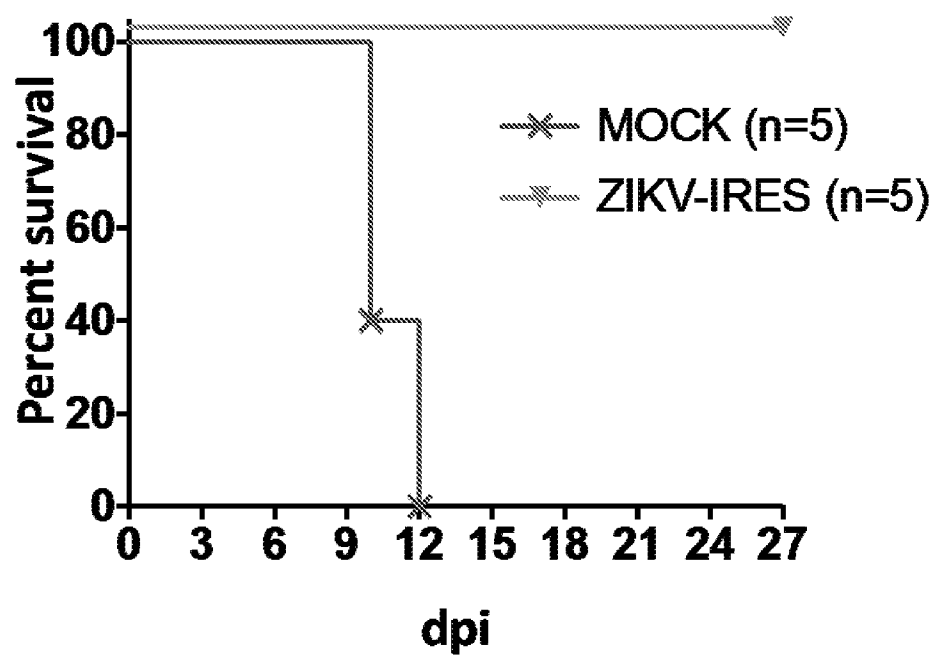


FIG. 27B

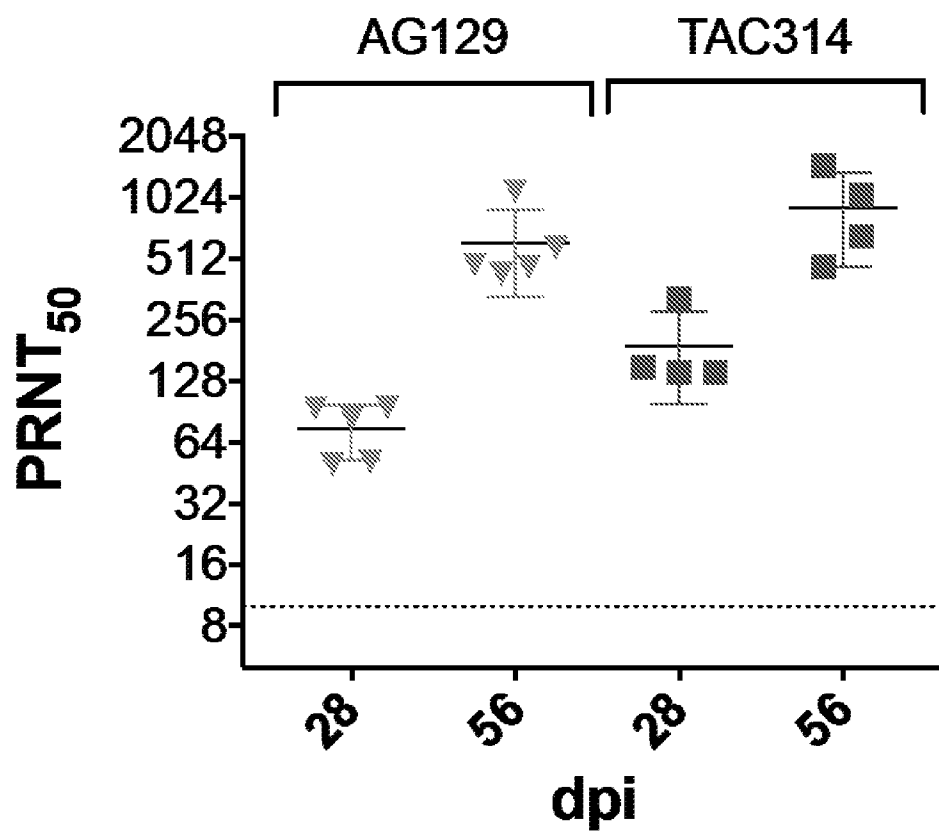


FIG. 27C

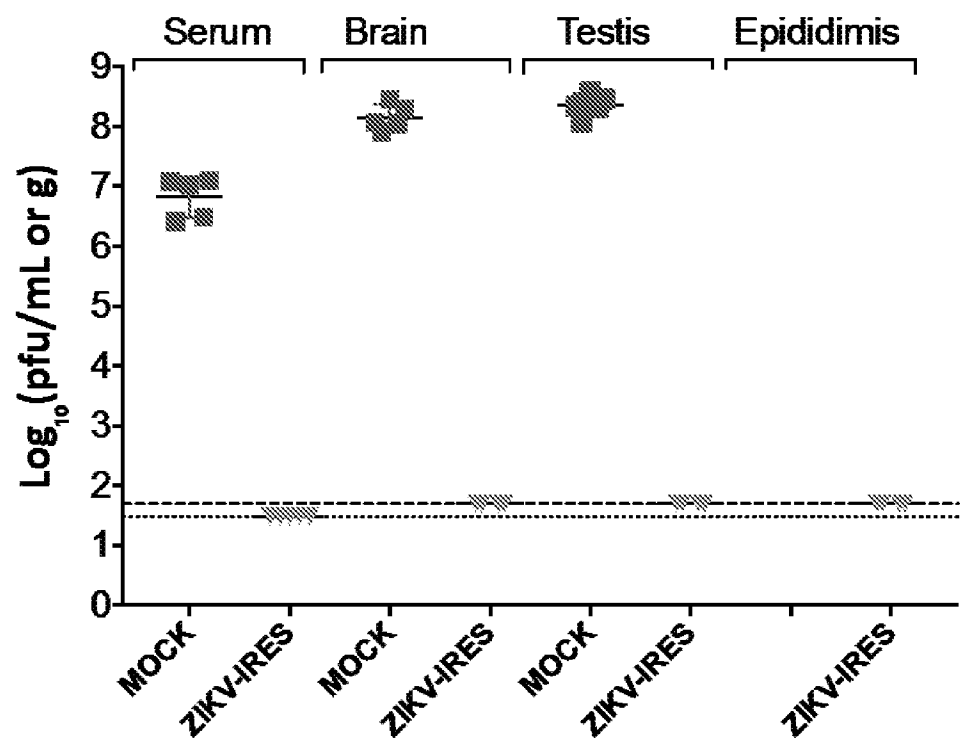


FIG. 28

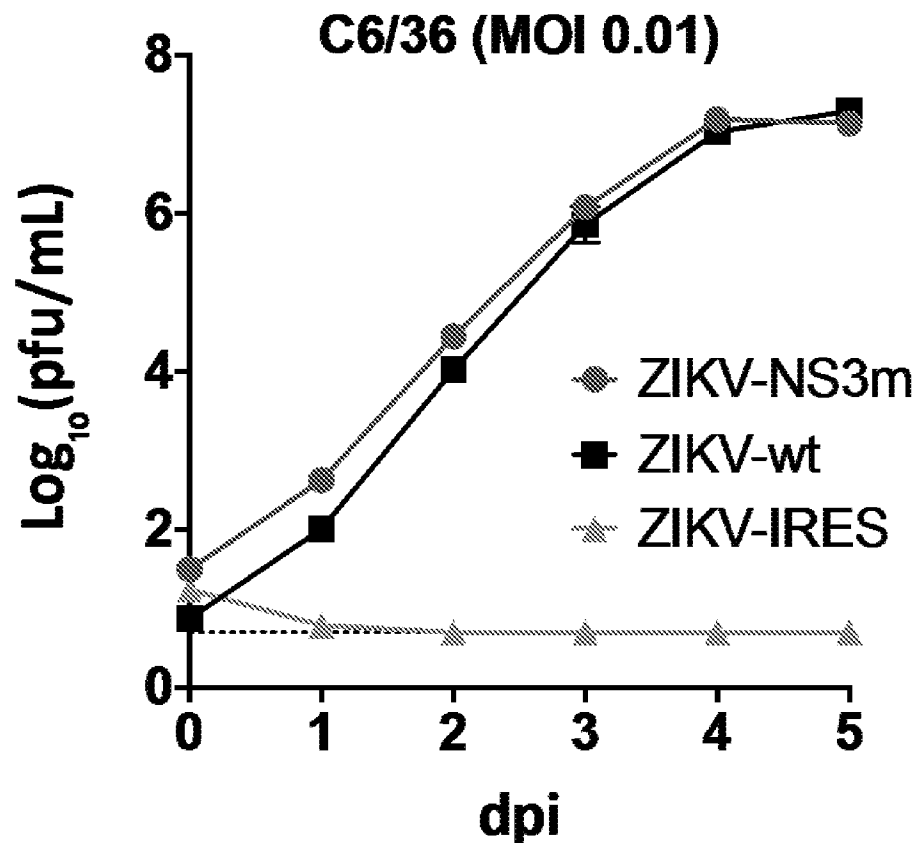
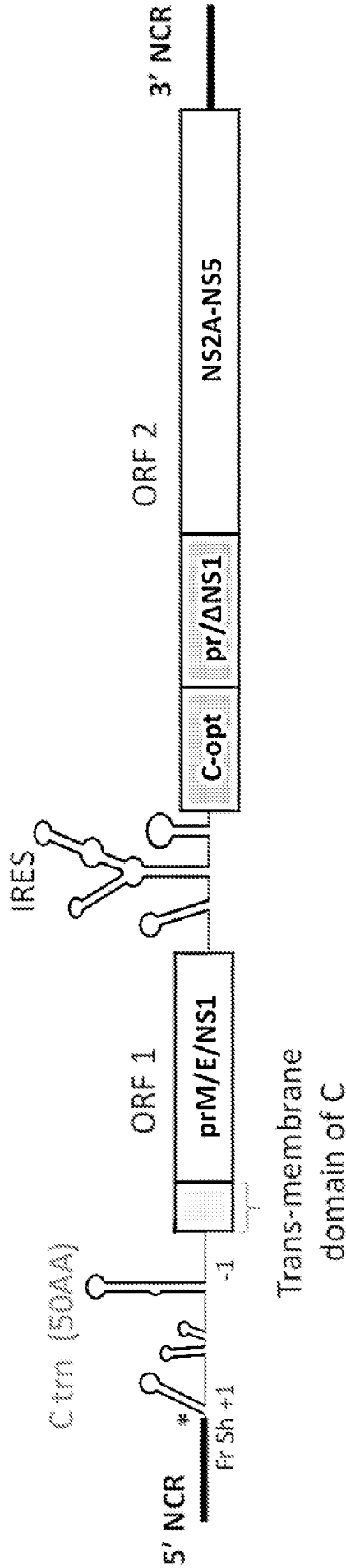


FIG. 29



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/012346

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/012346

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K39/12 C12N7/04 C12N15/11 C12N15/113 C12N15/86 ADD.								
According to International Patent Classification (IPC) or to both national classification and IPC								
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K C12N Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data, CHEM ABS Data, Sequence Search								
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>Y</td> <td> M. HUSSAIN ET AL: "MicroRNA-like viral small RNA from Dengue virus 2 autoregulates its replication in mosquito cells", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 7, 3 February 2014 (2014-02-03), pages 2746-2751, XP055467378, ISSN: 0027-8424, DOI: 10.1073/pnas.1320123111 the whole document ----- -/-- </td> <td>1-68</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	Y	M. HUSSAIN ET AL: "MicroRNA-like viral small RNA from Dengue virus 2 autoregulates its replication in mosquito cells", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 7, 3 February 2014 (2014-02-03), pages 2746-2751, XP055467378, ISSN: 0027-8424, DOI: 10.1073/pnas.1320123111 the whole document ----- -/--	1-68
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.						
Y	M. HUSSAIN ET AL: "MicroRNA-like viral small RNA from Dengue virus 2 autoregulates its replication in mosquito cells", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 7, 3 February 2014 (2014-02-03), pages 2746-2751, XP055467378, ISSN: 0027-8424, DOI: 10.1073/pnas.1320123111 the whole document ----- -/--	1-68						
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.								
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family								
Date of the actual completion of the international search		Date of mailing of the international search report						
3 May 2018		09/05/2018						
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Meyer, Wolfram						

INTERNATIONAL SEARCH REPORT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANONYMOUS: "2010 RNA Symposium, Invited Lecture 8: MicroRNA-124 attenuates neurovirulence of flavivirus via a designed target in the 3'-untranslated region of nonpolyadenylated RNA genome", INTERNET CITATION, February 2010 (2010-02), pages 1-2, XP002668198, Retrieved from the Internet: URL: http://140.116.6.160:8080/RNA2010/program.pdf [retrieved on 2012-01-27] the whole document -----	1-68
Y	DWIGHT BARNES ET AL: "Harnessing endogenous miRNAs to control virus tissue tropism as a strategy for developing attenuated virus vaccines", CELL HOST AND MICROBE, CELL PRESS, US, vol. 4, no. 3, 11 September 2008 (2008-09-11), pages 239-248, XP002544788, ISSN: 1934-6069, DOI: 10.1016/J.CHOM.2008.08.003 [retrieved on 2008-09-10] the whole document -----	1-68
X	WO 2012/051491 A1 (US OF AMERICA AS REPRESENTED BY THE SECRETARY NAT INST OF HEALTH [US];) 19 April 2012 (2012-04-19) the whole document -----	1-68

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2018/012346

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 2012051491 A1	19-04-2012	NONE	
