COMPOSITIONS AND METHODS FOR DENGUE VIRUS CHIMERIC CONSTRUCTS IN VACCINES

PRIORITY

[0001] This PCT Application claims priority to U.S. Provisional Application No. 61/800,204 filed March 15, 2013. This application is incorporated herein in its entirety by reference for all purposes.

FEDERALLY FUNDED RESEARCH

[0002] Some embodiments disclosed herein were supported in part by grant number R43 AI084291-01 from the National Institutes of Health. The U.S. Government may have certain rights to practice the subject invention.

FIELD

[0003] Embodiments herein report compositions, methods, uses and manufacturing procedures for dengue virus constructs and vaccine compositions thereof. Some embodiments concern a composition that includes, but is not limited to, chimeric flavivirus virus constructs that alone or in combination with other constructs can be used in a vaccine composition. In certain embodiments, compositions can include constructs of more than one serotypes of dengue virus, such as dengue-1 (DEN-1) virus, dengue-2 (DEN-2) virus, dengue-3 (DEN-3) virus and/or dengue-4 (DEN-4) virus. In other embodiments, manufacturing strategy that can improve the safety and genetic stability of recombinant live-attenuated chimeric dengue vaccine (DENVax) viruses. Certain embodiments include at least one live, attenuated dengue virus in combination with dengue virus chimeric constructs identified to be both safe and effective in vaccine compositions where the constructs have undergone additional passages in cell cultures. **BACKGROUND**

[0004] Infection with dengue virus can lead to a painful fever of varying severity. To date, four serotypes of dengue virus have been identified: dengue-1 (DEN-1), dengue-2 (DEN-2), or dengue-3 (DEN-3) in combination with dengue-4 (DEN-4). Dengue fever is caused by infection of a dengue virus. Other subtypes may be discovered in the future (e.g. DEN-5). Dengue virus serotypes 1-4 can also cause dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). The most severe consequences of infection, DHF and DSS, can be life threatening. Dengue viruses cause 50-100 million cases of debilitating dengue fever, 500,000 cases of DHF/DSS, and more than 20,000 deaths each year. To date, there is no

effective vaccine to protect against dengue fever and no drug treatment for the disease. Mosquito control efforts have been ineffective in preventing dengue outbreaks in endemic areas or in preventing further geographic spread of the disease. It is estimated that 3.5 billion people are threatened by infection with dengue virus. In addition, dengue virus is a leading cause of fever in travelers to endemic areas, such as Asia, Central and South America, and the Caribbean.

[0005] All four dengue virus serotypes are endemic throughout the tropical regions of the world and constitute the most significant mosquito-borne viral threat to humans in tropical regions, worldwide. Dengue viruses are transmitted to humans primarily by *Aedes aegypti* mosquitoes. Infection with one dengue virus serotype results in life-long protection from re-infection by that serotype, but does not prevent secondary infection by one of the other three dengue virus serotypes. In fact, previous infection with one dengue virus serotype leads to an increased risk of severe disease (DHF/DSS) upon secondary infection with a different serotype. The development of an effective vaccine represents an important approach to the prevention and control of this global emerging disease. Multiple immunizations make complete vaccine coverage difficult both for public health efforts in dengue virus endemic countries as well as travelers.

SUMMARY

[0006] In one aspect, the present disclosure provides a polynucleotide molecule encoding a modified live, attenuated dengue-2 virus strain PDK-53-V polypeptide molecule, wherein the polynucleotide molecule comprises: an adenine to guanine mutation at position 592 encoding a glutamic acid instead of a lysine in the polypeptide molecule at amino acid position 166 corresponding to prM-52; and an adenine to guanine mutation at position 8803 encoding a valine instead of an isoleucine in the polypeptide molecule at amino acid position 2903 corresponding to NS5-412. In another aspect, the present disclosure provides a polypeptide molecule encoded by a polynucleotide molecule. In yet another aspect, the present disclosure provides a modified live, attenuated dengue-2 strain PDK-53-V virus, comprising the polypeptide molecule as described herein, or represented by a polynucleotide molecule as described herein.

[0006a] In a further aspect, the present disclosure provides a pharmaceutical composition comprising a polynucleotide molecule as described herein, a polypeptide molecule as described herein, or a dengue-2 virus as described herein, and a pharmaceutically acceptable excipient. In a further aspect, the present disclosure provides the pharmaceutical composition as described herein for use as a medicament. In a further aspect, the present disclosure

provides a use of the pharmaceutical composition as described herein in the manufacture of a medicament for inducing an immune response in a subject.

[0006b] In a further aspect, the present disclosure provides a vector encoding a polynucleotide molecule as described herein. In a further aspect, the present disclosure provides a cell comprising the polynucleotide molecule as described herein or the vector as described herein. In a further aspect, the present disclosure provides an immunogenic composition comprising a modified live, attenuated dengue-2 strain PDK-53-V virus represented by a polynucleotide molecule as described herein and a pharmaceutically acceptable carrier. In a further aspect, the present disclosure provides a composition comprising one or more live attenuated viruses as described herein and a pharmaceutically acceptable carrier.

[0006c] Examples herein concern compositions, methods and uses of chimeric dengue virus constructs. In some examples, a composition can include chimeric dengue virus constructs having an attenuated dengue virus backbone with structural genes from at least one other dengue virus serotype. Other examples concern at least one live, attenuated virus in combination with one or more chimeric dengue viruses. Other examples can include a composition of chimeric dengue viruses having a modified DEN-2 backbone (e.g. PDK-53 as a starting backbone in P1 (passage-1) and passage variability (after passage and growth *in vitro* on a permissive cell line) as indicated for P2, P3,…P8..P10 etc.) and one or more structural components of DEN-1, DEN-2, DEN-3 or DEN-4. In other examples, an immunogenic composition is generated where when introduced to a subject, the composition produces an immune response to one or more dengue viruses in the subject. Therefore, constructs contemplated herein can be generated and passaged *in vitro*, and each of the passages provides an attenuated dengue virus contemplated of use in a pharmaceutically acceptable vaccine composition. In certain examples a live, attenuated virus can be a live, attenuated dengue-2 virus alone or in combination with one or more chimeric dengue viruses.

[0007] In certain examples, chimeric dengue virus constructs of dengue virus serotypes can include passage 7 (P7) live, attenuated viruses or chimeric viruses having nucleic acid sequences identified by SEQ ID NOS: 1, 4, 7 and 10 or polypeptide sequences indicated by SEQ ID NOS: 2, 3, 5, 6, 8, 9, 11 and 12. It is contemplated herein that any of the passages for any of the live, attenuated viruses described herein can be used in an immunogenic composition to induce immune responses to the represented dengue viruses (e.g. serotypes 1-4). In accordance with these examples, an immunogenic composition that includes a P-8 isolated live, attenuated virus can be administered to a subject to induce an immunogenic response against one or more dengue virus serotypes depending on the construct selected. In addition, a live, attenuated virus can be combined with one or more of these chimeric viruses. This is contemplated for each of the live, attenuated viruses isolated/produced in each subsequent cell passages (*e.g.* African Green Monkey Vero cell production, hereinafter: Vero cells). It is contemplated herein that any cell line (e.g. GMP-produced cell bank, FDA or EMA-approved) capable of producing dengue viruses is of use to passage any of the viral constructs at a manufacturing scale or as appropriate contemplated herein for subsequent use in a vaccine or immunogenic composition against Dengue virus.

[0008] In other examples, compositions contemplated herein can be combined with other immunogenic compositions against other Flaviviruses such as West Nile virus, Japanese encephalitis or any other flavivirus chimeric construct and/or live, attenuated virus. In certain examples, a single composition can be used against multiple flaviviruses.

[0009] In certain examples, an immunogenic composition of the present summary can include chimeric dengue viruses against one or more of DEN-1, DEN-2, DEN-3 and/or DEN-4, alone or in combination with a live, attenuated dengue virus composition.

[00010] In other examples, a construct can include a construct having adaptive mutations in the structural or non-structural regions of the virus that increase growth or production without affecting attenuation or safety of the virus when introduced to a subject. In certain examples, any of the contemplated chimeric dengue virus constructs can include a live, attenuated DEN-2 virus having specific mutations used as a backbone where the live attenuated DEN-2 PDK virus further includes structural proteins of one or more of prM (premembrane) and E (envelope) structural proteins of the other dengue virus serotypes. In addition, a DEN-2 backbone can include additional mutations in order to increase production of or enhance the immune response to a predetermine composition in a subject upon administration (e.g. chimeric Dengue virus 2/1, 2/3 or 2/4).

[00011] In some examples, structural protein genes can include prM and E genes of DEN-1, DEN-2, DEN-3 or DEN-4 on a DEN-2 backbone having one or two mutations that are part of a live, attenuated dengue virus. For example, a dengue construct, in certain examples can include those constructs termed DENVax-1-A, DENVax-2-F, DENVax-3-F, and DENVax-4-F (see Example section) where the DEN-2 backbone has one or more mutations (e.g. not found in the P1 or other previous passaged virus or PDK-53) from the DEN-2 live, attenuated virus previously demonstrated to be safe and effective to induce an immune response. The DEN-2 live, attenuated virus of the instant application is an improved version of the originally used DEN-2 live, attenuated virus. A chimeric construct of the instant summary can include a modified attenuated DEN-2 PDK-53 backbone, having one or more structural proteins of the second dengue virus serotype wherein the structural proteins can include additional mutations to increase an immunogenic response to the chimeric construct. In some examples, certain mutations acquired by attenuated DEN-2 PDK-53 can produce a conservative amino acid change or not in a constructs different from the P1 construct which can result in desirable traits for production etc.

[00012] In other examples, a live, attenuated DEN-2 genome can be used to generate constructs of dengue virus serotype 1 (DEN-1) and dengue virus serotype 3 (DEN-3), dengue virus serotype 4 (DEN-4) where one or more structural protein genes of the DEN-2 viral genome can be replaced by one or more structural protein genes of DEN-1, DEN-3 or DEN-4, respectively. In some examples, a structural protein can be the C, prM or E protein of a second dengue virus. In certain examples, structural protein genes include the prM and E genes of DEN-1, DEN-3 or DEN-4. These hybrid viruses express the surface antigens of DEN-1, DEN-3 or DEN-4 while retaining the attenuation phenotypes of the parent attenuated DEN-2.

[00013] Constructs disclosed herein can include chimeric constructs of DEN-4, DEN-2, DEN-1, and DEN-3 expressing surface antigens of DEN-1, DEN-3 and DEN-4 using attenuated DEN-2 virus as a backbone.

[00014] In certain examples, compositions of the instant summary can include a composition that comprises a single chimeric dengue virus construct disclosed herein and a pharmaceutically acceptable carrier or excipient. Alternatively, compositions of the instant summary can include a composition that comprises two or more, or three or more chimeric dengue virus constructs disclosed herein, and a pharmaceutically acceptable carrier or excipient. In accordance with these examples, a one or more dengue virus chimeric constructs contemplated herein can be combined with one or more, live attenuated dengue

viruses. In certain examples, a live, attenuated virus can be a live, attenuated DEN-2 virus wherein additional mutations in the NCR, NS1 regions or other regions increase the immune response, increase viral growth or other improvement for an improved live, attenuated dengue virus.

[00015] In certain examples, the attenuation loci, nucleotide 5'NCR-57-T, NS1-53-Asp, and NS3-250-Val, of the DENV-2 vaccine have been previously determined, and all of these changes are shared by the common PDK-53 virus-specific genetic background of the four DENVax viruses. The genetic sequence of the three attenuation loci as well as the previously established *in vitro* and *in vivo* attenuation phenotypes of these vaccine candidates were carefully monitored for the cGMP-manufactured DENVax seeds. This report describes strategies used to generate master virus seeds (MVS) as well as their genetic and phenotypic characterization of use in the manufacture of dengue virus vaccine compositions. These MVS can be used for manufacture of clinical materials and ultimately commercial vaccine supplies.

Brief Description of the Drawings

[00016] The following drawings form part of the present specification and are included to further demonstrate certain embodiments. Some embodiments may be better understood by reference to one or more of these drawings alone or in combination with the detailed description of specific embodiments presented.

[00017] **Fig. 1** represents an exemplary chart reflecting an exemplary chimeric construct of the instant invention, DEN-2/DEN-4 compared to previously generated constructs and wild type dengue viruses.

[00018] **Fig. 2** represents an exemplary histogram plot comparing various responses using a live, attenuated DEN-2 backbone (with additional mutations) and a second dengue virus serotype as structural components substituted for the dengue-2 structural components (e.g. DENVax-1 MVS). This plot illustrates plaque sizes of the DENVax MVS. Wild-type Dengue viruses and previously published research-grade vaccine candidate viruses were included for control and comparison. This plot illustrates improved production of the dengue virus constructs compared to control dengue virus chimeric constructs.

[00019] **Fig. 3** represents an exemplary histogram plot that represents temperature sensitivities of DENVax MVS (Master Virus Seed). Wild type dengue viruses and previously published research-grade vaccine candidate viruses were included for comparison with the MVS grade.

[00020] **Fig. 4** represents an exemplary histogram plot that represents viral growth of DENVax MVS in C6/36 cells compared to controls. Wild-type dengue viruses and research-grade vaccine candidate viruses were included for comparison with the DENVax MVS.

[00021] **Figs. 5A-5C** represent exemplary plots of neurovirulence in newborn mice. Pooled results of several experiments summarizing the neurovirulence of wt DENV-2 16681 virus in CDC-ICR (n=72) and Taconic-ICR (n=32) newborn mice challenged ic with 10^4 pfu of the virus (A). Neurovirulence of DENVax MVS tested in Taconic-ICR mice with a dose of 10^4 pfu (B) or 10^3 pfu (C). The numbers of animals tested per group in one experiment (n=16) or two pooled experiments (n=31 or 32) are indicated.

[00022] **Fig. 6** represents an exemplary histogram illustrating plaque size of the DENVax MVS, WVS, and BVS. Mean plaque diameters ± SD (error bars) of the virus plaques in Vero or LLC-MK_2 cells under agarose overlay measured on day 9 pi. Wild type DENVs and previously published research-grade vaccine candidate viruses were included for control and comparison.

[00023] **Fig. 7** represents an exemplary histogram plot illustrating growth of DENVax MSV, WVS, and BVS in C6/36cells at two incubation temperatures to verify their retention of this *in vitro* attenuation marker after large scale manufacturing.

[00024] **Fig. 8** represents an exemplary histogram plotting restricted growth of DENVax MVS, WVS, and BVS in C6/36 cells. Mean titers ± SD (error bars) of the viruses replicated in C6/36 cells 7 days pi. The wt Dengue viruses and previously published research-grade vaccine candidate viruses were included for comparison.

[00025] **Figs. 9A-9B** represent exemplary graphs of data of neurovirulence of DENVax MVS in newborn ICR mice. (A) IC inoculations of the virus at dose of 10^4 PFU. (B) IC inoculation of the virus at dose of 10^3 PFU.

[00026] **Fig. 10** represents an exemplary chart comparing new live, attenuated viruses to previously generated live, attenuated dengue viruses.

Definitions

[00027] As used herein, "a" or "an" may mean one or more than one of an item.

[00028] As used herein the specification, "subject" or "subjects" may include, but are not limited to, mammals such as humans or mammals, domesticated or wild, for example dogs, cats, other household pets (e.g. hamster, guinea pig, mouse, rat), ferrets, rabbits, pigs, horses, cattle, prairie dogs, wild rodents, or zoo animals.

[00029] As used herein, the terms "virus chimera," "chimeric virus," "flavivirus

chimera" and "chimeric flavivirus" can mean a construct comprising a portion of the nucleotide sequence of a dengue-2 virus and further nucleotide sequence that is not from dengue-2 virus or is from a different flavivirus. A "dengue chimera" comprises at least two different dengue virus serotypes but not a different flavivirus. Thus, examples of other dengue viruses or flaviviruses include, but are not limited to, sequences from dengue-1 virus, dengue-3 virus, dengue-4 virus, West Nile virus, Japanese encephalitis virus, St. Louis encephalitis virus, tick-borne encephalitis virus, yellow fever virus and any combination thereof.

[00030] As used herein, "nucleic acid chimera" can mean a construct of the invention comprising nucleic acid comprising a portion of the nucleotide sequence of a dengue-2 virus and further nucleotide sequence that is not of the same origin as the nucleotide sequence of the dengue-2 virus. Correspondingly, any chimeric flavivirus or flavivirus chimera disclosed herein can be recognized as an example of a nucleic acid chimera.

[00031] As used herein, "a live, attenuated virus" can mean a wild-type virus, mutated or selected for traits of use in vaccine or other immunogenic compositions wherein some traits can include reduced virulence, safety, efficacy or improved growth etc.

DESCRIPTION

[00032] In the following sections, various exemplary compositions and methods are described in order to detail various embodiments. It will be obvious to one skilled in the art that practicing the various embodiments does not require the employment of all or even some of the specific details outlined herein, but rather that concentrations, times and other specific details may be modified through routine experimentation. In some cases, well-known methods or components have not been included in the description.

[00033] In accordance with embodiments of the present invention, there may be employed conventional molecular biology, protein chemistry, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Animal Cell Culture, R. I. Freshney, ed., 1986).

[00034] Embodiments herein concern compositions, methods and uses for inducing immune responses against one or more dengue virus serotypes in a subject, individually or simultaneously. In accordance with these embodiments, attenuated dengue viruses and nucleic acid chimeras are generated and used in vaccine compositions disclosed herein. Some embodiments concern modified or mutated dengue constructs or chimeras. Other

embodiments concern introducing mutations to modify the amino acid sequences of structural proteins of dengue viruses wherein the mutation increase immunogenicity to the virus.

[00035] Live, attenuated dengue viruses of all four serotypes have been developed by passaging wild-type viruses in cell culture. These are some of the most promising live, attenuated vaccine candidates for immunization against flavivirus and in particular dengue virus infection and/or disease. These vaccine candidates have been designated by a combination of their dengue serotype, the cell line through which they were passaged and the number of times they were passaged. Thus, a dengue serotype 1 wild-type virus passaged in PDK cells 13 times is designated as DEN-1 PDK-13 virus. Other vaccine candidates are DEN-2 PDK-53, DEN-3 PGMK-30/FRhL-3 (e.g. thirty passages in primary green monkey kidney cells, followed by three passages in fetal rhesus lung cells and DEN-4 PDK-48). These four candidate vaccine viruses were derived by tissue culture passage of wild-type parental DEN-1 16007, DEN-2 16681, DEN-3 16562 and DEN-4 1036 viruses, respectively.

[00036] In certain embodiments, live, attenuated dengue-2 PDK-53 vaccine virus contained a mixture of viruses, with the population containing varying nucleotide differences. After genetic characterization of the attenuating mutations, certain attenuating characteristics were outlined and engineered into a cDNA infectious clone. RNA was transcribed from this infectious clone and introduced into Vero cells as a passage 1 of the newly characterized and derived PDK-53-Vero-DEN-2-P 1 virus (see for example, Table 1). This attenuated virus was created for each DEN serotype, but for DEN-1, DEN-3 and DEN-4, the prM and E genes were engineered into 3 separate cDNA infectious clones, thus generating four separate PDK-53-Vero viruses (termed herein as: PDK-53-Vero-DEN-2-P 1, PDK-53-Vero-DEN-1-P 1, PDK-53-Vero-DEN-3-P 1, and PDK-53-Vero-DEN-4-P 1). These attenuated vaccine virus strains were passaged in Vero cells 10 times (Table 1), and each separate lineage acquired mutations upon their adaptation to grow in Vero cells (Table 3). Certain embodiments here are directed to derivation and uses for these live, attenuated dengue viruses.

[00037] Previous human clinical trials with these attenuated viruses have indicated that DEN-2 PDK-53 has the lowest infectious dose (50% minimal infectious dose of 5 plaque forming units or PFU) in humans, is strongly immunogenic, and produces no apparent safety concerns. The DEN-1 PDK-13, DEN-3 PGMK-30/FRhL-3 and DEN-4 PDK-48 vaccine virus candidates have higher 50% minimal infectious doses of 10,000,

3500, and 150 PFU, respectively, in humans. Although only one immunization with monovalent DEN-2 PDK-53 virus or DEN-4 PDK-48 virus was required to achieve 100% seroconversion in human subjects, a booster was needed to achieve the same seroconversion rate for DEN-1 PDK-13 and DEN-3 PGMK-30/FRhL-3 viruses, which have the two highest infectious doses for humans.

[00038] DEN-2 PDK-53 virus vaccine candidate, also abbreviated PDK-53, has several measurable biological markers associated with attenuation, including temperature sensitivity, small plaque size, decreased replication in mosquito C6136 cell culture, decreased replication in intact mosquitoes, loss of neurovirulence for suckling mice and decreased incidence of viremia in monkeys. Clinical trials of the candidate PDK-53 vaccine have demonstrated its safety and immunogenicity in humans. Furthermore, the PDK-53 vaccine induces dengue virus-specific T-cell memory responses in human vaccine recipients. Some embodiments herein describe an improvement on the DEN-2 PDK-53 used in chimeric constructs disclosed herein.

[00039] Immunogenic flavivirus chimeras having a dengue-2 virus backbone and at least one structural protein of another dengue virus serotype can be used for preparing the dengue virus chimeras and methods for producing the dengue virus chimeras are described. The immunogenic dengue virus chimeras are provided, alone or in combination, in a pharmaceutically acceptable carrier as immunogenic compositions to minimize, inhibit, or immunize individuals against infection by one or more serotypes, such as dengue virus serotypes DEN-1, DEN-2, DEN-3 and DEN-4, alone or in combination. When combined, the immunogenic dengue virus chimeras may be used as multivalent vaccines (e.g. bi-, tri- and tetravalent) to confer simultaneous protection against infection by more than one species or strain of flavivirus. In certain embodiments, the dengue virus chimeras are combined in an immunogenic composition useful as a bivalent, trivalent or tetravalent vaccine against the known dengue virus serotypes or confer immunity to other pathogenic flaviviruses by including nucleic acids encoding one or more proteins from a different flavivirus.

[00040] In some embodiments, avirulent, immunogenic dengue virus chimeras provided herein contain the nonstructural protein genes of the attenuated dengue-2 virus (e.g. PDK-53), or the equivalent thereof, and one or more of the structural protein genes or immunogenic portions thereof of the flavivirus against which immunogenicity is to be induced in a subject. For example, some embodiments concern a chimera having attenuated dengue-2 virus PDK-53 genome as the viral backbone, and one or more structural protein

genes encoding capsid, premembrane/membrane, or envelope of the PDK-53 genome, or combinations thereof, replaced with one or more corresponding structural protein genes from DEN-1, DEN-3 or DEN-4 or other flavivirus to be protected against, such as a different flavivirus or a different dengue virus serotype. In accordance with these embodiments, a nucleic acid chimera disclosed herein can have functional properties of the attenuated dengue-2 virus and is avirulent, but expresses antigenic epitopes of the structural gene products of DEN-1, DEN-3 or DEN-4 in addition to other flaviviruses and is immunogenic (e.g. induces an immune response to the gene products in a subject). Then, these DNA constructs are used to transcribe RNA from an infectious clone, this RNA is introduced into Vero cells again producing a new progeny virus at P1. These new progeny viruses are distinguishable from PDK-53. (See *e.g.* P1-P10).

[00041] In another embodiment, a nucleic acid chimera can be a nucleic acid chimera having, but not limited to, a first nucleotide sequence encoding nonstructural proteins from an attenuated dengue-2 virus, and a second nucleotide sequence encoding a structural protein from dengue-4 virus alone or in combination with another flavivirus. In other embodiments, the attenuated dengue-2 virus can be vaccine strain PDK-53 having one or more mutated amino acids (see Examples). These additional mutations confer desirable traits of use as live, attenuated dengue-2 or as chimeric constructs described herein. Some embodiments include structural proteins of one or more of C, prM or E protein of a second dengue virus.

[00042] Other aspects include that chimeric viruses can include nucleotide and amino acid substitutions, deletions or insertions for example, in the control PDK-53 dengue-2 genome to reduce interference with immunogenicity responses to a targeted dengue virus serotype. These modifications can be made in structural and nonstructural proteins alone or in combination with the example modifications disclosed herein and can be generated by passaging the attenuated virus and obtaining an improved composition for inducing an immune response against one or more dengue virus serotypes.

[00043] Certain embodiments disclosed herein provide for method for making the chimeric viruses of this invention using recombinant techniques, by inserting the required substitutions into the appropriate backbone genome. Other embodiments herein concern passaging a confirmed (e.g. safe and effective) live, attenuated chimeric virus for additional improvements. In certain embodiments, a dengue-2 backbone used herein can include one or more mutations presented in Table 3. In other embodiments, a dengue-dengue chimera of the instant application can include one or more mutations as presented in Table 3. In yet

other embodiments, a dengue-dengue chimera can include all of the mutations for each chimera as represented in Table 3 for Den-2/Den-1, Den-2/Den-3 or Den-2/Den-4. Pharmaceutical compositions that include a live, attenuated virus represented by the constructs of Table 3 are contemplated. For example, mono-, di-, tri- or tetravalent compositions are contemplated of use herein using chimeras and live, attenuated dengue-2 viruses as presented in Table 3.

[00044] In certain embodiments, a live, attenuated DEN-2 variant contemplated herein can be formulated into a pharmaceutical composition wherein the pharmaceutical composition can be administered alone or in combination with dengue-dengue chimeras or dengue-flavivirus chimeras. In certain embodiments, a bi , tri or tetravalent compositions can be administered in a single application or in multiple applications to a subject.

Flavivirus Chimeras

[00045] Dengue virus types 1-4 (DEN-1 to DEN-4) are mosquito-borne flavivirus pathogens. The flavivirus genome contains a 5'-noncoding region (5'-NC), followed by a capsid protein (C) encoding region, followed by a premembrane/membrane protein (prM) encoding region, followed by an envelope protein (E) encoding region, followed by the region encoding the nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) and finally a 3' noncoding region (3'NC). The viral structural proteins are C, prM and E, and the nonstructural proteins are NS1-NS5. The structural and nonstructural proteins are translated as a single polyprotein and processed by cellular and viral proteases.

[00046] Flavivirus chimeras can be constructs formed by fusing non-structural protein genes from one type, or serotype, of dengue virus or virus species of the flaviviridae, with protein genes, for example, structural protein genes, from a different type, or serotype, of dengue virus or virus species of the flaviviridae. Alternatively, a flavivirus chimera of the invention is a construct formed by fusing non-structural protein genes from one type, or serotype, of dengue virus or virus species of the flaviviridae, with further nucleotide sequences that direct the synthesis of polypeptides or proteins selected from other dengue virus serotypes or other viruses of the flaviviridae.

[00047] In other embodiments, avirulent, immunogenic flavivirus chimeras provided herein contain the nonstructural protein genes of the attenuated dengue-2 virus, or the equivalent thereof, and one or more of the structural protein genes, or antigenic portions thereof, of the flavivirus against which immunogenicity is to be conferred. Suitable flaviviruses include, but are not limited to those listed in Table 1.

[00048] Other suitable dengue viruses for use in constructing the chimeras can be

wild-type, virulent DEN-1 16007, DEN-2 16681, DEN-3 16562 and DEN-4 1036 and attenuated, vaccine-strain DEN-1 PDK-13, DEN-2 PDK-53, DEN-3 PMK-30/FRhL-3 and DEN-4 PDK-48. Genetic differences between the DEN-1, DEN-2, DEN-3 and DEN-4 wild type/attenuated virus pairs are contemplated along with changes in the amino acid sequences encoded by the viral genomes.

[00049] Sequence listings for DEN-2 PDK-53 correspond to the DEN-2 PDK-53-V variant, wherein genome nucleotide position 5270 is mutated from an A to a T and amino acid position 1725 of the polyprotein or amino acid position 250 of the NS3 protein contains a valine residue. The DEN-2 PDK-53 variant without this nucleotide mutation, DEN-2 PDK-53-E, differs from PDK-53-V only in this one position. DEN-2 PDK-53-E has an A at nucleotide position 5270 and a glutamate at polyprotein amino acid position 1725, NS3 protein amino acid position 250. It is understood that embodiments herein include modified PDK 53 that include one or more passages in a separate host cell (e.g. Vero cells, see Table 1) where desirable traits of use in vaccine compositions contemplated herein are generated.

[00050] In certain embodiments, designations of the chimeras can be based on the DEN-2 virus-specific infectious clone modified backbones and structural genes (prM-E or C-prM-E) insert of other dengue viruses or other flaviviruses. DEN-2 for the dengue-2 backbone, followed by the strain from which the structural genes are inserted. One DEN-2 backbone variant is reflected in the next letter after the number designation. One particular DEN-2 backbone variant from which the chimera was constructed is indicated by the following letter placed after a hyphen, parent 16681 (P), PDK-53-E (E), or PDK-53-V (V); the last letter indicates the C-prM-E structural genes from the parental (P) strain or its vaccine derivative (V) or the prM-E structural genes from the parental (P) or its vaccine derivative (V1). For example; DEN-2/1-VP denotes the chimera comprising the attenuated DEN-2 PDK-53V backbone comprising a valine at NS3-250 and the C-prM-E genes from wild-type DEN-1 16007; DEN-2/1-VV denotes the DEN-2 PDK-53V backbone with the vaccine strain of dengue-1, DEN-1 PDK-13; DEN-2/1-VP1 denotes the DEN-2 PDK-53V backbone and the prM-E genes from wild-type DEN-1 16007; DEN-2/3-VP1 denotes the DEN-2 PDK-53V backbone and the prM-E genes from wild-type DEN-3 16562; DEN-2/4VP1 denotes the DEN-2 PDK-53V backbone and the prM-E genes from wild-type DEN-4 1036. Other chimeras disclosed herein are indicated by the same manner.

[00051] In one embodiment, chimeras disclosed herein contain attenuated dengue-2 virus PDK-53 genome as the viral backbone, in which the structural protein genes encoding

C, prM and E proteins of the PDK-53 genome, or combinations thereof, can be replaced with the corresponding structural protein genes from dengue-1, dengue-3 or dengue-4 virus and optionally, another flavivirus to be protected against, such as a different flavivirus or a different dengue virus strain.

[00052] In the nonstructural protein regions, a Gly-to-Asp (wild type-to-PDK-53) mutation was discovered at nonstructural protein NS1-53 (genome nucleotide position 2579); a Leu-to-Phe (wild type-to-PDK-53) mutation was discovered at nonstructural protein NS2A-181 (genome nucleotide position 4018); a Glu-to-Val (wild type-to-PDK-53) mutation was discovered at nonstructural protein NS3-250 (genome nucleotide position 5270); and a Gly-to-Ala mutation (wild type-to-PDK-53) was discovered at nonstructural protein NS4A-75 (genome nucleotide position 6599). The live, attenuated DEN-2 virus of the instant invention further includes mutations as presented in any chimera or live, attenuated dengue-2 virus of Table 3.

[00053] PDK-53 virus strain has a mixed genotype at genome nucleotide 5270. A significant portion (approximately 29%) of the virus population encodes the non-mutated NS3-250-Glu that is present in the wild type DEN-2 16681 virus rather than the NS3-250-Val mutation. As both genetic variants are avirulent, this mutation may not be necessary in an avirulent chimera.

[00054] Previously, it was discovered that avirulence of the attenuated PDK-53 virus strain can be attributed to mutations in the nucleotide sequence encoding nonstructural proteins and in the 5' noncoding region. For example, a single mutation at NS1-53, a double mutation at NS1-53 and at 5'NC-57, a double mutation at NS1-53 and at NS3-250 and a triple mutation at NS1-53, at 5'NC-57 and at NS3-250, result in attenuation of the DEN-2 virus. Therefore, the genome of any dengue-2 virus containing such non-conservative amino acid substitutions or nucleotide substitutions at these loci can be used as a base sequence for deriving the modified PDK-53 viruses disclosed herein. Another mutation in the stem of the stem/loop structure in the 5' noncoding region will provide additional avirulent phenotype stability, if desired. Mutations to this region disrupt potential secondary structures important for viral replication. A single mutation in this short (only 6 nucleotide residues in length) stem structure in both DEN and Venezuelan equine encephalitis viruses disrupts the formation of the hairpin structure. Further mutations in this stem structure decrease the possibility of reversion at this locus, while maintaining virus viability.

[00055] Mutations disclosed herein can be achieved by any method known in the art including, but not limited to, naturally-occurring or selected clones having additional

features once passaged in a cell line of interest (*e.g.* Vero cells). It is 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 avirulent backbone structures.

Construction of Flavivirus Chimeras

[00056] Flavivirus chimeras described herein can be produced by splicing one or more of the structural protein genes of the flavivirus against which immunity is desired into a PDK-53 dengue virus genome backbone, or other methods known in the art, using recombinant engineering to remove the corresponding PDK-53 gene and replace it with a dengue-1, dengue-3 or dengue-4 virus gene or other gene known in the art.

[00057] Alternatively, using the sequences provided in the sequence listing, the nucleic acid molecules encoding the flavivirus proteins may be synthesized using known nucleic acid synthesis techniques and inserted into an appropriate vector. Avirulent, immunogenic virus is therefore produced using recombinant engineering techniques known to those skilled in the art.

[00058] A target gene can be inserted into the backbone that encodes a flavivirus structural protein of interest for DEN-1, DEN-3, DEN-4 or other flavivirus. A flavivirus gene to be inserted can be a gene encoding a C protein, a PrM protein and/or an E protein. The sequence inserted into the dengue-2 backbone can encode both PrM and E structural proteins. The sequence inserted into the dengue-2 backbone can encode all or one of C, prM and E structural proteins.

[00059] Suitable chimeric viruses or nucleic acid chimeras containing nucleotide sequences encoding structural proteins of other flaviviruses or dengue virus serotypes can be evaluated for usefulness as vaccines by screening them for the foregoing phenotypic markers of attenuation that indicate avirulence and by screening them for immunogenicity. Antigenicity and immunogenicity can be evaluated using in vitro or in vivo reactivity with flavivirus antibodies or immunoreactive serum using routine screening procedures known to those skilled in the art.

Dengue Virus Vaccines

[00060] In certain embodiments, chimeric viruses and nucleic acid chimeras can provide live, attenuated viruses useful as immunogens or vaccines. Some embodiments include chimeras that exhibit high immunogenicity to dengue-4 virus while producing no dangerous pathogenic or lethal effects.

[00061] To reduce occurrence of DHF/DSS in subjects, a tetravalent vaccine is

needed to provide simultaneous immunity for all four serotypes of the virus. A tetravalent vaccine is produced by combining a live, attenuated dengue-2 virus of the instant application with dengue-2/1, dengue-2/3, and dengue-2/4 chimeras described above in a suitable pharmaceutical carrier for administration as a multivalent vaccine.

[00062] The chimeric viruses or nucleic acid chimeras of this invention can include structural genes of either wild-type or live, attenuated virus in a virulent or an attenuated DEN-2 virus backbone. For example, the chimera may express the structural protein genes of wild-type DEN-4 1036 virus, its candidate vaccine derivative in either DEN-2 backgrounds.

[00063] Viruses used in the chimeras described herein can be grown using techniques known in the art. Virus plaque titrations are then performed and plaques counted in order to assess the viability and phenotypic characteristics of the growing cultures. Wild type viruses can be passaged through cultured cell lines to derive attenuated candidate starting materials.

[00064] Chimeric infectious clones can be constructed from the various dengue serotype clones available. The cloning of virus-specific cDNA fragments can also be accomplished, if desired. The cDNA fragments containing the structural protein or nonstructural protein genes are amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from dengue virus RNA with various primers. Amplified fragments are cloned into the cleavage sites of other intermediate clones. Intermediate, chimeric dengue virus clones are then sequenced to verify the accuracy of the inserted dengue virus-specific cDNA.

[00065] Full genome-length chimeric plasmids constructed by inserting the structural protein and/or nonstructural protein gene region of dengue serotype viruses into vectors are obtainable using recombinant techniques well known to those skilled in the art.

Nucleotide and Amino Acid Analysis

[00066] The NS1-53 mutation in the DEN-2 PDK-53 vaccine virus is significant for the attenuated phenotype of this virus, because the NS1-53-Gly of the DEN-2 16681 virus is conserved in nearly all flaviviruses, including the tick-borne viruses, sequenced to date. DEN-4 vaccine virus can also contain an amino acid mutation in the NS1 protein at position 253. This locus, which is a Gln-to-His mutation in DEN-4 PDK-48 vaccine virus, is Gln in all four wild serotypes of dengue virus. This Gln residue is unique to the dengue viruses within the flavivirus genus. The NS1 protein is a glycoprotein that is secreted from flavivirus-infected cells. It is present on the surface of the infected cell and NS1-specific antibodies are present in the serum of virus-infected individuals. Protection of animals immunized with NS1 protein or passively with NS1-specific antibody has been reported.

The NS1 protein appears to participate in early viral RNA replication.

[00067] The mutations that occurred in the NS2A, NS2B, NS4A, and NS4B proteins of the DEN-1, -2, -3 and -4 attenuated strains are conservative in nature. The NS4A-75 and NS4A-95 mutations of DEN-2 and DEN-4 vaccine viruses, respectively, occurred at sites of amino acid conservation among dengue viruses, but not among flaviviruses in general.

[00068] The flaviviral NS3 protein possesses at least two recognized functions: the viral proteinase and RNA helicase/NTPase. The 698-aa long (DEN-2 virus) NS3 protein contains an amino-terminal serine protease domain (NS3-51-His, -75-Asp, -135-Ser catalytic triad) that is followed by sequence motifs for RNA helicase/NTPase functions (NS3-196-GAGKT (SEQ ID NO:147), -284-DEAH, -459-GRIGR). None of the mutations in the NS3 proteins of DEN-1, DEN-2, or DEN-3 virus occurred within a recognized motif. The NS3-510 Tyr-to-Phe mutation in DEN-1 PDK-13 virus was conservative. Since the wild-type DEN-2, -3 and -4 viruses contain Phe at this position, it is unlikely that the Tyr-to-Phe mutation plays a role in the attenuation of DEN-1 virus. The NS3-182 Glu-to-Lys mutation in DEN-1 PDK-13 virus occurred at a position that is conserved as Asp or Glu in most mosquito-borne flaviviruses and it may play some role in attenuation. This mutation was located 15 amino acid residues upstream of the GAGKT helicase motif. As noted in previous reports, the NS3-250-Glu in DEN-2 16681 virus is conserved in all mosquito-borne flaviviruses except for yellow fever virus.

[00069] Nucleic acid probes selectively hybridize with nucleic acid molecules encoding the DEN-1, DEN-3 and DEN-4 viruses 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 dengue virus. Therefore, in the design of hybridizing nucleic acids, selectivity will depend upon the other components present in a 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 of this invention can have at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, and 99% complementarity with the segment of the sequence to which it hybridizes, preferably 85% or more.

[00070] Sequences, probes and primers which selectively hybridize to the encoding nucleic acid or the complementary, or opposite, strand of the nucleic acid are contemplated. Specific hybridization with nucleic acid can occur with minor modifications or substitutions

in the nucleic acid, so long as functional species-specific hybridization capability is maintained. By "probe" 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 can vary in length from about 5 to 100 nucleotides, or preferably from about 10 to 50 nucleotides, or most preferably about 18-24 nucleotides.

[00071] If used as primers, the composition preferably 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 the 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.

[00072] Nucleic acid sequences encoding the DEN-4, DEN-3 or DEN-1 virus (e.g. structural elements) can be inserted into a vector, such as a plasmid, and recombinantly expressed in a living organism (e.g. into a dengue-2 backbone) to produce recombinant dengue virus peptides and/or polypeptides and/or viruses.

Nucleic Acid Detection Methods

[00073] A rapid genetic test that is diagnostic for each of the vaccine viruses described herein is provided by the current invention. This embodiment of the invention enhances analyses of viruses isolated from the serum of vaccinated humans who developed a viremia, as well as enhancing characterization of viremia in nonhuman primates immunized with the candidate vaccine viruses.

[00074] These sequences include a diagnostic TaqMan probe that serves to report the detection of the cDNA amplicon amplified from the viral genomic RNA template by using a reverse-transciptase/polymerase chain reaction (RT/PCR), as well as the forward and reverse amplimers that are designed to amplify the cDNA amplicon, as described below. In certain instances, one of the amplimers has been 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.

[00075] Automated PCR-based nucleic acid sequence detection system can be used, or other known technology for nucleic acid detection. The TaqMan assay is a highly

specific and sensitive assay that permits automated, real time visualization and quantitation of PCR-generated amplicons from a sample nucleic acid template. TaqMan can determine the presence or absence of a specific sequence. In this assay, a forward and a reverse primer are designed to anneal upstream and downstream of the target mutation site, respectively. A specific detector probe, which is designed to have a melting temperature of about 10.degree. C. higher than either of the amplimers and containing the vaccine virus-specific nucleotide mutation or its complement (depending on the strand of RT/PCR amplicon that is being detected), constitutes the third primer component of this assay.

[00076] A probe designed to specifically detect a mutated locus in one of the vaccine viral genomes will contain the vaccine-specific nucleotide in the middle of the probe. This probe will result in detectable fluorescence in the TaqMan assay if the viral RNA template is vaccine virus-specific. However, genomic RNA templates from wild-type DEN viruses will have decreased efficiency of probe hybridization because of the single nucleotide mismatch (in the case of the parental viruses DEN viruses) or possibly more than one mismatch (as may occur in other wild-type DEN viruses) and will not result in significant fluorescence. The DNA polymerase is more likely to displace a mismatched probe from the RT/PCR amplicon template than to cleave the mismatched probe to release the reporter dye (TaqMan Allelic Discrimination assay, Applied Biosystems).

[00077] One strategy for diagnostic genetic testing makes use of molecular beacons. The molecular beacon strategy also utilizes primers for RT/PCR amplification of amplicons, and detection of a specific sequence within the amplicon by a probe containing reporter and quencher dyes at the probe termini. In this assay, the probe forms a stem-loop structure. The molecular beacons assay employs quencher and reporter dyes that differ from those used in the TaqMan assay.

Pharmaceutical Compositions

[00078] Embodiments herein provide for administration of compositions to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo*. By "biologically compatible form suitable for administration in vivo" is meant a form of the active agent (e.g. pharmaceutical chemical, protein, gene, of the embodiments) to be administered in which any toxic effects are outweighed by the therapeutic effects of the active agent. Administration of a therapeutically active amount of the therapeutic compositions is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a compound may vary according to factors such as the disease state, age, sex, and weight of the

individual, and the ability of antibody to elicit a desired response in the individual. Dosage regima may be adjusted to provide the optimum therapeutic response.

[00079] In one embodiment, the compound (e.g. pharmaceutical chemical, protein, peptide etc. of the embodiments) may be administered in a convenient manner, for example, subcutaneous, intravenous, by oral administration, inhalation, intradermal, transdermal application, intravaginal application, topical application, intranasal or rectal administration. Depending on the route of administration, the active compound may be contained in a protective buffer (e.g. FTA, F127/trehalose/albumin). In one embodiment, a composition may be orally administered. In another embodiment, the composition may be administered intravenously. In one embodiment, the composition may be administered intranasally, such as inhalation. In yet another embodiment, the composition may be administered intradermally using a needle-free system (e.g. Pharmajet®) or other intradermal administration system.

[00080] A composition may be administered to a subject in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. The term "pharmaceutically acceptable carrier" as used herein is intended to include diluents such as saline and aqueous buffer solutions. It may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. The active agent may also be administered parenterally, or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms or other stabilizing formulation (e.g. FTA).

[00081] Pharmaceutical compositions suitable for injectable use may be administered by means known in the art. For example, sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion may be used. In all cases, the composition can be sterile and can be fluid to the extent that easy syringability exists. It might be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms such as bacteria and fungi. The pharmaceutically acceptable carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of

dispersion and by the use of surfactants. Prevention of microorganisms can be achieved by heating, exposing the agent to detergent, irradiation or adding various antibacterial or antifungal agents.

[00082] Sterile injectable solutions can be prepared by incorporating active compound (e.g. a compound that induces an immune response to one or more dengue virus serotypes) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

[00083] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above. It is contemplated that compositions are especially suitable for intramuscular, subcutaneous, intradermal, intranasal and intraperitoneal administration. A particular ratio may be sought such as a 1:1, 1:2 or other ratio (e.g. PFUs of a given dengue virus serotype)

[00084] The active therapeutic agents may be formulated within a mixture predetermined ratios. Single dose or multiple doses can also be administered on an appropriate schedule for a given situation (e.g. prior to travel, outbreak of dengue fever).

[00085] In another embodiment, nasal solutions or sprays, aerosols or inhalants may be used to deliver the compound of interest. Additional formulations that are suitable for other modes of administration include suppositories and pessaries.

[00086] Certain formulations can include excipients, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like.

[00087] A pharmaceutical composition may be prepared with carriers that protect active ingredients against rapid elimination from the body, such as time-release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, microencapsulated delivery systems, and biodegradable, biocompatible polymers, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others are known.

[00088] Pharmaceutical compositions are administered in an amount, and with a frequency, that is effective to inhibit or alleviate side effects of a transplant and/or to reduce or prevent rejection. The precise dosage and duration of treatment may be determined empirically using known testing protocols or by testing the compositions in model systems known in the art and extrapolating therefrom. Dosages may also vary with the severity of

the condition. A pharmaceutical composition is generally formulated and administered to exert a therapeutically useful effect while minimizing undesirable side effects. In general, dose ranges from about 10^2 to 10^6 PFU can be administered initially and optionally, followed by a second administration within 30 days or up to 180 days later, as needed. In certain embodiments, a subject can receive dual administration of a mono, bi-, tri or tetravalent composition disclosed herein wherein the composition is a single composition mixture or has predetermined compositions of different dengue virus serotypes. In some embodiments, a DEN2/4 chimera can be present in higher concentrations than other dengue virus serotypes such as a live, attenuated dengue-1.

[00089] It will be apparent that, for any particular subject, specific dosage regimens may be adjusted over time according to the individual need.

[00090] In one embodiment, a composition disclosed herein can be administered to a subject subcutaneously or intradermally.

[00091] The pharmaceutical compositions containing live, attenuated dengue viruses may be administered to individuals, particularly humans, for example by subcutaneously, intramuscularly, intranasally, orally, topically, transdermally, parenterally, gastrointestinally, transbronchially and transalveolarly. Topical administration is accomplished via a topically applied cream, gel, rinse, etc. containing therapeutically effective amounts of inhibitors of serine proteases. Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the inhibitors of serine proteases to penetrate the skin and enter the blood stream. In addition, osmotic pumps may be used for administration. The necessary dosage will vary with the particular condition being treated, method of administration and rate of clearance of the molecule from the body.

[00092] In certain embodiments of the methods of the present invention, the subject may be a mammal such as a human or a veterinary and/or a domesticated animal or livestock or wild animal.

Therapeutic Methods

[00093] In one embodiment of the present invention, methods provide for inducing an immune response to dengue virus serotype(s) using a mono, bi-, tri or tetravalent formulation of live, attenuated and/or chimeric viral constructs contemplated herein.

[00094] Embodiments of the present invention is further illustrated by the following non-limiting examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading

the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention or the scope of the appended claims.

EXAMPLES

[00095] The following examples are included to demonstrate certain embodiments presented herein. It should be appreciated by those of skill in the art that the techniques disclosed in the Examples which follow represent techniques discovered to function well in the practices disclosed herein, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in particular embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope herein.

Example 1

[00096] In some exemplary methods, compositions used to generate as referred to herein as "master virus seeds (MVS)" are disclosed. These compositions may be derived from one or more live, attenuated dengue viruses, such as DEN-1, DEN-2, DEN-3, and DEN-4. In certain methods, compositions may be derived from one or more live attenuated Dengue viruses that include but are not limited to, specific constructs disclosed herein referred to as DENVax-1, DENVax-2, DENVax-3, and DENVax-4. In other exemplary methods, strategies used to generate and characterize these compositions are provided. In yet other embodiments, tetravalent dengue virus formulations and genetic and phenotypic characterization of these formulations are provided.

Production and analysis of pre-master DENVax viruses

[00097] Certain procedures were performed to generate pre-master dengue virus seeds, such as serial amplification and purification of dengue viruses (e.g. DENVax). First, DENVax viruses were re-derived by transfection of viral RNA transcribed from the full-length recombinant DENVax cDNA into production-certified cells (e.g. Vero cells), resulting in P1 (passage 1) virus seed. The four P1 viruses from each of dengue-1 to dengue-4 were then amplified and plaque purified to obtain the candidate pre-master vaccine P7 seeds (see Table 1). Certain tests were performed to analyze passages of dengue viruses. For example, full-length genome sequencing demonstrated that all four of the P2 (passage 2) seed viruses were genetically identical to their homologous progenitor, research-derived, research-grade candidate vaccine virus. The original plaque phenotypes were also retained in the P2 viruses. Six plaque purified viruses (P3 A-F) were isolated for each serotype of dengue virus (e.g. DENVax1-4) from the P2 seeds, and each isolated plaque was directly plaque purified two more times. The third plaque purification (P5) of

each virus was amplified twice (P6 A-F and P7 A-F) in Vero cells to produce the potential pre-master P7 DENVax seeds (Table 1).

Table 1 Example of a cGMP Rederivation of DENVax Viruses in WCB-Vero Cells

Passage	Seed Production/Purification	Characterizations
P1	Transfect WCB-Vero with transcribed viral RNAs	Plaque titrate
P2	Amplify P1 virus	Full genome sequence
P3	Pick 6 plaques (A-F)/serotype from P2 plaque assay	Plaque purification
P4	Pick plaques A-F from P3 plaque assay	Plaque purification
P5	Pick plaques A-F from P4 plaque assay	Plaque purification
P6	Amplify P5 A-F plaques	Plaque titrate
P7	Pre-master seeds: Amplify P6 A-F	Full genome sequence, TaqMAMA, Plaque phenotypes
P8*	MVS: Amplify selected P7 virus seed	Full genetic and phenotypic characterization
P9	WVS: Amplify P8 Master Seed viruses	Full genome sequence, TaqMAMA
P10	BVS: Amplify P9 Working Seed viruses	Full genome sequence, TaqMAMA
* One optimal P7 seed (A, B, C, D, E, or F) was selected based on the genetic and plaque analysis to make P8 MVS		

[00098] Some tests were further performed to characterize P7 DENVax seeds, such as analysis of genome sequences and plaque phenotypes of the P7 seeds, and comparison with P2 seeds (Table 2). Plaque phenotypes of the P7 viruses were generally similar to those of the P2 seeds. In some exemplary experiments, virus titers were monitored. Virus titers reached over 6.0 log pfu/ml for most of the P7 seeds, except for 5 viruses. Genome sequencing of more than 60 candidate vaccine virus seeds after 10 or more serial passages in Vero cells identified no reversion event at NS1-53 and NS3-250 of the three major attenuation determinants of the DENV-2 PDK-53 genetic vector, suggesting that these 2 loci are quite stable in candidate vaccine virus seeds. All sequence chromatograms of the 24 candidate strains generated from both forward and reverse sequencing for these two sites were homogenous without any minor nucleotide populations evident at the NS1-53 and NS3-250 genetic loci. In contrast to the NS1 and NS3 sites, different levels of reversions at the 5'NCR-57 attenuation locus were identified from multiple serially passaged research grade vaccine viruses, suggesting this locus might not be as stable as NS1 and NS3 after multiple passages in cell culture. Therefore, a sensitive mismatch amplification assay

(TaqMAMA) was developed to accurately measure the reversion rate at the 5'NCR-57 locus by real-time RT-PCR. In some studies, the 5'NCR-57 reversion rates of all 24 of the P7 seeds were measured by the TaqMAMA. Depending on the concentration of the input viral RNA for each virus in the assay, the sensitivity limit of the TaqMAMA ranged between 0.01% and 0.07% reversion, which is much more sensitive than the 10-30% reversion sensitivity limit detectable by consensus genome sequence analysis. The resulting data illustrates that 15 of the 24 P7 viruses had minimal or undetectable reversion (< 0.07%), one virus (DENVax-3-D) had almost 100% reversion, and 8 viruses (1 DENVax-1, 1 DENVax-2, 2 DENVax-3, and 4 DENVax-4) had partial reversion ranging from 0.08% to 12.85% (Table 2). Full-length genome sequencing was conducted for 16 of the 24 P7 viruses with low levels of 5'NCR57 reversion as measured by TaqMAMA. All the sequenced viruses maintained the other two DENVax attenuation determinants (NS1-53, NS3-250), and all had acquired additional mutations that were not present in the original, engineered recombinant cDNA clones (Table 2). In one exemplary target vaccine composition, DENVax-1-A, DENVax-2-F, DENVax-3-F, and DENVax-4-F were selected as target pre-master seed for each serotype because their genotypes and plaque phenotypes most closely resembled those of the originally designed vaccine recombinants. The DENVax-1-A, DENVax-2-F, and DENVax-4-F had two non-synonymous mutations, and the DENVax-3-F had one. The evidence suggests these additional mutations observed in these 4 pre-master seeds do not cause safety concerns or immunogenicity alterations for the viruses. These pre-master seeds were further amplified to generate the MVS (master seed, designated as P7, Table 1).

[00099] Exemplary methods provided herein used purified in-vitro transcribed viral RNA from cloned cDNA plasmid as the pure source to transfect vaccine-certified Vero cells to generate vaccine virus. Serial plaque purifications and full-genome sequence analyses were incorporated into the manufacturing procedures to ensure manufactured vaccine seeds with optimal purity and genetic stability. Six cloned viruses were prepared as potential pre-master seeds for each serotype of DENVax. Through genomic analysis, including TaqMAMA and complete genomic sequencing, as well as characterization of viral plaque phenotypes, pre-master seeds were chosen to advance to master virus seeds production for each serotype (serotypes 1-4). The selected pre-master seeds had undetectable reversions (<0.01% or <0.07%) at the 5'NCR-57 locus, with 1 or 2 amino acid substitutions in their genomes, and retained the small plaque phenotypes previously observed.

Table 2. Characterizations of pre-master (P7) seeds

Virus	Clone[a]	TaqMAMA[b]	Log_{10} pfu/ml	Plaque[c]	Mutations identified in genome[d]
DENVax-1	**A**	**	6.85	P2	NS2A-116 I-L, NS2B-92 E-D, one silent
	B	*	6.93	P2	nd[e]
	C	*	6.93	D	nd
	D	**	7.02	D	C-67 K-A; one silent
	E	0.57%	7.28	P2	nd
	F	**	7.18	P2	E473 T-M; one silent
DENVax-2	A	0.03%	6.33	P2	NS1-341 K-N
	B	*	6.33	P2	E-305 K-T, two silent
	C	*	5.84	L	NS4A-18 T-A, four silent
	D	0.08%	6.20	P2	NS2B-99 I-L, one 3'NCR
	E	0.03%	6.31	P2	prM-52 K-E, NS5-412 I-V, two silent
	F	**	6.15	P2	prM-52 K-E, NS5-412 I-V
DENVax-3	A	*	6.00	P2	NS5-200 K-N, one silent, one 3'NCR
	B	0.05%	6.27	P2	NS2A-33 I-T, NS2A-59 M-T
	C	0.30%	6.25	P2	nd
	D	100.00%	6.27	P2	nd
	E	0.31%	6.00	P2	nd
	F	**	6.30	P2	E-223 T-S, one silent
DENVax-4	A	0.47%	5.60	P2	E323 K-R/K, NS2B-21 L-F/L, NS2B-39 T-S, one silent
	B	*	5.65	D	NS2A-126 A-V; NS4A-5 N-D;NS5-383 K-R, one silent
	C	4.50%	5.90	P2	nd
	D	12.85%	5.97	D	nd
	E	0.52%	6.85	S	prM-85 E-D, NS2B-45 T-A, NS5-320 M-T, NS5-551 E-G, two silent
	F	0.02%	6.93	S	NS2A-66 D-G, NS4A-21 A-V, four silent

[a] Cloned viruses (by serial plaque purifications) selected for further development of MVS are designated bold.
[b] *: Reversion rate < 0.07% (detection limit). **: Reversion rate < 0.01% (detection limit)
[c] Plaque phenotypes: P2: similar to P2 virus; L = larger than P2 virus, D = similar size, but appear somewhat different in clearness of the plaques; S = smaller than P2.
[d] Substitutions differing from the engineered DENVax cDNA clones. Amino acid mutations are listed with residue position of the virus protein and the changes (wt-mutation). Total number of silent mutations in structural and non-structural genes of each seed is listed. Mutations at non-coding region (NCR) are also noted.
[e] nd = Not done. These clones had higher 5'NCR-57 reversion rates (by TaqMAMA) than other clones, so were excluded from further sequence analysis.

Example 2

[000100] In some exemplary methods, compositions of master virus seeds, working virus seeds and bulk virus seeds as well as their genetic and phenotypic characterization are described. These compositions are provided for manufacture of clinical materials and ultimately commercial vaccine supplies. Serial plaque purifications and full-genome sequence analyses were incorporated into the manufacturing process to ensure compositions of vaccine seeds with optimal safety and genetic stability for manufacture of clinical trial materials.

Production and manufacturing quality controls for MVS, WVS, and BVS

[000101] In some studies, MVS of the 4 DENVax were produced by amplifying the pre-master P7 seed in certified Vero cells. In other studies, MVS were used to make large amount of WVS in cell factories. Further, the BVS stocks of DENVax were amplified from the WVS and were formulated into tetravalent drug product mixtures to be used used for human clinic trials. Quality controls for product release were performed in some exemplary methods, including, but not limited to, testing all of the MVS, WVS, and BVS for identity, infectious titer, sterility, mycoplasma, and in vitro and in vivo adventitious agents. All seeds passed the virus identity test using serotype-specific RT-PCR assays, which showed positive amplification corresponding to its serotype and negative for heterologous serotypes (data not shown). No detectable mycoplasma or adventitious agents were detected in the MVS, WVS, or BVS stocks.

Genetic analysis of the MVS, WVS, and BVS

[000102] In certain exemplary methods, after generation of MVS from the selected pre-MVS (P7) strains selected above were produced and the respective viral RNA was sequenced again. Full-length genome sequencing revealed that the MVS for DENVax-1 was identical to its pre-master seed, while the WVS and subsequent BVS acquired 2 additional substitutions at E-483 and NS4B-108 (see Tables 2 and 3). The Ala substitution at E-483 represented part of the genotype in the MVS, but became the dominant genotype in BVS. DENVax-2 and DENVax-3 were identical to their respective pre-master seeds (Table 2 and 3). The DENVax-2 MVS was identical to its pre-master seed, and the WVS and BVS had 2 additional mutations at NS4A-36 and NS4B-111. Both mutations were partial in WVS and were the major genotype in the BVS. The MVS of DENVax-3 was again identical to the pre-master seed, but the WVS and BVS contained an additional aa substitution at NS4A-23. The DENVax-4 MVS acquired an additional amino acid mutation, at locus NS2A-99 (from Lys to Lys/Arg mixed genotype) during production of the MVS (**Table 3**). Its WVS and BVS retained the NS2A-99 Lys/Arg mixed genotype, and the BVS

had an extra NS4B-238 Ser/Phe mixed genotype. Consensus sequence results also confirmed that MVS, WVS as well as BV retained the three genetic determinants of attenuation at the 5'NCR-57, NS1-53, and NS3-250 loci. Analysis of the least stable attenuating locus by TaqMAMA demonstrated that the 5'NCR-57 reversion rate between <0.7% to and 0.13% among MVS, ≤0.07% among WVS, and between <0.07 and 0.21% among BVS. A 3% reversion at the 5'NCR-57 locus was considered the maximum permissible rate for acceptance of a vaccine lot (**Table 3**).

Table 3. Nucleotide and amino acid substitutions in DENVax seeds

DENVax	Nucleotides	Amino Acids	Pre-master	MVS[a]	WVS[a]	BVS[a]
DENVax-1	2384 G-C	E-483 Gly-Ala	-	-	**Gly/Ala**	**Ala**
	3823 A-C	NS2A-116 Ile-Leu	Leu	Leu	Leu	Leu
	4407 A-T	NS2B-92 Glu-Asp	Asp	Asp	Asp	Asp
	7148 C-T	NS4B-108 Thr-Ile	-	-	**Ile**	**Ile**
	7311 A-G	silent	G	G	G	G
	TaqMAMA 5'NCR-57 reversion %[b]		--	-	-	-
DENVax-2	592 A-G	prM-52 Lys-Glu	Glu	Glu	Glu	Glu
	6481 G-C	NS4A-36 Ala-Pro	-	-	**Ala/Pro**	**Pro**
	7156 C-T	NS4B-111 Leu-Phe	-	-	**Leu/Phe**	**Phe**
	8803 A-G	NS5-412 Ile-Val	Val	Val	Val	Val
	TaqMAMA 5'NCR-57 reversion %[b]		--	-	0.07%	0.21%
DENVax-3	1603 A-T	E-223 Thr-Ser	Ser	Ser	Ser	Ser
	6436 G-A	NS4A-23 Asp-Asn	-	-	**Asn**	**Asn**
	7620 A-G	silent	G	G	G	G
	TaqMAMA 5'NCR-57 reversion %[b]		--	-	-	-
DENVax-4	225 A-T	silent	T	T	T	T
	3674 A-G	NS2A-66 Asp-Gly	Gly	Gly	Gly	Gly
	3773 A-A/G	NS2A-99 Lys-Lys/Arg	-	**Lys/Arg**	**Lys/Arg**	**Lys/Arg**
	5391 C-T	silent	T	T	T	T
	6437 C-T	NS4A-21 Ala-Val	Val	Val	Val	Val
	7026 T-C	silent	T/C	T/C	T/C	T/C
	7538 C-C/T	NS4B-238 Ser-Ser/Phe	-	-	**Ser/Phe**	**Ser/Phe**
	9750 A-C	silent	C	C	C	C
	TaqMAMA 5'NCR-57 reversion %[b]		-	0.13%	-	-

[a] Bold: Changes started at MVS stocks.

[b] "--" indicates reversion rate < 0.01% (detection limit), "-" indicates reversion rate <0.07% (detection limit)

[000103] Full-genome sequence analysis revealed that an additional amino acid mutation developed in the DENVax-4 MVS, while the other three DENVax MVS lots retained the consensus genome sequence of their pre-master seeds. Overall, from deriving of the P1 seeds to the pre-master (P7) seeds, only 1 or 2 non-synonymous mutations occurred in a given seed. From P1 to MVS (P8) seeds, 2 to7 nucleotide substitutions were identified in any given DENVax seed and only 2 to 3 of these substitutions resulted in amino acid changes. Thus, minor changes occurred. RNA viruses are error-prone in their

genome replication, so genetic substitutions in flavivirus genome during cell passages are not unexpected. None of the silent mutations in the MVS were within the 5' or 3'NCR that may affect virus replication. Only the change in prM-52 Lys-Glu of the DENVax-2, and the substitution in NS2A-66 Asp-Gly of DENVax-4 are not conservative changes. The NS2A-66 mutation of the DENVax-4 is in the nonstructural backbone part of the DENV-2 PDK-53. Although NS2A-66 locus is usually Asp among various strains of DENV-2, it is usually Gly for DENV-4. It is possible that the Asp to Gly change in the DENVax-4 is relevant for fitness of the DENVax-4 in Vero cells. The DENVax-2 prM-52 mutation resides in the C-terminal portion of the prM that is cleaved out from the mature virus particles. In some exemplary methods, phenotypic characterization was performed to confirm that none of the mutations in the MVS seeds significantly altered the attenuation phenotypes of the vaccine.

[000104] The DENVax viruses demonstrated high genetic stability during the manufacturing process. The three defined DENV-2 PDK-53 attenuation loci located in 5'NCR, NS1-53, and NS3-250 remained stable in the consensus genome sequence upon serial passage of the DENVax from pre-Master strains to bulk vaccine preparations. The highly sensitive TaqMAMA of the 5'NCR-57 locus demonstrated minimal or undetectable reversion in the MVS, WVS (P9/Working), and BVS (Bulk Virus Seed for vaccines) of dengue virus serotypes. The 5'NCR-57 reversion rates of the DENVax BVS preparations (P10-equivalent) were significantly lower than the 5'NCR-57 reversion rates that evolved in research-grade vaccine candidates after 10-serial passages in Vero cells (4-74% reversion). The strategy for large-scale manufacturing of the DENVax seeds provided herein resulted in a genetically stable vaccine seed which retained the attenuation markers in the candidate vaccine viruses.

Plaque phenotype of DENVax MVS

[000105] In one exemplary method, plaque phenotypes of the DENVax MVS were compared with wild type Dengue viruses and their homologous research-grade chimeric viruses in Vero cells (Fig. 2). All of the MVS of DENVax-1, -2, and -3 produced plaques that were significantly smaller than their wild type homologs and very similar (within 0.4-mm differences) to their homologous research-grade viruses in Vero cells. DENVax-4 MVS was also significantly smaller than the wild type DENV-4, but was slightly larger (0.9 mm difference) than the original lab derived D2/4-V chimera.

[000106] Fig. 2 represents an exemplary histogram illustrating plaque sizes of the DENVax MVS in contrast with control wild type viruses and research-grade vaccine

candidate viruses. Mean plaque diameters (mm) ± SD (error bars) of the virus plaques in Vero cells under agarose overlay measured on day 9 pi. The wild type DEN viruses, represented by black bars, and previously published research-grade vaccine candidate viruses, represented by white bars, were included for control and comparison to the DENVax master vaccine seeds represented by grey bars.

Temperature sensitivity of DENVax MVS

[000107] In another exemplary method, temperature sensitivity was tested in Vero cells for the DENVax MVS and compared with their homologous wild type and the original research-grade chimeric vaccine virus. The wild type (wt) DENV-3 16562 was not temperature sensitive. The wt dengue virus serotype 1 and dengue virus serotype-4 were moderately temperature sensitive at 39°C (titers were approximately 1.0 $\log_{10}$ pfu/ml lower at 39°C than at 37°C, Fig. 3). Wt Dengue virus serotype-2 16681 was the most temperature sensitive of the wt Dengue viruses tested, and resulted in a 100-fold titer drop at 39°C. DENVax-1, -2, and -3 were as temperature sensitive as their original homologous research-grade chimeric vaccine viruses (Fig. 2). Titers at 39°C dropped between 2.0 and 3.0 $\log_{10}$ pfu/ml for these DENVax strains. DENVax-4 also was temperature sensitive, demonstrating a 5-fold reduction in titer. However, the original research-grade D2/4-V demonstrated about a 10-fold reduction in titer. The final stabilized DENVax-4 MVS contained F127 (and other agents known to stabilize these formulations (FTA)), which was shown to enhance thermal stability of the Dengue viruses. The presence of the F127 in DENVax-4 MVS likely contributed to the less pronounced temperature sensitivity of the virus in the Vero culture assay. In a separate experiment, temperature sensitivity of an MSV-derived DENVax-4 strain in the absence of F127 was further evaluated. To remove the F127 from the strain, viral RNA was isolated from a DENVax-4 bulk virus preparation and was transfected into Vero cells. This DENVax-4 virus appeared to be as temperature sensitive as the D2/4 V research strain (titer reduced 1.5 $\log_{10}$ pfu/ml) on day 3 pi in the absence of F127 (Fig. 3).

[000108] Fig. 3 illustrates an exemplary histogram illustrating temperature sensitivities of DENVax MVS. The wild type Dengue viruses and previously published research-grade vaccine candidate viruses were included for comparison. The DENVax-4 MVS contains additional F-127 that can mask the temperature sensitivity results of the virus in this assay. A separate experiment analyzing a surrogate DENVax-4 in the absence of F127 was also included. Mean titers ± SD (error bars) of the viruses replicated in Vero cells at 37°C or

39°C.

DENVax MVS replication in mosquito C6/36 cells

[000109] In some exemplary methods, the DENVax MVS were grown in C6/36 cells to verify their retention of the *in vitro* attenuation phenotype, with the knowledge that the research-grade chimeric vaccine viruses retained the attenuation phenotype of the backbone DENV-2 PDK53 virus in these mosquito cells. Compared to the wt Dengue viruses, DENVax-1, DENVax-2 and DENVax-4 MVS showed significant growth reduction (at least 3 $\log_{10}$ pfu/ml reduction) in C6/36 cells on day 6 pi (Fig. 4). The DENVax-3 MSV also exhibited reduced growth compared to the wt DENV-3 16562, but the reduction was not as marked (1-2 $\log_{10}$ pfu/ml reduction). However, the C6/36 titers of the DENVax-3 seed lots were similar (within 1 $\log_{10}$ pfu/ml difference) to the C6/36 titer of the original research-grade chimeric D2/3-V vaccine virus.

[000110] Fig. 4 illustrates an exemplary histogram plotting restricted growth of DENVax MVS (grey bars) in C6/36 cells in comparison with wt Dengue viruses (black bars) and research-grade vaccine viruses (white bars). Mean titers ± SD (error bars) of the viruses replicated in C6/36 cells 6 days pi.

Virus infection, dissemination, and transmission rates in whole mosquitoes

[000111] In some exemplary methods, the infection and dissemination rates of the DENVax were compared with their parental wt Dengue viruses. In certain exemplary experiments, oral infection experiments were conducted in *Ae. aegypti* mosquitoes. Infectious blood meals were back-titrated to measure the virus titers and only the experiments with similar virus titers in the blood meal (less than 1 $\log_{10}$ pfu/ml differences) between parental Dengue viruses and DENVax for each serotype were included for comparisons in Table 4. DENVax-1, DENVax-2, and research-grade D2 PDK-53-VV45R did not infect mosquitoes through oral feeding, which is significantly different ($p < 0.0001$) from their parental viruses, DENV-1 16007 (44% infection) and DENV-2 16681 (43.3% infection). Because no mosquito was infected by DENVax-1 and -2, there was little to no dissemination concern for these two vaccine viruses. While DENVax-4 did infect some mosquitoes through oral feeding (2 out of 55), the infection rate was significantly lower ($p < 0.05$) than its parental wt virus, DENV-4 1036 (8 out of 50). DENVax-3 did not infect any mosquitoes in two experiments with blood meal viral titers of 5.2±0.02 $\log_{10}$ pfu/ml (Table 4), and in a separate experiment with blood meal viral titer of 6.0 $\log_{10}$ pfu/ml, only 1 out of 30 mosquitoes became infected (data not shown). However, wt Dengue virus-3 16562 also

had a very low infection rate (8%) at 5.2 $\log_{10}$ pfu/ml, and the rate did not increase in a separate experiment with a higher blood meal viral titer at 6.2 $\log_{10}$ pfu/ml (3%, 1 positive out of 30 mosquitoes, data not shown). Although the wild type (wt) Dengue virus-3 and Dengue virus-4 had significantly lower infection rates than the wt Dengue virus-1 and Dengue virus-2, the mean virus titers in the infected mosquitoes were similar (3.1 to 3.9 $\log_{10}$ pfu/mosquito). In contrast, the DENVax-4 titers from the two infected mosquitoes were both minimal (0.7 $\log_{10}$ pfu/mosquito), which was 1,000-fold lower than the titer from the mosquitoes infected by wt Dengue virus serotype-4 1036 (3.9 ± 1.5 pfu/mosquito).

[000112] For those mosquitoes that were infected, dissemination out of the midgut could be assessed by determining whether virus was present in the legs. The four parental DENVs resulted in dissemination rates ranging between 36.3% and 62.5%, and their mean virus titers (in $\log_{10}$ pfu) from the legs were between 0.9±0.3 and 2.2±0.7 (excluding negative samples). Neither of the two DENVax-4 infected mosquitoes resulted in virus dissemination to the legs (Table 4). While disseminated virus was detectable in the legs, none of the four wt Dengue viruses was detectable in saliva of orally infected mosquitoes, suggesting that oral feeding conditions may not be sufficiently sensitive to measure the transmission rate of these DENVs. Therefore, in other exemplary methods, highly stringent artificial mosquito infections by direct IT inoculation were subsequently performed (Table 4). Except for DENVax-4, all viruses (wt and DENVax) achieved 100% infection of the IT inoculated *Ae. aegypti*. The DENVax-4 inoculum had a slightly lower viral titer than the other three viral inocula, but it still successfully infected 70% of the inoculated mosquitoes. Despite the high body infection rates achieved by IT inoculation, all four DENVax viruses exhibited significantly lower ($p < 0.005$) or non-detectable transmission rates (0-10%) compared to the wt Dengue viruses (43-87%, Table 4). The DENVax viruses demonstrated little to no infection and dissemination after oral feeding, and the highly stringent IT results affirmed the minimal transmission capacity of these DENVax viruses in *Ae. aegypti*.

Table 4: Virus infection, dissemination, and transmission rates in whole mosquitoes

Virus	Oral Feed					IT inoculation				
	Blood Meal[a]	Infection[b]	Body Titer[c]		Dissemination[e]	Inoculum	Infection[b]	Body Titer[c]	Saliva[f]	
	Mean± SD	% (P/N)	Mean± SD	p^d	% (P/N)[f]	pfu/dose	% (P/N)	Mean± SD	% (P/N)	p^d

DENV-1 16007	6.6	44.0% (11/25)	3.6 ± 1.5		36.3% (4/11)	53.9	100% (30/30)	4.7 ± 0.48	43% (13/30)	
DENVax-1	6.9	0% (0/30)	NA	<0.0001	NA	67.8	100% (30/30)	3.4 ± 0.39	10% (3/30)	<0.005
DENV-2 16681	6.6	43.3% (13/30)	3.1 ± 1.5		38.5% (5/13)	67.8	100% (30/30)	5.2 ± 0.34	87% (26/30)	
D2 PDK53-VV45R	6.4	0% (0/30)	NA	<0.0001	NA	56.4	100% (30/30)	4.0 ± 0.20	0% (0/30)	<0.0001
DENVax-2	6.4	0% (0/30)	NA	<0.0001	NA	52.7	100% (30/30)	3.5 ± 0.27	7% (2/30)	<0.0001
DENV-3 16562	5.2	8% (2/25)	3.8 ± 0.2		50% (1/23)	34.0	100% (30/30)	4.2 ± 0.50	67% (20/30)	
DENVax-3	5.2 ± 0.02	0% (0/50)	NA	0.108	NA	37.3	100% (30/30)	3.3 ± 0.36	3% (1/30)	<0.0001
DENV-4 1036	5.8 ± 0.5	16% (8/50)	3.9 ± 1.5		62.5% (5/8)	69.4	100% (30/30)	5.2 ± 0.45	70% (21/30)	
DENVax-4	5.4 ⊥ 0.4	3.6% (2/55)	0.7 ± 0.0	0.033	0% (0/2)	11.8	70% (21/30)	1.1 ± 0.46	0% (0/21)	<0.0001

[a] Virus titers or Mean±standard deviation if from more than 1 experiment in blood meal (log_{10} pfu/ml) by back titration
[b] Rate of virus detected in mosquito bodies. *P/N* = positive/total mosquitoes
[c] Mean virus titers ⊥ standard deviation (log_{10} pfu/mosquito) in mosquito body, only positive sample are included for calculation
[d] Statistic analysis of the differences between wt DENV and DENVax by Fisher Exact probability
[e] Rate of virus detected in legs of the positively infected mosquitoes
[f] Rate of virus detected in saliva of the positively infected mosquitoes. Used to measure transmission efficiency

[000113] Vector competence is an important safety component for live-attenuated flavivirus vaccine viruses. Previously, the research-grade DENV-2 PDK-53-VV45R virus and wt derivatives were tested in *Ae. aegypti,* and found that the NS1-53-Asp attenuating mutation was the dominant determinant for impaired mosquito replication. The other two major attenuation loci of the DENV-2 PDK-53 vaccine, nucleotide 5'NCR-57-T and NS3-250-Val, also exhibited some inhibiting effect on replication in mosquitoes, thus providing additional, redundant restrictions for mosquito vector competence. Some exemplary methods described herein were used to test the mosquito oral and IT infection and replication for all four DENVax strains. DENVax-1, -2, and -3 did not infect any *Ae. aegypti* mosquitoes through oral infection (Table 4). The DENVax-4 infected only 3.6% of orally exposed mosquitoes, a level significantly lower than that of the wt DENV-4 with a replicative mean titer in the mosquito bodies lower than that of wt DENV-4 infected mosquitoes. Surprisingly, DENVax-4 was detected in the legs of the infected mosquitoes, suggesting that DENVax-4 was not able to disseminate from the mosquito midgut following oral infection. The infection rates for the DENVax-1, -2, and -4 were all significantly less than their wt counterparts, but the difference was not significant between DENVax-3 and wt DENV-3 16562 due to the very low infection rates for both viruses. Compared to other wt strains of DENV assessed in *Ae. aegypti* collected from the same Mae Sot Province, Thailand, the parental wt Dengue virus strains used for engineering DENVax appeared to have lower infectious and dissemination rates by oral infection. The wt DENV-1 PUO359,

DENV-2 PUO218, DENV-3 PaH881/88, and DENV-4 1288 used for engineering the Yellow Fever (YF) 17D vaccine-based ChimeriVax-DEN vaccines had infection rates ranging 47-77%. In contrast, the YF 17D vaccine cannot infect *Ae. aegypti*. Although the ChimeriVax strains contained the prM-E from these highly infectious wt DENV, the ChimeriVax retain the mosquito attenuation phenotype of their YF 17D replicative backbone. Results provided herein also indicated that the mosquito attenuation of DENV-2 PDK-53 backbone was maintained in the DENVax strains. In addition, using the wt Dengue virus strains with lower mosquito-infectivity in constructs included in compositions described herein provides an additional safety feature.

[000114] The oral infection results illustrate that the DENVax had minimum mosquito infectivity and dissemination capacity. In addition, the more sensitive and stringent IT infection experiments were performed to further analyze the potential of DENVax to be transmitted by *Ae. aegypti*. The IT results demonstrated that all four DENVax viruses had non-detectable or minimal mosquito transmission potential compared to their wt counterparts. DENVax transmission could only theoretically occur if (1) vector feeds on a vaccinee with a sufficient viremia titer to infect mosquito midgut, (2) the virus is capable of replicating in the midgut epithelium and able to subsequently disseminate out of the midgut, and (3) the disseminated virus can replicate in salivary gland and expectorate sufficient virus in saliva for transmission. The threshold of human viremia required to infect mosquitoes has not been established adequately, but human viremia can be 10^5-10^8 mosquito infectious dose$_{50}$ (MID_{50})/ml after natural wt DENV infection. This MID_{50} was based on direct IT inoculation of mosquitoes with diluted human plasma. Analysis of DENVax in nonhuman primates indicated that viremia titers following DENVax immunization were very low (less than 2.4 $\log_{10}$ pfu/ml) and lasted for 2-7 days. Given the low viremia levels and the low mosquito infection, dissemination, and transmission capacity of DENVax, it is unlikely that these vaccine viruses could be transmitted by mosquitoes in nature or cause viremia.

[000115] Therefore, it is proposed that any of the passages of any of the serotypes (P1-P10) could be used in a composition to generate a safe and effective vaccine against one, two, three or all four dengue virus serotypes.

Neurovirulence in suckling mice

[000116] The original research-grade vaccine viruses were highly attenuated for neurovirulence in newborn ICR mice maintained in-house at DVBD/CDC. All of these mice survived ic (intracerebral) challenge with 10^4 pfu of each vaccine virus. The wt

Dengue virus serotype-2 16681 virus, on the other hand, resulted in 62.5% - 100% mortality in these CDC-ICR mice in various experiments. In some experiments, commercial ICR mice obtained from Taconic Labs (Taconic-ICR) were used to study neurovirulence in newborn mice. It was observed that newborn Taconic-ICR mice were significantly more susceptible to Dengue virus serotype-2 infection than the previous CDC-ICR mice. Fig. 5A summarizes the neurovirulence of wt Dengue virus serotype-2 16681 in CDC-ICR colony and Taconic-ICR newborn mice challenged ic with 10^4 pfu of the virus. The Taconic-ICR mice (100% mortality in 32 mice, average survival time of 8.3 ± 0.5 days) were more susceptible to ic Dengue virus serotype-2 16681 challenge than the previous CDC-ICR mice (91% fatalities in 72 mice, average survival time of 14.6 ± 2.3 days).

[000117] In other exemplary methods, in order to evaluate neurovirulence of the DENVax MVS, the Taconic-ICR mice initially were challenged ic (intracerebrally)with a dose of approximately 10^4 pfu of wt Dengue virus serotype-2 16681, D2 PDK-53 VV45R, D2/3-V, or DENVax 1-4 virus in one (n=16) or two (n=31-32) experiments (Fig. 5B). At this dose, D2/3-V research grade virus, as well as DENVax-1, and DENVax-3 MVS exhibited fully attenuated neurovirulence phenotypes (no illness or mortality). As expected, wt Dengue virus serotype-2 was found to be "fatal", with average mouse survival time (AST) of 8.3 ± 0.8 days. In these Dengue virus serotype-2-sensitive Taconic-ICR mice, the D2 PDK-53-VV45R research grade virus resulted in 81.3% mortality. The DENVax-2 MVS and DENVax-4 MVS were uniformly fatal in the Taconic-ICR, showing AST values of 9.8 ± 1.7, 10.2 ± 1.4, and 11.3 ± 0.4 days, respectively.

[000118] In some exemplary methods, the neurovirulence of wt Dengue virus serotype-2 16681 virus was compared with that of D2 PDK-53 VV45R, DENVax-2 MVS and DENVax-4 MVS, as well as D2/4-V research grade virus, at a 10-fold lower dose (10^3 pfu, Fig. 5C). The wt Dengue virus serotype-2 retained a uniformly fatal neurovirulent phenotype, with AST of 9.0 ± 1.4 days, at this lower challenge dose. The other 4 viruses exhibited intermediate neurovirulence phenotypes, and the degree of neurovirulence was serotype-specific. The D2 PDK-53-VV45R virus and its DENVax-2 MVS cognate showed significant attenuation (32.3% survival with AST of 13.1 ± 3.8 days and 31.2% survival with AST of 10.5 ± 3.4 days, respectively). Both the DENVax-4 MVS and the research grade D2/4-V virus were highly attenuated for neurovirulence (81.3% survival with AST of 18.8 ± 5.8 days and 100% survival, respectively). The results suggested that MVS of DENVax-1 and -3 exhibited complete attenuation of neurovirulence, while DENVax-2 and -4 MVS lots retained attenuation phenotypes that closely resembled their homologous

research-grade virus vaccine candidates.

[000119] Figs. 5A-5C represent exemplary graphs illustrating neurovirulence in newborn mice tested with various compositions including wt Dengue virus serotype-2 and different attenuated Dengue viruses. Pooled results of numerous experiments summarizing the neurovirulence of wt Dengue virus serotype-2 16681 virus in CDC-ICR (n=72) and Taconic-ICR (n=32) newborn mice challenged ic with 10^4 pfu of the virus (A). Neurovirulence of DENVax MVS tested in Taconic-ICR mice with a dose of 10^4 pfu (B) or 10^3 pfu (C). The numbers of animals tested per group in one experiment (n=16) or two pooled experiments (n=31 or 32) are indicated.

Plaque phenotype of WVS, and BVS

[000120] Certain studies were performed to compare plaque phenotypes of WVS and BVS with MVS, wt Dengue viruses and their homologous lab derived, research-grade chimeras in Vero cells (Fig. 6). Mean plaque sizes were calculated from 10 plaques for each vaccine virus, but from reduced numbers of wt DENV-1, -3, and -4. All of the MVS viruses of DENVax-1, -2, and -3 produced plaques that were significantly smaller than their wt homologs and very similar (within 0.4-mm differences) to their homologous research-grade viruses in Vero cells. DENVax-4 MVS was also significantly smaller than the wt DENV-4, but was slightly (0.9 mm) larger than the original lab derived D2/4-V chimera. With the exception of the DENVax-2, all of the WVS and BVS of the DENVax-1, -3, -4 retained significantly smaller plaque sizes than those produced from their wt homologs. The DENVax-2 WVS and BVS produced plaques that were similar to the plaques of wt DENV-2 virus in Vero cells, but when tested in LLC-MK_2 cells all of the DENVax-2 manufactured seeds produced plaques that were somewhat smaller than those of the wt DENV-2 (1.4 ± 0.4) and similar to the lab derived D2 PDK-53-VV45R (1.0 ± 0.3) (Fig. 6).

[000121] Evaluation of the phenotypic markers of viral attenuation, including small plaque phenotype, temperature sensitivity, reduced replication in mosquito cells, reduced infection/dissemination/transmission by mosquitoes, and reduced neurovirulence in newborn ICR mice, were assessed for the compositions of MVS stocks. Results indicated that all of the DENVax retained the expected attenuation phenotypes similar to the original research-grade vaccine viruses. Given the mutations responsible for attenuation are conserved in all MVS, WVS and BV, it can be expected the attenuated phenotypes to be retained in the material manufactured for human clinical testing.

[000122] Fig. 6 represents an exemplary histogram illustrating plaque size of the DENVax MVS, WVS, and BVS. Mean plaque diameters ± SD (error bars) of the virus

plaques in Vero or LLC-MK_2 cells under agarose overlay measured on day 9 pi. The wt DENVs and previously published research grade vaccine candidate viruses were included for control and comparison.

Virus replication in mosquito C6/36 cells

[000123] Previous studies demonstrated that the research-grade PDK-53-based chimeric vaccine viruses retained the attenuation phenotype of the backbone DENV-2 PDK53 virus in C6/36 cells. In some exemplary methods, the DENVax MSV, WVS, and BVS were grown in C6/36cells to verify their retention of this in vitro attenuation marker after large scale manufacturing. Compared to the wt Dengue viruses, except for DENVax-3, the manufactured seeds showed marked growth reduction (at least 3 $\log_{10}$ PFU/ml reduction) in C6/36 cells on day 6 pi (Fig. 7). The DENVax-3 seeds also exhibited reduced growth compared to the wt DENV 3 16562, but the reduction was not as marked (1-2 $\log_{10}$ PFU/ml reduction). However, the titers of the DENVax-3 seed lots were similar (within 1 $\log_{10}$ PFU/ml difference) to the original research-grade chimeric D2/3-V vaccine virus.

[000124] Fig. 8 represents an exemplary histogram plotting restricted growth of DENVax MVS, WVS, and BVS in C6/36 cells. Mean titers ± SD (error bars) of the viruses replicated in C6/36 cells 7 days pi. The wt Dengue viruses and previously published research grade vaccine candidate viruses were included for comparison.

Neurovirulence in suckling mice

[000125] Additional experiments were performed to analyze neurovirulence in newborn ICR mice. At an intracranial dose of 10^4 PFU, the survival rates for wt DENV-2 16681 and the D2 PDK-53-VV45R were 0% and 18.8%, respectively (Fig. 9A) in the ICR mice, but were about 20% for wt DENV-2 16681 and 100% for the D2 PDK-53-VV45R in the CDC ICR mice. In this study, DENVax-1 and DENVax-3 MVS were attenuated (100% survival) for the mice at a dose of 10^4 PFU, but the MVS of DENVax-2 and DENVax-4 caused 100% mortality at the dose of over 10^4 PFU (Fig. 5A). However, when tested at a dose of 10^3 PFU of virus, the DENVax-2 (31.3% survival) and DENVax-4 (81.3% survival) showed reduced neurovirulence relative to wt Dengue virus serotype-2 16681 (0% survival), and their survival rates were similar to those of the research grade vaccine candidates D2 PKD-53-VV45R (32.3%) and D2/4 V (100%), respectively (Fig. 9B). Although, wt DENV-1, -3, or -4 were not included for comparison in this study, previous work demonstrated that wt DENV-1 16007 was attenuated in the CDC-ICR mice by the ic route, while both wt DENV-3 16562 and DENV-4 1036 were highly virulent (0% survival) for the CDC-ICR mice. It is likely that these 3 wt DENV would exhibit similar or greater virulence in the

more susceptible Taconic ICR mice. Therefore, inclusion of these wt Dengue viruses for comparison with their homologous DENVax MVSs was considered to be uninformative. This study indicated that all 4 DENVax MVSs and original laboratory derived candidate vaccine viruses exhibit comparable mouse attenuation phenotypes relative to the wt DENV-2 16681.

[000126] Figs. 9A-9B represent exemplary graphs of data of neurovirulence of DENVax MVS in newborn ICR mice. (A) IC inoculations of the virus at dose of 10^4 PFU. (B) IC inoculation of the virus at dose of 10^3 PFU

[000127] All seed lots of the DENVax were tested for the identity, sterility, and freedom from undesirable agents. Full-genome sequence analysis revealed that one extra amino acid mutation evolved in the DENVax-4 MVS, while the other 3 DENVax MVSs retained the consensus genome sequence of their pre-master seeds. In WVS lots, the DENVax-3 acquired an extra amino acid mutation and the other 3 serotypes accumulated 2 extra amino acid substitutions, relative to their pre-master seeds. Genome sequences of all the 4 BVS lots were identical to their WVS lots. Overall from the P2 seeds to the pre-master (P7) seeds, only 1 or 2 non-silent mutations occurred in a given seed. Between pre-master and BCS (P10) seeds, only 1 to 2 nucleotide substitutions were observed, all of which occurred in NS2A, 4A, or 4B, with the exception of single nucleotide change resulting in a conserved glycine and alanine at residue E-483. From P2 to BVS (P10) seeds, total 3 to 8 nucleotide substitutions were identified in any given DENVax seed, and only 2 to 4 of these substitutions resulted in amino acid changes. None of the silent mutations in the BVS were within the 5'- or 3'-NCR region which may affects virus replication. These results suggest that the DENVax viruses were genetically highly stable during manufacture. The three defined DENV-2 PDK-53 attenuation loci located in 5'NCR, NS1-53, and NS3-250 remained unchanged in the consensus genome sequence upon serial passage of the DENVax to generate BVS stocks. The highly sensitive TaqMAMA of the 5'-NCR-57 locus showed minimal or undetectable reversion in the MVS, WVS, and BVS of DENVax. The highest reversion rate of 0.21% was identified in the DENVax-2 BVS. The reversion rates of the P10-equivalent BVS (<0.07% to 0.21%) were significantly lower than the reversion rates that evolved in other vaccine candidates after serial passages in Vero cells (4-74% reversion by P10). This suggests that this strategy for large scale manufacturing of the DENVax seeds is successful, regarding maintaining genetic stability and retention of attenuation markers in the candidate vaccine viruses.

[000128] Since MVS stocks disclosed herein will be used for future manufacturing of

WVS and BVS lots, full panels of virus attenuation phenotype evaluations, including small plaque phenotype, temperature sensitivity, reduced replication in mosquito cells, reduced infection/dissemination/transmission in whole mosquitoes, and reduced neurovirulence in newborn ICR mice, were conducted for all MVS or their equivalent surrogate stocks. For the WVS and BVS stocks, plaque size, infectivity in mosquito cells, were also performed to confirm their attenuations. Results indicated that all the MVS stocks of the 4 serotypes of DENVax retained the expected attenuation phenotypes, such as small plaques, reduced replication in C6/36 cells, and reduced mouse neurovirulence, similar to the original lab-derived vaccine viruses (Figs. 6, 8, and 9). Except for the DENVax-4, all other 3 MVS stocks of DENVax were TS at 39°C as shown in Figs 3 and 7.

[000129] For the WVS and BVS stocks, two attenuation phenotypes, small plaques and restricted replication in C6/36 cells, were analyzed and confirmed. Since there are very little genetic changes between the MVS and BVS, it was expected that they would retain the attenuation phenotypes as MVS. In addition to the experiments described in this report, safety and immunogenicity of the manufactured DENVax in Ag129 mice and nonhuman primate have been tested.

[000130] Exemplary methods are provided herein to demonstrate manufacture of DENVax MVS, WVS, and BVS stocks under cGMP. The BVS stocks were used to formulate the tetravalent DENVax currently in human clinical trial evaluations. A unique manufacture strategy to optimize the genetic stability and safety of the manufactured MVS was provided in some exemplary methods. Since the main attenuation loci of the DENVax have been well characterized previously and a highly sensitive and quantifiable SNP assay, TaqMAMA was developed to integrate genome sequence and the TaqMAMA to identify optimal pre-master seeds for making the MVS. The genetic and phenotypic characterizations of the MVS were fully analyzed to confirm that these viruses retained desirable attenuations for safety of the vaccine. This may be the only live, attenuated viral vaccine that can be efficiently analyzed for all the major attenuation genetic loci during manufacturing from pre-master all the way to BVS stocks. Results provided herein exemplified the advantage of strategically designed live-attenuated vaccines in vaccine safety.

[000131] **Fig. 10** represents an exemplary table comparing new live, attenuated viruses to previously generated live, attenuated dengue viruses. Mutations are indicated where different from a control virus (e.g. 16681), or other live, attenuated dengue-2 viruses.

Materials and methods

Viruses and cells

[000132] DENV-1 16007, DENV-2 16681, DENV-3 16562, and DENV-4 1034 served as wild-type (wt) DENV controls, and they were the parental genotype viruses for the four recombinant DENVax vaccine candidates. DENVax progenitor research-grade viruses, designated as D2/1-V, D2 PDK-53-VV45R, D2/3-V, and D2/4-V, were prepared and characterized previously. Vero (African green monkey kidney) cells used for making the master and working cell banks for vaccine production were originated from the American Type Culture Collection (ATCC) CCL81 cell line that has been characterized by the World Health Organization (WHO) for vaccine manufacture (WCB-Vero cells).

Derivation of live recombinant DENVax viruses from cDNA clones

[000133] To re-derive the candidate vaccine viruses under cGMP manufacturing conditions, the previously engineered DENV infectious cDNA clones, pD2-PDK-53-VV45R, pD2/1-V, pD2/4-V, and *in vitro*-ligated pD2/3-V containing the full genome-length viral cDNAs were used to make fresh viral RNA transcripts by *in vitro* transcription as described previously. Briefly, XbaI-linearized DENV genomic cDNAs were treated with proteinase K, extracted with phenol/chloroform and precipitated in ethanol to remove any residual proteins, and then suspended in RNase-free Tris-EDTA buffer prior to transcription. The *in vitro* transcription was conducted using the AmpliScribe T7 High Yield Transcription kit (Epicentre Technologies) following the manufacturer's recommended protocol. The RNA A-cap analog, m7G(5')ppp(5')A (New England BioLabs), was incorporated during the 2-hr transcription reaction to add the 5'-terminal A-cap to the RNA transcript. The samples were then treated with DNase I to digest the template cDNA, followed by low pH phenol/chloroform extraction and ethanol precipitation to remove residual DNA and proteins. The purified RNA transcripts, suspended in RNase-free water, were distributed in 20-µl aliquots and stored at -80°C until ready for transfection of cells. The integrity and concentration of the RNA transcripts were analyzed by agarose gel electrophoresis. Each 20-µl aliquot was estimated to contain sufficient genome-length viral RNA to permit transfection of 0.4-1 x 10^7 production-certified Vero cells by electroporation.

[000134] Transfection of each RNA transcript into WCB-Vero cells was performed in the cGMP facility at Shantha Biotechnics. DENVax RNA transcripts were thawed, mixed with 400 µl of the Vero cell suspension (1 x 10^7 cells/ml), and transferred to a pre-chilled sterile electroporation cuvette (4-mm gap) for electroporation by a Gene Pulser Xcell total system (BioRad Laboratories). Each sample was pulsed once at 250V/∞ Ohms/500 µF,

incubated for 10-15 min at room temperature, transferred to a 75-cm^2 flask containing 30 ml of cell growth medium (MEM with 10% FBS), and incubated at 36°C ± 1°C, 5% CO_2 for 6 to 11 days. The culture medium was harvested, clarified by centrifugation, stabilized, and stored in small aliquots below -60°C. The viral titers of candidate vaccine stocks (termed P1 for passage level 1) resulting from transfection were determined by plaque titration assay in Vero cells and used for further propagation of the DENVax seeds.

Manufacture of DENVax virus seeds

[000135] P1 virus seeds were used to propagate DENVax pre-master, master, working, and bulk virus seed lots through a strategy designed to ensure the optimal genetic stability and safety of the manufactured lots. This strategy included three serial plaque purifications, as well as genetic analyses of viruses at various passage levels to select the optimal clonal virus population for continued seed production (Table 1). Briefly, the P1 seeds harvested from transfected cells were amplified once by infection of Vero cells at a MOI of 0.001 to generate the P2 seeds. Aliquots of the P2 seed stocks were evaluated by plaque morphology and complete viral genomic sequencing. The genetically confirmed P2 stocks were plated on Vero cell monolayers with overlay medium as described in the plaque titration section below to generate well-isolated plaques. After visualization with neutral red, six individual plaques from each of the 4 serotypes of vaccine viruses were isolated (plaque clones A to F) and mixed into 0.5 ml of culture medium (passage P3). Each of the six plaque suspensions was subjected to two additional rounds of plaque purification, resulting in twice- and thrice-plaque purified virus seeds at passages P4 and P5, respectively. The P5 viruses were amplified through two sequential Vero passages to produce P7 seed stocks.

[000136] Genetic analysis of the three major DENVax attenuation loci using spot sequencing and/or Taqman-based mismatched amplification mutation assay (TaqMAMA) as previously disclosed, and plaque phenotype analysis were conducted to screen all 24 P7 seeds. Seeds possessing appropriate initial characteristics were then further characterized by full genomic sequencing. As a result of these analyses, one of the 6 (clone A-F) P7 seeds of each DENVax serotype was selected to be the pre-master seed, based on the presence of the DENV-2 PDK-53 attenuating mutations, minimal genomic sequence alterations, and expected plaque phenotype. Each selected pre-master seed was expanded to master virus seed (MVS or P8) by a one-time passage of the virus at MOI of 0.001in multiple 175 cm^2 flasks of Vero cells. Except for the DENVax-4 MVS, the master virus seeds were harvested at 8-10 days post infection (pi). The MVS stocks were harvested at 6-10 days post infection (pi), clarified by centrifugation, stabilized by the addition of sucrose/phosphate/glutamate

solution (final concentration 7.5 % sucrose, 3.4 mM potassium dihydrogen phosphate, 7.2 mM dipotassium hydrogen phosphate, 5.4 mM monosodium glutamate, respectively) and 0.95 to 1.90% FBS (final concentration). DENVax-4 MVS was prepared differently to optimize its yield. Briefly, multiple flasks of cells were infected with DENVax-4 pre-master seed at a MOI of 0.001 in the presence of 0.1% F-127™, poloxamer 407, (other EO-PO block copolymers have been assessed and may substitute here, see issued patent) that have been demonstrated to enhance DENV virus thermal stability. Infectious media was harvested days 6-10 pi, and stabilized with 17% FBS (final concentration), pooled, and frozen. All four DENVax MVS stocks were stored as 1-ml aliquots below -60°C.

[000137] The DENVax working virus seeds (WVS) were prepared by one-time passage in Vero cell culture of the MVS at a MOI of 0.001. The procedures were similar to the production of MVS, except they were cultured in multiple-layer cell factories (6360 cm^2). The WVS stocks were filtered through 10 μM and 0.45μM filters, stabilized with the same stabilizers used for the MVS, aliquoted into 30ml PETG bottles or 2.0 ml cryovials, and stored below-60°C.

[000138] In certain methods, bulk virus seeds (BVS) were produced by infecting multiple cell factories (6360 cm^2 each) of confluent Vero cells with 90 mL of diluted WVS to attain a MOI of 0.001. A media used for dilution of the WVS inocula contained 0.1% F-127™ without serum. After 1.5 hr adsorption, cells were washed 3 times with PBS, and 800 ml of serum-free DMEM medium was added to each cell factory, and the factories were incubated at 36(±1)°C in 5(±0.5)% CO_2. After incubation for four days, small aliquots of medium were collected for sterility testing. Viruses were harvested between day 5 and day 10 pi, and immediately clarified by filtration through a 0.45 um pore size filter, and 1L of each clarified virus pool was stabilized by addition of 500 ml of 3x FTA buffer (final concentrations of 15% trehalose, 1.0% Pluronic® F-127™ poloxamer 407, 0.1% human albumin USP in PBS, pH 7.4). The stabilized virus was distributed into 1-L PETG bottles and stored frozen below -60°C for subsequent pooling and quality control testing. All stabilized virus harvests with a virus titer above 10^5 PFU/ml and an acceptable level of residual DNA were rapidly thawed in a water bath at 32°C, then aseptically pooled and mixed. Each pooled monovalent BVS was distributed into labeled PETG containers and stored at below -60°C until further use.

Manufacture product quality controls

[000139] The MVS, WVS, and BVS seeds were tested for identity, sterility, and

detectable adventitious agents. The identity of each vaccine stock was confirmed by RT-PCR with DENVax serotype-specific primers. The amplified cDNA fragments contained the E/NS1 chimeric junction site to permit identification of each of the four DENVax serotypes. Each seed was tested in all 4 serotype-specific RT-PCR reactions to confirm viral identity and freedom from cross contamination with heterologous DENVax serotypes. Sterility testing was performed in accordance with USP 71 (United States Pharmacopeia, section 71). Mycoplasma testing was performed.

[000140] The following *in vitro* and *in vivo* tests for viral contamination were all performed using unclarified, unstabilized DENVax harvests collected during manufacture of the seeds. Harvested infectious media were first neutralized with DENV rabbit polyclonal antiserum (Inviragen) at 36 ± 1°C for 1 hr to inactivate the DENV. For *in vitro* test, the neutralized seeds were inoculated into three indicator cells lines, MRC5, VERO and MA104, in 25 cm^2 flasks. Echo virus (CPE control) or mumps virus (hemadsorption control) were used as positive CPE or hemadsorption control, respectively. All cells were monitored daily for CPE for a total of 14 days. At the end of 14 days, the culture supernatant was removed and replaced with 10 mL of a guinea pig red blood cell (RBC) solution (3 mL of 0.5% guinea pig RBC in phosphate buffered saline, made up to 10 mL with cell growth medium). The flasks were then incubated at 5 ± 3°C for 30 minutes followed by incubation at room temperature for 30 minutes. The monolayers were washed with PBS and observed under 10 X magnification for the presence of any star-shaped clumps of RBCs for hemadsorption.

[000141] *In vivo* tests for adventitious agents were performed in suckling mice, post-weaning mice and guinea pigs. Suckling mice were inoculated with 0.1ml or 0.01 ml (10 mice in each dose group) of the DENV-antiserum neutralized seed sample through intraperitoneal (ip) injection. Similarly, 10 post-weaning mice were each inoculated ip with 0.5 ml or 0.03 ml of the sample. Guinea pigs (5/group) were each inoculated ip with 5.0 mL. Suckling mice were observed daily for morbidity and mortality for a total of 14 days following inoculation. Post-weaning mice were observed for a total of 28 days, and guinea pigs were observed for a total of 42 days following inoculation. The test articles met the acceptance criterion if >80% of the inoculated animals remained healthy throughout the observation period.

[000142] The *in vivo* testing for contaminants was also performed in embryonated chicken eggs and was conducted. For every sample, 10 embryonated hen eggs (9 days old) were each inoculated with 0.5 mL of the DENV antiserum-neutralized sample into the

allantoic fluid and incubated at 35°C for 3 days. The allantoic fluids from these 10 eggs were harvested, pooled and passaged into the allantoic fluid of 10 fresh embryonated eggs (10-11 days old; 0.5mL/egg) and incubated at 35°C for a further 3 days. Similarly, for each sample, 10 embryonated eggs (6-7 days old) were each inoculated with 0.5 mL per egg (DENVax-2 monovalent BVS) or 0.25 mL per egg (DENVax-1, DENVax-3 and DENVax-4 BVS) by injection into the yolk sac and incubated at 35°C for 9 days. The yolk sacs from these 10 eggs were harvested and pooled, and a 10% suspension was passaged into the yolk sacs of 10 fresh embryonated eggs (6-7 days old; 0.5 mL/egg) and incubated at 35°C for a further 9 days. Eggs inoculated into the allantoic fluid (both initial and passage inoculations) were observed for viability after 3 days incubation. Both pools of allantoic fluid were tested for hemagglutination activity using chicken, guinea pig and human type O erythrocytes at 4°C and 25°C. Eggs inoculated into the yolk sack (both initial and passage inoculations) were observed for viability after 9 days of incubation.

Virus plaque assay and immunofocus assay

[000143] Virus titers were measured by plaque assay or immunofocus assay using Vero cells. Plaque assays were performed in double agarose overlays in six-well plates of confluent Vero cells as previously described, and they were also used to evaluate the plaque phenotypes of the DENVax seeds. For accurate comparison, plaque sizes of all viruses were measured and compared in the same experiment. After visualization with neutral red on day 9 pi, up to 10 well isolated plaques for each virus were measured for mean plaque size calculation. Fewer plaques were measured for wt DENV-1, -3, and -4, whose larger plaque sizes often did not permit measurement of 10 well-separated plaques.

[000144] Because tetravalent DENVax contains all four DENV serotypes, a DENV serotype-specific immunofocus assay was developed to quantitate each DENVax component in the tetravalent formulations. Immunofocus assays of each individual DENVax MVS were compared with the plaque assays to ensure virus titration results were comparable between the two assays. The immunofocus assay was conducted in 6-well plates of confluent Vero cells infected with serially diluted viruses. Cells were overlayed with a balanced salt medium (BSS/YE-LAH medium) containing 0.7% high viscosity carboxymethyl cellulose (Sigma) and incubated for 7 days at 37°C with 5% CO_2. After removal of overlays, cell sheets were washed 3 times with PBS, fixed with cold 80% acetone for 30min at -20°C, washed once with PBS, and blocked with a blocking buffer containing 2.5% (w/v) nonfat dry milk, 0.5% Triton X-100, 0.05% Tween-20 in PBS at 37°C for 30 min. Blocked cells were incubated with diluted DENV serotype-specific MAbs,

1F1 (DENV-1), 3H5 (DENV-2), 8A-1 (DENV-3), or 1H10 (DENV-4) in blocking buffer at 37°C for 1 hour or 4°C overnight, washed 3 times with washing buffer (0.05% Tween-20 in PBS), and incubated with alkaline phosphatase- or horse radish peroxidase (HRP)-conjugated affinity-pure goat anti-mouse IgG (Jackson Immuno Research Laboratories) at 37°C for 45-60 min. Plates were washed 3 times before the appropriate substrate, 1-Step NBT/BCIP plus suppressor (Pierce) for alkaline phosphatase or Vector-VIP kit (Vector Labs) for HRP, was added for color development. Color development was stopped by rinsing with water when the foci were fully developed. Stained immunofoci were directly visualized and counted on a light box.

Genetic sequence

[000145] Full length genomes of the MVS and WVS were sequenced (see below). Briefly, viral RNA was extracted from DENVax seeds by using the QIAamp viral RNA kit (Qiagen), and overlapping cDNA fragments covering the entire genome were amplified using the Titan One Tube RT-PCR kit (Roche Applied Science, Inc.). The amplified cDNA fragments were gel purified before sequencing with both forward and reverse primers using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequence reactions were cleaned using the BigDye XTerminator Purification kit (Applied Biosystems), and run on the 3130xl Genetic analyzer (Applied Biosystems) at DVBD/CDC. The Lasergene SeqMan software (DNAStar, Inc) was used for genome analysis and comparison.

Taqman-based mismatch amplification mutation assay (TaqMAMA)

[000146] TaqMAMA is a sensitive, quantitative single nucleotide polymorphism assay developed to permit finer assessment of the level of reversion at the 5'NC-57 locus of attenuation, and was further optimized for this study. Extracted viral RNA from MVS and WVS were analyzed by the TaqMAMA with both sets of primers/Taqman probe that are specific to wt or the vaccine 5'NC-57 region. The forward primers used to detect DENV-2 wt and vaccine sequences were D2-41-GC and D2-40-TT, respectively. The 3'-terminal nucleotide of each forward primer matched the specific 5'NCR-57 nucleotide for each virus, while the nucleotide adjacent to the 3'-terminal nucleotide in each primer differed from the DENV-2 viral genomic sequence to enhance the mismatch effect. The reverse primer, CD-207, and the Taqman probe, CD-169F, for both wt and vaccine sets were identical. Sequences of the primers and probe as well as cycling conditions were described previously. The real time RT-PCR was performed with the iQ5 or CFX-95 system (BioRad), using a BioRad iScript RT-PCR (for probes) kit, in a 25-µl reaction containing 5 µl of viral RNA

template, 0.4 uM of each primer, and 0.2 uM of the probe. Triplicate reactions for each wt- and vaccine-specific assay were conducted for each sample. Genome copy numbers were determined relative to a standard curve prepared for each viral genotype, where the RNA standards were transcripts derived from plasmids containing nt 1-2670 of each genotype-specific cDNA. In addition, the specificity of the assay was confirmed by testing each RNA standard with the heterologous genotype primer/probe sets to ensure minimum cross-reactivity in every experiment. The results were reported as the percentage of viral genomes showing reversion. Previously, due to higher cross-reactive backgrounds that limited the input RNA levels for this assay, the original detection sensitivity was about 0.1% reversion (discrimination power). Since then, the assay has been further optimized using improved real-time PCR equipment and reaction kits, and the cross-reactive background was decreased considerably at much high levels (7-8 $\log_{10}$ copies) of RNA template input. This optimization resulted in significant improvement of the detection sensitivity, down to 0.01-0.07% reversion.

Virus replication in mosquito C6/36 cells and temperature sensitivity in mammalian Vero cells

[000147] The replication phenotypes of the four DENVax MVS stocks and wt DENV-1, -2, -3, and -4 viruses were evaluated in C6/36 mosquito cells (*Aedes albopictus*). C6/36 cells grown in 6-well plates were infected in duplicate with each virus at a MOI of 0.001 and incubated with 4 ml/well of DMEM medium containing 2% FBS in a 5% CO_2 incubator at 28°C. Small aliquots of the culture supernatant were collected for each virus on day 6 pi, mixed with an equal volume of medium containing 40% FBS, and stored at -80°C until ready for virus plaque titration.

[000148] Temperature sensitivity was conducted by comparing viral growth at 39°C versus growth at 37°C at five days pi of Vero cells in 6-well plates. Cells were infected in quadruplicate with each virus at a MOI of 0.001 at 37°C. Following adsorption of virus, the infected cultures were incubated with 4 ml/well of DMEM medium containing 2% FBS in 2 separate 5% CO_2 incubators, one set (duplicate plates) at 37°C and the other at 39°C. Aliquots (50-µl) of the culture supernatant were collected on day 5 pi, mixed with an equal volume of DMEM containing 40% of FBS, and stored at -80°C until ready for virus plaque titration. Incubator temperatures were calibrated with NIST-traceable factory-calibrated thermometers (-1 to 51 °C; ERTCO).

Mosquito infection, dissemination, and transmission

[000149] *Aedes aegypti* mosquitoes used for the study were from a colony established in 2002 from a village near Mae Sot (16' N, 33' E), Thailand. After emerging from larvae, adult mosquitoes were maintained at 28°C at a 16:8 (light:dark) photoperiod with 10% sucrose solution provided *ad libitum*. Five-to-seven day old female mosquitoes were used for infectious blood meal feeding or intrathoracic (IT) inoculations. Aliquots of freshly cultured DENVax and wt DENV were used immediately upon harvest (without any freeze-thaw cycle) to make virus blood meals as indicated below for oral infection. Remaining virus supernatants were supplemented with FBS to a final concentration of 20%, and aliquots were stored at -80°C for future virus plaque titration and IT inoculation experiments. The freshly prepared DENVax seeds for these experiments were amplified from the pre-master seeds in Vero cells, and were considered DENVax MVS equivalents.

[000150] Infectious blood meals were prepared by mixing fresh virus at a ratio of 1:1 with defibrinated chicken blood (Colorado Serum Company) on the day of oral infection. Mosquitoes were sugar-starved overnight and then offered the virus:blood mixture for 1 hour using a Hemotek membrane feeding system (Discovery Workshops). A 50-µl aliquot of the blood meal was retained at -80°C for back-titration of virus doses. Fully-engorged females were sorted under cold anesthesia and placed into cartons with 10% sucrose solution provided *ad libitum*. Cartons were placed at 28°C with a photoperiod of 16:8 h (light:dark). After 14 days, 25-30 mosquitoes from each virus group were anesthetized via exposure to triethylamine (Flynap ®, Carolina Biological Supply Company) and one hind leg was removed and placed in 0.5 ml of DMEM with 10% FBS and 5% penicillin/streptomycin (100U/ml and 100µg/ml respectively). Saliva was collected by inserting the proboscis of the anesthetized mosquito into a capillary tube containing 2.5% FBS and 25% sucrose solution. Mosquitoes were allowed to salivate for at least 15 minutes and then capillary tubes and bodies were placed into separate tubes containing DMEM. Mosquito bodies, legs and saliva were stored at -80°C until they were triturated and assayed for infectious virus. For IT inoculation, mosquitoes were cold-anesthetized and inoculated with approximately 50 pfu of virus in 0.34 µl inoculum. Inoculated mosquitoes were kept for 7 days in the same conditions as described above. Mosquitoes were then anesthetized, and their saliva and bodies were collected as described above. Samples were stored at -80°C until further processing.

[000151] To process the samples for virus titration, body and leg samples were homogenized with copper coated BBs (Crossman Corporation, NY) at 24 cycles/second for 4 min using a mixer mill, and then clarified by centrifuging at 3,000 x g for 3 min. Saliva

samples were centrifuged at 3,000 x g for 3 minutes to expel fluid from capillary tubes. Ten-fold dilutions of the body and leg homogenates and saliva samples were tested for presence of infectious virus by plaque assay. Results from bodies, legs, and saliva were used for determining the infection, dissemination, and transmission rates, respectively.

Mouse neurovirulence

[000152] Timed pregnant female ICR mice were obtained from Taconic Labs, and monitored several times each day to determine approximate birth times of pup litters. In a given experiment, approximately 12-24 hours after birth, two litters of eight pups per virus (n=16), was challenged with 10^3 to 10^4 pfu of virus in 20 µl of diluent by intracranial (ic) inoculation using a 30-gauge needle. Animals were monitored at least 3 times daily for at least 32 days following challenge. At the first sign of illness (rough fur, hunched back, weight loss, abnormal movement, paralysis, or lethargy) animals were euthanized by lethal anesthetization with isoflurane gas, followed by cervical dislocation. The post-infection day of euthanasia represented the "time to illness/morbidity" or "survival time" for the animal. The animal experiments were conducted following a DVBD/CDC IACUC-approved animal protocol.

Derivation of Master Seed Viruses

DENvax-1 Master Virus Seed (MVS)

[000153] Nucleotide sequence of the chimeric viral genome and deduced amino acid sequence of the translated protein are provided herein. Most of the prM-E gene (nt 457 to -2379, underlined) is wild-type (wt) DEN-1 16007 virus specific; the remaining genome is DEN-2 PDK-53 virus specific. All engineered substitutions differ from wt virus (D1 16007 or D2 16681), as well as extra mutations (changes from engineered cDNA clone) detected in the MVS are marked.

Substitutions Included in the Genome and Protein:

[000154] Junction sites between D1 (prM-E) and D2 backbone:

a. MluI (nt 451-456): engineered silent mutation, nt-453 A-to-G
b. NgoMIV (nt 2380-2385): engineered mutations, nt-2381/2382 TG-to-CC (resulted in E-482 Val-to-Ala change)

[000155] D2 PDK-53 virus backbone (change from wt D2 16681): all in bold

a. 5'-noncoding region(NCR)-57 (nt-57 C-to-T): major attenuation locus (in red)
b. NS1-53 Gly-to-Asp (nt-2579 G-to-A): major attenuation locus (in red)
c. NS2A-181 Leu-to-Phe (nt-4018 C-to-T)
d. NS3-250 Glu-to-Val (nt-5270 A-to-T): major attenuation locus (in red)
e. nt-5547 (NS3 gene) T-to-C silent mutation

f. NS4A-75 Gly-to-Ala (nt-6599 G-to-C)

* nt-8571 C-to-T silent mutation of PDK-53 is not engineered in the vaccine virus

[000156] DEN-1 prM-E (change from wt D1 16007)

a. Engineered nt-1575 T-to-C silent mutation to remove native XbaI site

[000157] Additional substitutions found in vaccine seed (0.03% nt different from original clone)

a. NS2A-116 Ile-to-Leu (nt-3823 A-to-C, in bold)

b. NS2B-92 Glu-to-Asp (nt-4407 A-to-T, in bold)

c. nt-7311 A-to-G silent mutation (in bold)

NCE-57-T, D2 PDK-53 attenuation locus (wt D2 16681: C)

```
>5'-Noncoding Region                                                                                    >C
       10        20        30        40        50        60        70        80        90        100
AGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAATGTAGTTCTAACAGTTTTTTAATTAGAGAGCAGATCTCTGATGA
                                                                                                  M  N

      110       120       130       140       150       160       170       180       190       200
ATAACCAACGGAAAAAGGCGAAAAACACGCCTTTCAATATGCTGAAACGCGAGAGAAACCGCGTGTCGACTGTGCAACAGCTGACAAAGAGATTCTCACT
  N  Q  R  K  K  A  K  N  T  P  F  N  M  L  K  R  E  R  N  R  V  S  T  V  Q  Q  L  T  K  R  F  S  L

      210       220       230       240       250       260       270       280       290       300
TGGAATGCTGCAGGGACGAGGACCATTAAAACTGTTCATGGCCCTGGTGGCGTTCCTTCGTTTCCTAACAATCCCACCAACAGCAGGGATATTGAAGAGA
 G  M  L  Q  G  R  G  P  L  K  L  F  M  A  L  V  A  F  L  R  F  L  T  I  P  P  T  A  G  I  L  K  R

      310       320       330       340       350       360       370       380       390       400
TGGGGAACAATTAAAAAATCAAAAGCTATTAATGTTTTGAGAGGGTTCAGGAAAGAGATTGGAAGGATGCTGAACATCTTGAATAGGAGACGCAGATCTG
W  G  T  I  K  K  S  K  A  I  N  V  L  R  G  F  R  K  E  I  G  R  M  L  N  I  L  N  R  R  R  R  S  A

                                  >prM                Beginning of D1 16007 sequence
      410       420       430       440       450      |460       470       480       490       500
CAGGCATGATCATTATGCTGATTCCAACAGTGATGGCGTTCCATTTAACCACGCGTGGGGGAGAGCCGCATATGATAGTTAGCAAGCAGGAAAGAGGAAA
  G  M  I  I  M  L  I  P  T  V  M  A  F  H  L  T  T  R  G  G  E  P  H  M  I  V  S  K  Q  E  R  G  K
     |
                                                Engineered MluI splicing site (nt-453 A-to-G silent)

      510       520       530       540       550       560       570       580       590       600
GTCACTTTTGTTCAAGACCTCTGCAGGTGTCAACATGTGCACCCTCATTGCGATGGATTTGGGAGAGTTGTGTGAGGACACGATGACCTACAAATGCCCC
 S  L  L  F  K  T  S  A  G  V  N  M  C  T  L  I  A  M  D  L  G  E  L  C  E  D  T  M  T  Y  K  C  P

      610       620       630       640       650       660       670       680       690       700
CGGATCACTGAGGCGGAACCAGATGACGTTGACTGTTGGTGCAATGCCACGGACACATGGGTGACCTATGGAACGTGCTCTCAAACTGGCGAACACCGAC
R  I  T  E  A  E  P  D  D  V  D  C  W  C  N  A  T  D  T  W  V  T  Y  G  T  C  S  Q  T  G  E  H  R  R

          >M
      710       720       730       740       750       760       770       780       790       800
GAGACAAACGTTCCGTCGCATTGGCCCCACACGTGGGGCTTGCCTAGAAACAAGAGCCGAAACGTGGATGTCCTCTGAAGGTGCTTGGAAACAGATACA
  D  K  R  S  V  A  L  A  P  H  V  G  L  G  L  E  T  R  A  E  T  W  M  S  S  E  G  A  W  K  Q  I  Q

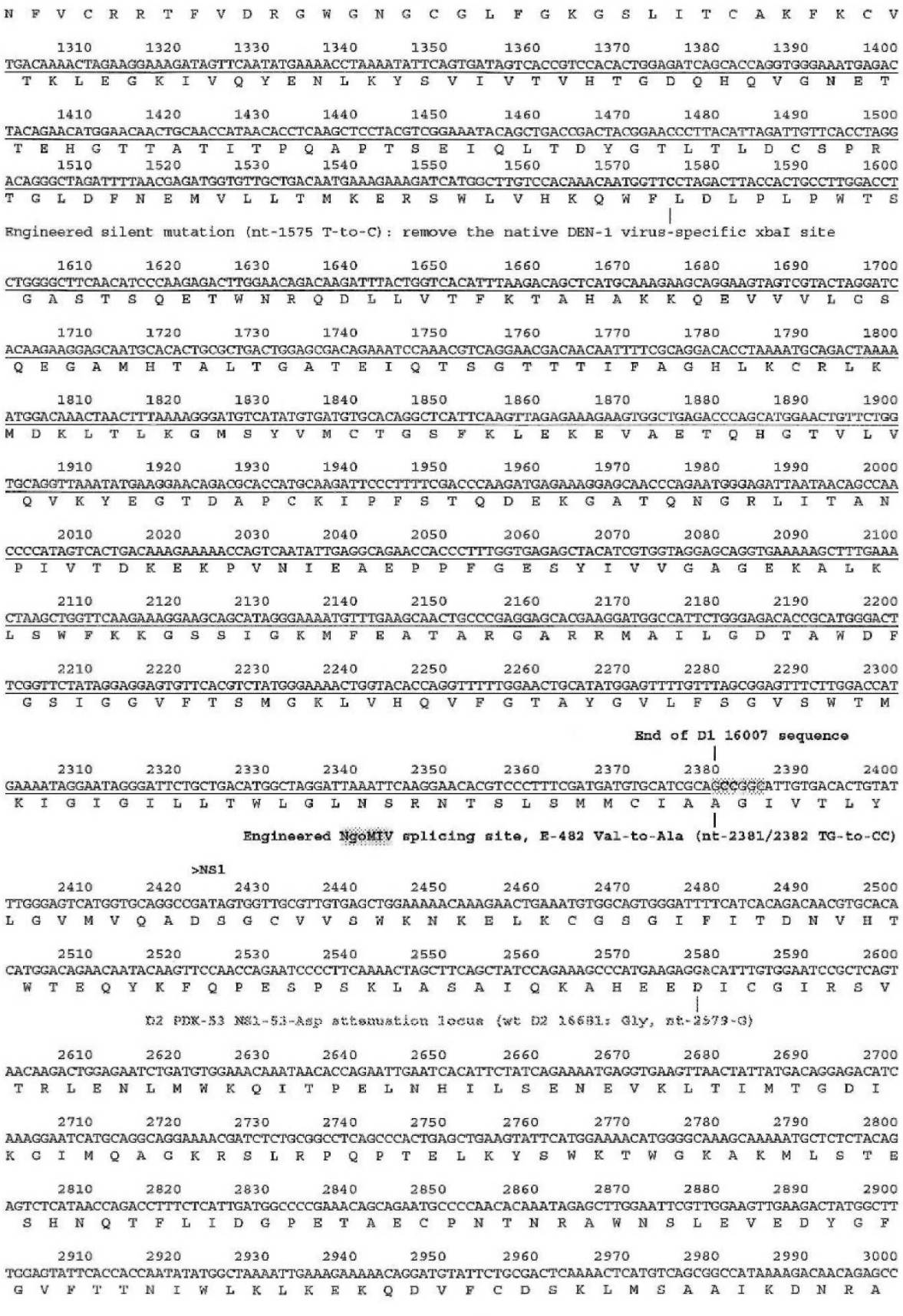
      810       820       830       840       850       860       870       880       890       900
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 K  V  E  T  W  A  L  R  H  P  G  F  T  V  I  A  L  F  L  A  H  A  I  G  T  S  I  T  Q  K  G  I  I

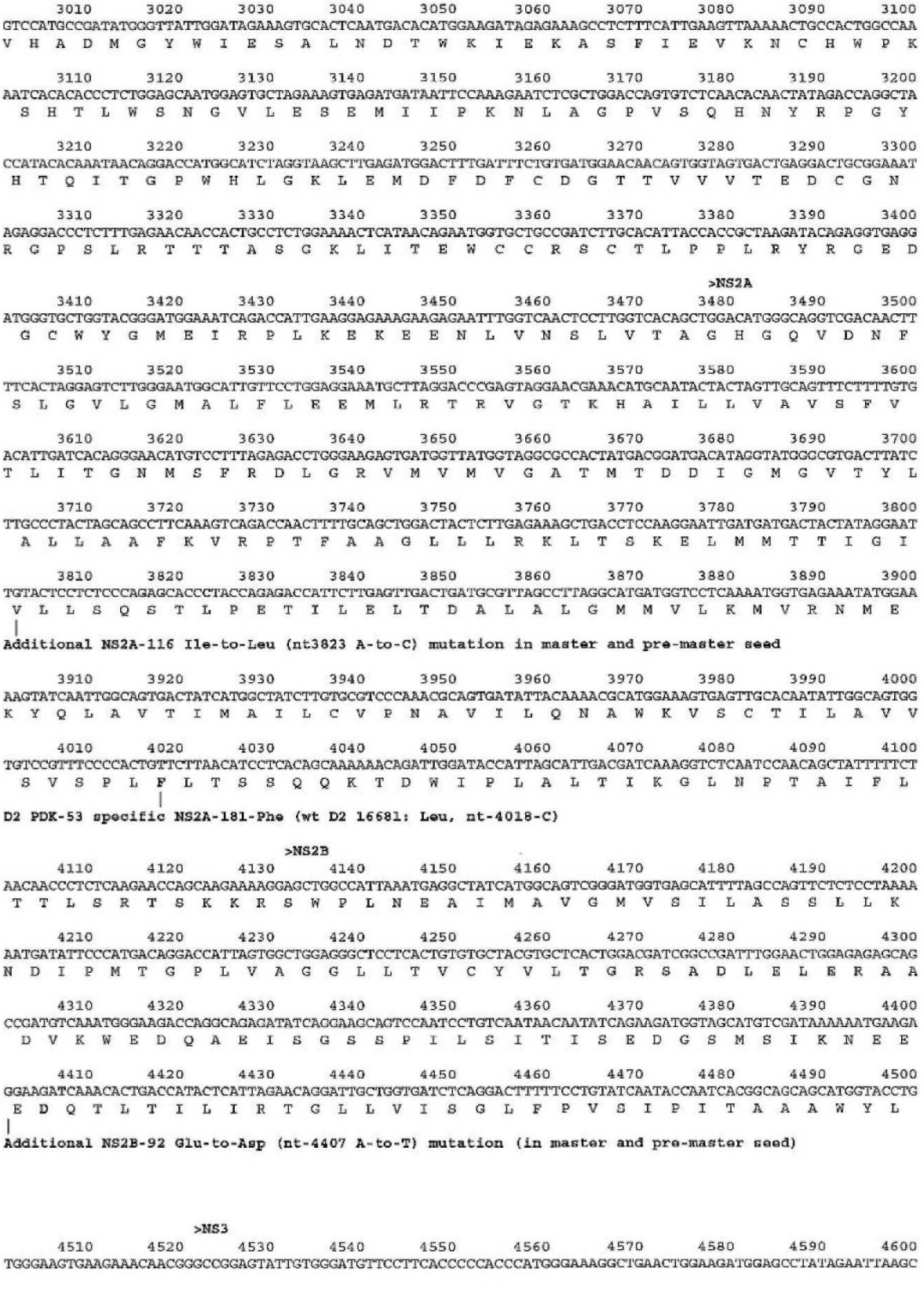
                                  >E
      910       920       930       940       950       960       970       980       990      1000
TTCATTTTGCTGATGCTGGTAACACCATCTATGGCCATGCGATGCGTGGGAATAGGCAACAGAGACTTCGTGGAAGGACTGTCAGGAGCAACATGGGTGG
F  I  L  L  M  L  V  T  P  S  M  A  M  R  C  V  G  I  G  N  R  D  F  V  E  G  L  S  G  A  T  W  V  D

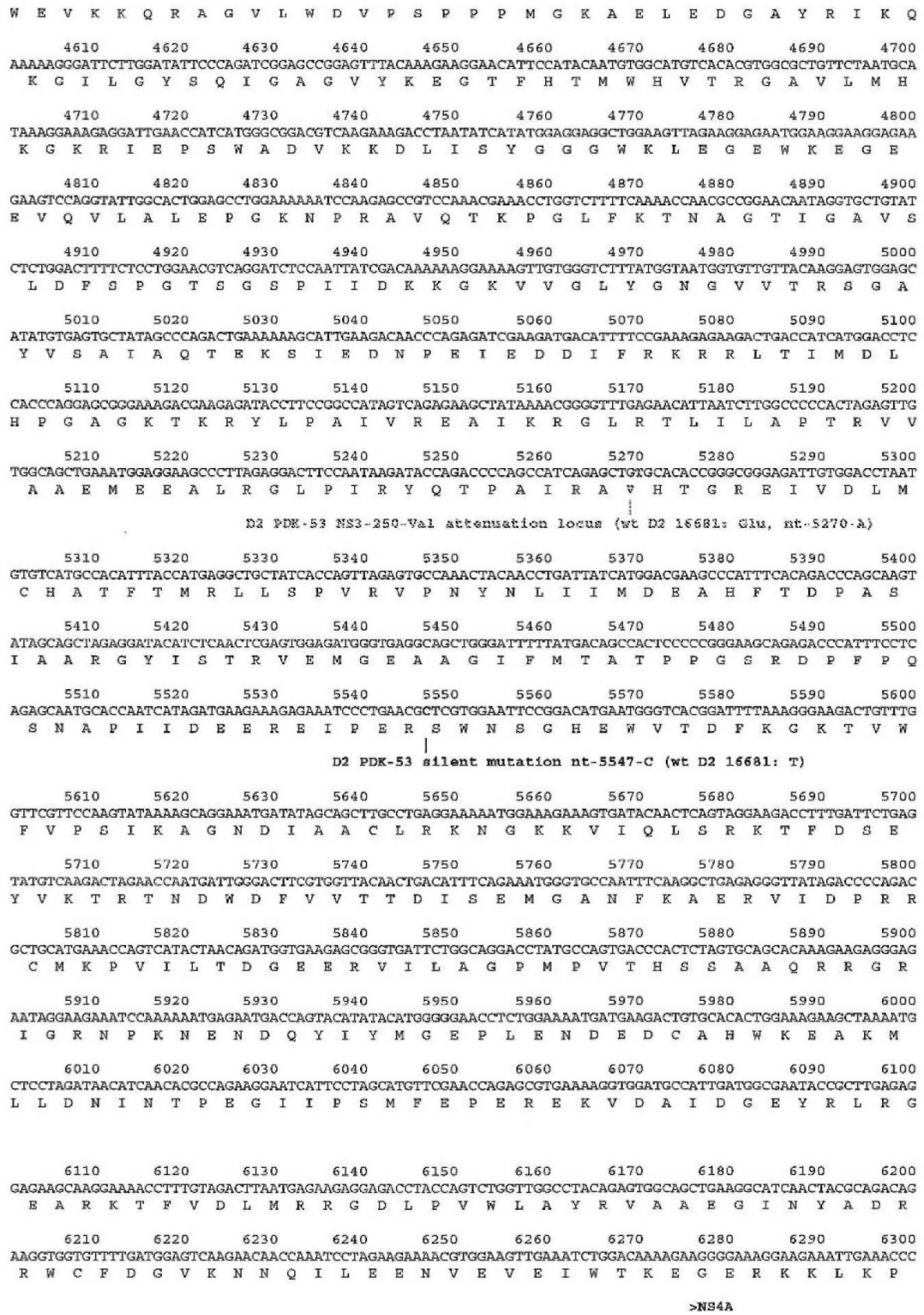
     1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
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  V  V  L  E  H  G  S  C  V  T  T  M  A  K  N  K  P  T  L  D  I  E  L  L  K  T  E  V  T  N  P  A  V

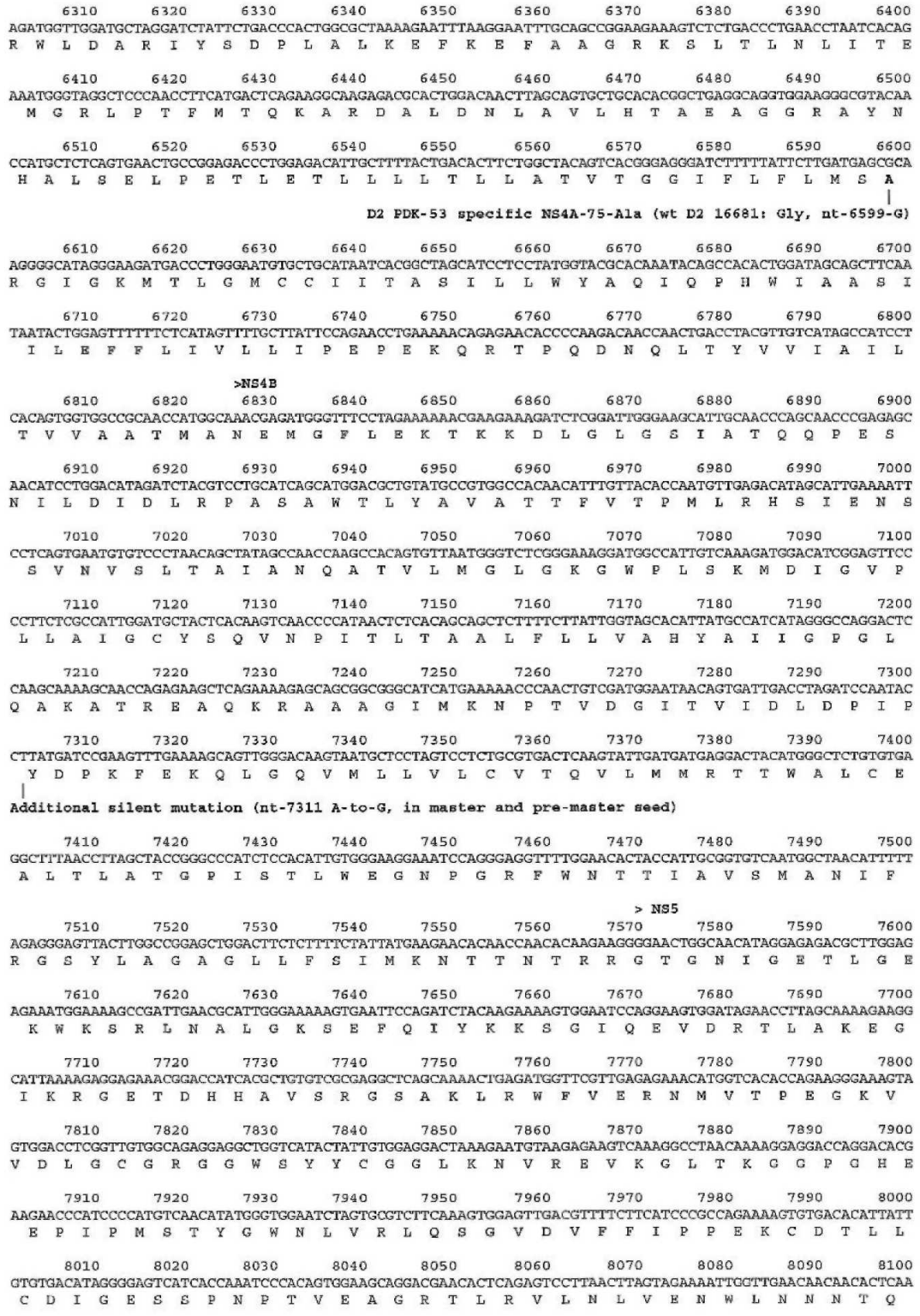
     1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
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 L  R  K  L  C  I  E  A  K  I  S  N  T  T  T  D  S  R  C  P  T  Q  G  E  A  T  L  V  E  E  Q  D  A

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
AACTTTGTGTGCCGACGAACGTTCGTGGACAGAGGCTGGGGCAATGGCTGTGGGCTATTCGGAAAAGGTAGTCTAATAACGTGTGCCAAGTTTAAGTGTG
```


N F V C R R T F V D R G W G N G C G L F G K G S L I T C A K F K C V
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
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T K L E G K I V Q Y E N L K Y S V I V T V H T G D Q H Q V G N E T
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
TACAGAACATGGAACAACTGCAACCATAACACCTCAAGCTCCTACGTCGGAAATACAGCTGACCGACTACGGAACCCTTACATTAGATTGTTCACCTAGG
T E H G T T A T I T P Q A P T S E I Q L T D Y G T L T L D C S P R
1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
ACAGGGCTAGATTTTAACGAGATGGTGTTGCTGACAATGAAAGAAAGATCATGGCTTGTCCACAAACAATGGTTCCTAGACTTACCACTGCCTTGGACCT
T G L D F N E M V L L T M K E R S W L V H K Q W F L D L P L P W T S
Engineered silent mutation (nt-1575 T-to-C): remove the native DEN-1 virus-specific xbaI site
1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
CTGGGGCTTCAACATCCCAAGAGACTTGGAACAGACAAGATTTACTGGTCACATTTAAGACAGCTCATGCAAAGAAGCAGGAAGTAGTCGTACTAGGATC
G A S T S Q E T W N R Q D L L V T F K T A H A K K Q E V V V L G S
1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
ACAAGAAGGAGCAATGCACACTGCGCTGACTGGAGCGACAGAAATCCAAACGTCAGGAACGACAACAATTTTCGCAGGACACCTAAAATGCAGACTAAAA
Q E G A M H T A L T G A T E I Q T S G T T T I F A G H L K C R L K
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
ATGGACAAACTAACTTTAAAAGGGATGTCATATGTGATGTGCACAGGCTCATTCAAGTTAGAGAAAGAAGTGGCTGAGACCCAGCATGGAACTGTTCTGG
M D K L T L K G M S Y V M C T G S F K L E K E V A E T Q H G T V L V
1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
TGCAGGTTAAATATGAAGGAACAGACGCACCATGCAAGATTCCCTTTTCGACCCAAGATGAGAAAGGAGCAACCCAGAATGGGAGATTAATAACAGCCAA
Q V K Y E G T D A P C K I P F S T Q D E K G A T Q N G R L I T A N
2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
CCCCATAGTCACTGACAAAGAAAAACCAGTCAATATTGAGGCAGAACCACCCTTTGGTGAGAGCTACATCGTGGTAGGAGCAGGTGAAAAAGCTTTGAAA
P I V T D K E K P V N I E A E P P F G E S Y I V V G A G E K A L K
2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
CTAAGCTGGTTCAAGAAAGGAAGCAGCATAGGGAAAATGTTTGAAGCAACTGCCCGAGGAGCACGAAGGATGGCCATTCTGGGAGACACCGCATGGGACT
L S W F K K G S S I G K M F E A T A R G A R R M A I L G D T A W D F
2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
TCGGTTCTATAGGAGGAGTGTTCACGTCTATGGGAAAACTGGTACACCAGGTTTTTGGAACTGCATATGGAGTTTTGTTTAGCGGAGTTTCTTGGACCAT
G S I G G V F T S M G K L V H Q V F G T A Y G V L F S G V S W T M
End of D1 16007 sequence
2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
GAAAATAGGAATAGGGATTCTGCTGACATGGCTAGGATTAAATTCAAGGAACACGTCCCTTTCGATGATGTGCATCGCAGCCGGCATTGTGACACTGTAT
K I G I G I L L T W L G L N S R N T S L S M M C I A A G I V T L Y
Engineered NgoMIV splicing site, E-482 Val-to-Ala (nt-2381/2382 TG-to-CC)
>NS1
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
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L G V M V Q A D S G C V V S W K N K E L K C G S G I F I T D N V H T
2510 2520 2530 2540 2550 2560 2570 2580 2590 2600
CATGGACAGAACAATACAAGTTCCAACCAGAATCCCCTTCAAAACTAGCTTCAGCTATCCAGAAAGCCCATGAAGAGGACATTTGTGGAATCCGCTCAGT
W T E Q Y K F Q P E S P S K L A S A I Q K A H E E D I C G I R S V
D2 PDK-53 NS1-53-Asp attenuation locus (wt D2 16681: Gly, nt-2579-G)
2610 2620 2630 2640 2650 2660 2670 2680 2690 2700
AACAAGACTGGAGAATCTGATGTGGAAACAAATAACACCAGAATTGAATCACATTCTATCAGAAAATGAGGTGAAGTTAACTATTATGACAGGAGACATC
T R L E N L M W K Q I T P E L N H I L S E N E V K L T I M T G D I
2710 2720 2730 2740 2750 2760 2770 2780 2790 2800
AAAGGAATCATGCAGGCAGGAAAACGATCTCTGCGGCCTCAGCCCACTGAGCTGAAGTATTCATGGAAAACATGGGGCAAAGCAAAAATGCTCTCTACAG
K G I M Q A G K R S L R P Q P T E L K Y S W K T W G K A K M L S T E
2810 2820 2830 2840 2850 2860 2870 2880 2890 2900
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S H N Q T F L I D G P E T A E C P N T N R A W N S L E V E D Y G F
2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
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G V F T T N I W L K L K E K Q D V F C D S K L M S A A I K D N R A


3010 3020 3030 3040 3050 3060 3070 3080 3090 3100
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V H A D M G Y W I E S A L N D T W K I E K A S F I E V K N C H W P K
3110 3120 3130 3140 3150 3160 3170 3180 3190 3200
AATCACACACCCTCTGGAGCAATGGAGTGCTAGAAAGTGAGATGATAATTCCAAAGAATCTCGCTGGACCAGTGTCTCAACACAACTATAGACCAGGCTA
S H T L W S N G V L E S E M I I P K N L A G P V S Q H N Y R P G Y
3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
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H T Q I T G P W H L G K L E M D F D F C D G T T V V V T E D C G N
3310 3320 3330 3340 3350 3360 3370 3380 3390 3400
AGAGGACCCTCTTTGAGAACAACCACTGCCTCTGGAAAACTCATAACAGAATGGTGCTGCCGATCTTGCACATTACCACCGCTAAGATACAGAGGTGAGG
R G P S L R T T T A S G K L I T E W C C R S C T L P P L R Y R G E D
>NS2A
3410 3420 3430 3440 3450 3460 3470 3480 3490 3500
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G C W Y G M E I R P L K E K E E N L V N S L V T A G H G Q V D N F
3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
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S L G V L G M A L F L E E M L R T R V G T K H A I L L V A V S F V
3610 3620 3630 3640 3650 3660 3670 3680 3690 3700
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3710 3720 3730 3740 3750 3760 3770 3780 3790 3800
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A L L A A F K V R P T F A A G L L L R K L T S K E L M M T T I G I
3810 3820 3830 3840 3850 3860 3870 3880 3890 3900
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V L L S Q S T L P E T I L E L T D A L A L G M M V L K M V R N M E
Additional NS2A-116 Ile-to-Leu (nt3823 A-to-C) mutation in master and pre-master seed
3910 3920 3930 3940 3950 3960 3970 3980 3990 4000
AAGTATCAATTGGCAGTGACTATCATGGCTATCTTGTGCGTCCCAAACGCAGTGATATTACAAAACGCATGGAAAGTGAGTTGCACAATATTGGCAGTGG
K Y Q L A V T I M A I L C V P N A V I L Q N A W K V S C T I L A V V
4010 4020 4030 4040 4050 4060 4070 4080 4090 4100
TGTCCGTTTCCCCACTGTTCTTAACATCCTCACAGCAAAAAACAGATTGGATACCATTAGCATTGACGATCAAAGGTCTCAATCCAACAGCTATTTTTCT
S V S P L F L T S S Q Q K T D W I P L A L T I K G L N P T A I F L
D2 PDK-53 specific NS2A-181-Phe (wt D2 16681: Leu, nt-4018-C)
>NS2B
4110 4120 4130 4140 4150 4160 4170 4180 4190 4200
AACAACCCTCTCAAGAACCAGCAAGAAAAGGAGCTGGCCATTAAATGAGGCTATCATGGCAGTCGGGATGGTGAGCATTTTAGCCAGTTCTCTCCTAAAA
T T L S R T S K K R S W P L N E A I M A V G M V S I L A S S L L K
4210 4220 4230 4240 4250 4260 4270 4280 4290 4300
AATGATATTCCCATGACAGGACCATTAGTGGCTGGAGGGCTCCTCACTGTGTGCTACGTGCTCACTGGACGATCGGCCGATTTGGAACTGGAGAGAGCAG
N D I P M T G P L V A G G L L T V C Y V L T G R S A D L E L E R A A
4310 4320 4330 4340 4350 4360 4370 4380 4390 4400
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D V K W E D Q A E I S G S S P I L S I T I S E D G S M S I K N E E
4410 4420 4430 4440 4450 4460 4470 4480 4490 4500
GGAAGATCAAACACTGACCATACTCATTAGAACAGGATTGCTGGTGATCTCAGGACTTTTTCCTGTATCAATACCAATCACGGCAGCAGCATGGTACCTG
E D Q T L T I L I R T G L L V I S G L F P V S I P I T A A A W Y L
Additional NS2B-92 Glu-to-Asp (nt-4407 A-to-T) mutation (in master and pre-master seed)
>NS3
4510 4520 4530 4540 4550 4560 4570 4580 4590 4600
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W E V K K Q R A G V L W D V P S P P P M G K A E L E D G A Y R I K Q
4610 4620 4630 4640 4650 4660 4670 4680 4690 4700
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K G I L G Y S Q I G A G V Y K E G T F H T M W H V T R G A V L M H
4710 4720 4730 4740 4750 4760 4770 4780 4790 4800
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K G K R I E P S W A D V K K D L I S Y G G G W K L E G E W K E G E
4810 4820 4830 4840 4850 4860 4870 4880 4890 4900
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E V Q V L A L E P G K N P R A V Q T K P G L F K T N A G T I G A V S
4910 4920 4930 4940 4950 4960 4970 4980 4990 5000
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L D F S P G T S G S P I I D K K G K V V G L Y G N G V V T R S G A
5010 5020 5030 5040 5050 5060 5070 5080 5090 5100
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Y V S A I A Q T E K S I E D N P E I E D D I F R K R R L T I M D L
5110 5120 5130 5140 5150 5160 5170 5180 5190 5200
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H P G A G K T K R Y L P A I V R E A I K R G L R T L I L A P T R V V
5210 5220 5230 5240 5250 5260 5270 5280 5290 5300
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A A E M E E A L R G L P I R Y Q T P A I R A V H T G R E I V D L M
D2 PDK-53 NS3-250-Val attenuation locus (wt D2 16681: Glu, nt-5270-A)
5310 5320 5330 5340 5350 5360 5370 5380 5390 5400
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C H A T F T M R L L S P V R V P N Y N L I I M D E A H F T D P A S
5410 5420 5430 5440 5450 5460 5470 5480 5490 5500
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I A A R G Y I S T R V E M G E A A G I F M T A T P P G S R D P F P Q
5510 5520 5530 5540 5550 5560 5570 5580 5590 5600
AGAGCAATGCACCAATCATAGATGAAGAAAGAGAAATCCCTGAACGCTCGTGGAATTCCGGACATGAATGGGTCACGGATTTTAAAGGGAAGACTGTTTG
S N A P I I D E E R E I P E R S W N S G H E W V T D F K G K T V W
D2 PDK-53 silent mutation nt-5547-C (wt D2 16681: T)
5610 5620 5630 5640 5650 5660 5670 5680 5690 5700
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F V P S I K A G N D I A A C L R K N G K K V I Q L S R K T F D S E
5710 5720 5730 5740 5750 5760 5770 5780 5790 5800
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Y V K T R T N D W D F V V T T D I S E M G A N F K A E R V I D P R R
5810 5820 5830 5840 5850 5860 5870 5880 5890 5900
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C M K P V I L T D G E E R V I L A G P M P V T H S S A A Q R R G R
5910 5920 5930 5940 5950 5960 5970 5980 5990 6000
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6010 6020 6030 6040 6050 6060 6070 6080 6090 6100
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6110 6120 6130 6140 6150 6160 6170 6180 6190 6200
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6210 6220 6230 6240 6250 6260 6270 6280 6290 6300
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R W C F D G V K N N Q I L E E N V E V E I W T K E G E R K K L K P
>NS4A


6310 6320 6330 6340 6350 6360 6370 6380 6390 6400
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R W L D A R I Y S D P L A L K E F K E F A A G R K S L T L N L I T E
6410 6420 6430 6440 6450 6460 6470 6480 6490 6500
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M G R L P T F M T Q K A R D A L D N L A V L H T A E A G G R A Y N
6510 6520 6530 6540 6550 6560 6570 6580 6590 6600
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H A L S E L P E T L E T L L L L T L L A T V T G G I F L F L M S A
D2 PDK-53 specific NS4A-75-Ala (wt D2 16681: Gly, nt-6599-G)
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700
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R G I G K M T L G M C C I I T A S I L L W Y A Q I Q P H W I A A S I
6710 6720 6730 6740 6750 6760 6770 6780 6790 6800
TAATACTGGAGTTTTTTCTCATAGTTTTGCTTATTCCAGAACCTGAAAAACAGAGAACACCCCAAGACAACCAACTGACCTACGTTGTCATAGCCATCCT
I L E F F L I V L L I P E P E K Q R T P Q D N Q L T Y V V I A I L
>NS4B
6810 6820 6830 6840 6850 6860 6870 6880 6890 6900
CACAGTGGTGGCCGCAACCATGGCAAACGAGATGGGTTTCCTAGAAAAAACGAAGAAAGATCTCGGATTGGGAAGCATTGCAACCCAGCAACCCGAGAGC
T V V A A T M A N E M G F L E K T K K D L G L G S I A T Q Q P E S
6910 6920 6930 6940 6950 6960 6970 6980 6990 7000
AACATCCTGGACATAGATCTACGTCCTGCATCAGCATGGACGCTGTATGCCGTGGCCACAACATTTGTTACACCAATGTTGAGACATAGCATTGAAAATT
N I L D I D L R P A S A W T L Y A V A T T F V T P M L R H S I E N S
7010 7020 7030 7040 7050 7060 7070 7080 7090 7100
CCTCAGTGAATGTGTCCCTAACAGCTATAGCCAACCAAGCCACAGTGTTAATGGGTCTCGGGAAAGGATGGCCATTGTCAAAGATGGACATCGGAGTTCC
S V N V S L T A I A N Q A T V L M G L G K G W P L S K M D I G V P
7110 7120 7130 7140 7150 7160 7170 7180 7190 7200
CCTTCTCGCCATTGGATGCTACTCACAAGTCAACCCCATAACTCTCACAGCAGCTCTTTTCTTATTGGTAGCACATTATGCCATCATAGGGCCAGGACTC
L L A I G C Y S Q V N P I T L T A A L F L L V A H Y A I I G P G L
7210 7220 7230 7240 7250 7260 7270 7280 7290 7300
CAAGCAAAAGCAACCAGAGAAGCTCAGAAAAGAGCAGCGGCGGGCATCATGAAAAACCCAACTGTCGATGGAATAACAGTGATTGACCTAGATCCAATAC
Q A K A T R E A Q K R A A A A G I M K N P T V D G I T V I D L D P I P
7310 7320 7330 7340 7350 7360 7370 7380 7390 7400
CTTATGATCCGAAGTTTGAAAAGCAGTTGGGACAAGTAATGCTCCTAGTCCTCTGCGTGACTCAAGTATTGATGATGAGGACTACATGGGCTCTGTGTGA
Y D P K F E K Q L G Q V M L L V L C V T Q V L M M R T T W A L C E
Additional silent mutation (nt-7311 A-to-G, in master and pre-master seed)
7410 7420 7430 7440 7450 7460 7470 7480 7490 7500
GGCTTTAACCTTAGCTACCGGGCCCATCTCCACATTGTGGGAAGGAAATCCAGGGAGGTTTTGGAACACTACCATTGCGGTGTCAATGGCTAACATTTTT
A L T L A T G P I S T L W E G N P G R F W N T T I A V S M A N I F
> NS5
7510 7520 7530 7540 7550 7560 7570 7580 7590 7600
AGAGGGAGTTACTTGGCCGGAGCTGGACTTCTCTTTTCTATTATGAAGAACACAACCAACACAAGAAGGGGAACTGGCAACATAGGAGAGACGCTTGGAG
R G S Y L A G A G L L F S I M K N T T N T R R G T G N I G E T L G E
7610 7620 7630 7640 7650 7660 7670 7680 7690 7700
AGAAATGGAAAAGCCGATTGAACGCATTGGGAAAAAGTGAATTCCAGATCTACAAGAAAAGTGGAATCCAGGAAGTGGATAGAACCTTAGCAAAAGAAGG
K W K S R L N A L G K S E F Q I Y K K S G I Q E V D R T L A K E G
7710 7720 7730 7740 7750 7760 7770 7780 7790 7800
CATTAAAAGAGGAGAAACGGACCATCACGCTGTGTCGCGAGGCTCAGCAAAACTGAGATGGTTCGTTGAGAGAAACATGGTCACACCAGAAGGGAAAGTA
I K R G E T D H H A V S R G S A K L R W F V E R N M V T P E G K V
7810 7820 7830 7840 7850 7860 7870 7880 7890 7900
GTGGACCTCGGTTGTGGCAGAGGAGGCTGGTCATACTATTGTGGAGGACTAAAGAATGTAAGAGAAGTCAAAGGCCTAACAAAAGGAGGACCAGGACACG
V D L G C G R G G W S Y Y C G G L K N V R E V K G L T K G G P G H E
7910 7920 7930 7940 7950 7960 7970 7980 7990 8000
AAGAACCCATCCCCATGTCAACATATGGGTGGAATCTAGTGGGTCTTCAAAGTGGAGTTGACGTTTTCTTCATCCCGCCAGAAAAGTGTGACACATTATT
E P I P M S T Y G W N L V R L Q S G V D V F F I P P E K C D T L L
8010 8020 8030 8040 8050 8060 8070 8080 8090 8100
GTGTGACATAGGGGAGTCATCACCAAATCCCACAGTGGAAGCAGGACGAACACTCAGAGTCCTTAACTTAGTAGAAAATTGGTTGAACAACAACACTCAA
C D I G E S S P N P T V E A G R T L R V L N L V E N W L N N N T Q

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      8110       8120       8130       8140       8150       8160       8170       8180       8190       8200
TTTTGCATAAAGGTTCTCAACCCATATATGCCCTCAGTCATAGAAAAAATGGAAGCACTACAAAGGAAATATGGAGGAGCCTTAGTGAGGAATCCACTCT
F  C  I  K  V  L  N  P  Y  M  P  S  V  I  E  K  M  E  A  L  Q  R  K  Y  G  G  A  L  V  R  N  P  L  S

      8210       8220       8230       8240       8250       8260       8270       8280       8290       8300
CACGAAACTCCACACATGAGATGTACTGGGTATCCAATGCTTCCGGGAACATAGTGTCATCAGTGAACATGATTTCAAGGATGTTGATCAACAGATTTAC
  R  N  S  T  H  E  M  Y  W  V  S  N  A  S  G  N  I  V  S  S  V  N  M  I  S  R  M  L  I  N  R  F  T

      8310       8320       8330       8340       8350       8360       8370       8380       8390       8400
AATGAGATACAAGAAAGCCACTTACGAGCCGGATGTTGACCTCGGAAGCGGAACCCGTAACATCGGGATTGAAAGTGAGATACCAAACCTAGATATAATT
  M  R  Y  K  K  A  T  Y  E  P  D  V  D  L  G  S  G  T  R  N  I  G  I  E  S  E  I  P  N  L  D  I  I

      8410       8420       8430       8440       8450       8460       8470       8480       8490       8500
GGGAAAAGAATAGAAAAAATAAAGCAAGAGCATGAAACATCATGGCACTATGACCAAGACCACCCATACAAAACGTGGGCATACCATGGTAGCTATGAAA
G  K  R  I  E  K  I  K  Q  E  H  E  T  S  W  H  Y  D  Q  D  H  P  Y  K  T  W  A  Y  H  G  S  Y  E  T

      8510       8520       8530       8540       8550       8560       8570       8580       8590       8600
CAAAACAGACTGGATCAGCATCATCCATGGTCAACGGAGTGGTCAGGCTGCTGACAAAACCTTGGGACGTCGTCCCCATGGTGACACAGATGGCAATGAC
  K  Q  T  G  S  A  S  S  M  V  N  G  V  V  R  L  L  T  K  P  W  D  V  V  P  M  V  T  Q  M  A  M  T

      8610       8620       8630       8640       8650       8660       8670       8680       8690       8700
AGACACGACTCCATTTGGACAACAGCGCGTTTTTAAAGAGAAAGTGGACACGAGAACCCAAGAACCGAAAGAAGGCACGAAGAAACTAATGAAAATAACA
  D  T  T  P  F  G  Q  Q  R  V  F  K  E  K  V  D  T  R  T  Q  E  P  K  E  G  T  K  K  L  M  K  I  T

      8710       8720       8730       8740       8750       8760       8770       8780       8790       8800
GCAGAGTGGCTTTGGAAAGAATTAGGGAAGAAAAAGACACCCAGGATGTGCACCAGAGAAGAATTCACAAGAAAGGTGAGAAGCAATGCAGCCTTGGGGG
A  E  W  L  W  K  E  L  G  K  K  K  T  P  R  M  C  T  R  E  E  F  T  R  K  V  R  S  N  A  A  L  G  A

      8810       8820       8830       8840       8850       8860       8870       8880       8890       8900
CCATATTCACTGATGAGAACAAGTGGAAGTCGCCACGTGAGGCTGTTGAAGATAGTAGGTTTTGGGAGCTGGTTGACAAGGAAAGGAATCTCCATCTTGA
  I  F  T  D  E  N  K  W  K  S  A  R  E  A  V  E  D  S  R  F  W  E  L  V  D  K  E  R  N  L  H  L  E

      8910       8920       8930       8940       8950       8960       8970       8980       8990       9000
AGGAAAGTGTGAAACATGTGTGTACAACATGATGGGAAAAAGAGAGAAGAAGCTAGGGGAATTCGGCAAGGCAAAAGGCAGCAGAGCCATATGGTACATG
G  K  C  E  T  C  V  Y  N  M  M  G  K  R  E  K  K  L  G  E  F  G  K  A  K  G  S  R  A  I  W  Y  M

      9010       9020       9030       9040       9050       9060       9070       9080       9090       9100
TGGCTTGGAGCACGCTTCTTAGAGTTTGAAGCCCTAGGATTCTTAAATGAAGATCACTGGTTCTCCAGAGAGAACTCCCTGAGTGGAGTGGAAGGAGAAG
W  L  G  A  R  F  L  E  F  E  A  L  G  F  L  N  E  D  H  W  F  S  R  E  N  S  L  S  G  V  E  G  E  G

      9110       9120       9130       9140       9150       9160       9170       9180       9190       9200
GGCTGCACAAGCTAGGTTACATTCTAAGAGACGTGAGCAAGAAACAGGGAGGAGCAATGTATGCCGATGACACCGCAGGATGGGATACAAGAATCACACT
  L  H  K  L  G  Y  I  L  R  D  V  S  K  K  E  G  G  A  M  Y  A  D  D  T  A  G  W  D  T  R  I  T  L

      9210       9220       9230       9240       9250       9260       9270       9280       9290       9300
AGAAGACCTAAAAAATGAAGAAATGGTAACAAACCACATGGAAGGAGAACACAAGAAACTAGCCGAGGCCATTTTCAAACTAACGTACCAAAACAAGGTG
E  D  L  K  N  E  E  M  V  T  N  H  M  E  G  E  H  K  K  L  A  E  A  I  F  K  L  T  Y  Q  N  K  V

      9310       9320       9330       9340       9350       9360       9370       9380       9390       9400
GTGCGTGTGCAAAGACCAACACCAAGAGGCACAGTAATGGACATCATATCGAGAAGAGACCAAAGAGGTAGTGGACAAGTTGGCACCTATGGACTCAATA
V  R  V  Q  R  P  T  P  R  G  T  V  M  D  I  I  S  R  R  D  Q  R  G  S  G  Q  V  G  T  Y  G  L  N  T

      9410       9420       9430       9440       9450       9460       9470       9480       9490       9500
CTTTCACCAATATGGAAGCCCAACTAATCAGACAGATGGAGGGAGAAGGAGTCTTTAAAAGCATTCAGCACCTAACAATCACAGAAGAAATCGCTGTGCA
  F  T  N  M  E  A  Q  L  I  R  Q  M  E  G  E  G  V  F  K  S  I  Q  H  L  T  I  T  E  E  I  A  V  Q
      9510       9520       9530       9540       9550       9560       9570       9580       9590       9600
AAACTGGTTAGCAAGAGTGGGGCGCGAAAGGTTATCAAGAATGGCCATCAGTGGAGATGATTGTGTTGTGAAACCTTTAGATGACAGGTTCGCAAGCGCT
N  W  L  A  R  V  G  R  E  R  L  S  R  M  A  I  S  G  D  D  C  V  V  K  P  L  D  D  R  F  A  S  A

      9610       9620       9630       9640       9650       9660       9670       9680       9690       9700
TTAACAGCTCTAAATGACATGGGAAAGATTAGGAAAGACATACAACAATGGGAACCTTCAAGAGGATGGAATGATTGGACACAAGTGCCCTTCTGTTCAC
L  T  A  L  N  D  M  G  K  I  R  K  D  I  Q  Q  W  E  P  S  R  G  W  N  D  W  T  Q  V  P  F  C  S  H

      9710       9720       9730       9740       9750       9760       9770       9780       9790       9800
ACCATTTCCATGAGTTAATCATGAAAGACGGTCGCGTACTCGTTGTTCCATGTAGAAACCAAGATGAACTGATTGGCAGAGCCCGAATCTCCCAAGGAGC
  H  F  H  E  L  I  M  K  D  G  R  V  L  V  V  P  C  R  N  Q  D  E  L  I  G  R  A  R  I  S  Q  G  A

      9810       9820       9830       9840       9850       9860       9870       9880       9890       9900
AGGGTGGTCTTTGCGGGAGACGGCCTGTTTGGGGAAGTCTTACGCCCAAATGTGGAGCTTGATGTACTTCCACAGACGCGACCTCAGGCTGGCGGCAAAT
G  W  S  L  R  E  T  A  C  L  G  K  S  Y  A  Q  M  W  S  L  M  Y  F  H  R  R  D  L  R  L  A  A  N

      9910       9920       9930       9940       9950       9960       9970       9980       9990      10000
GCTATTTGCTCGGCAGTACCATCACATTGGGTTCCAACAAGTCGAACCTGGTCCATACATGCTAAACATGAATGGATGACAACGGAAGACATGCTGA
A  I  C  S  A  V  P  S  H  W  V  P  T  S  R  T  T  W  S  I  H  A  K  H  E  W  M  T  T  E  D  M  L  T

     10010      10020      10030      10040      10050      10060      10070      10080      10090      10100
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CAGTCTGGAACAGGGTGTGGATTCAAGAAAACCCATGGATGGAAGACAAAACTCCAGTGGAATCATGGGAGGAAATCCCATACTTGGGGAAAAGAGAAGA
  V  W  N  R  V  W  I  Q  E  N  P  W  M  E  D  K  T  P  V  E  S  W  E  E  I  P  Y  L  G  K  R  E  D

     10110      10120      10130      10140      10150      10160      10170      10180      10190      10200
CCAATGGTGCGGCTCATTGATTGGGTTAACAAGCAGGGCCCACCTGGGCAAAGAACATCCAAGCAGCAATAAATCAAGTTAGATCCCTTATAGGCAATGAA
 Q  W  C  G  S  L  I  G  L  T  S  R  A  T  W  A  K  N  I  Q  A  A  I  N  Q  V  R  S  L  I  G  N  E

                                                                                    >3'-Noncoding Region
     10210      10220      10230      10240      10250      10260      10270      10280      10290      10300
GAATACACAGATTACATGCCATCCATGAAAAGATTCAGAAGAGAAGAGGAAGAAGCAGGAGTTCTGTGGTAGAAAGCAAAACTAACATGAAACAAGGCTA
E  Y  T  D  Y  M  P  S  M  K  R  F  R  R  E  E  E  E  A  G  V  L  W  *

     10310      10320      10330      10340      10350      10360      10370      10380      10390      10400
GAAGTCAGGTCGGATTAAGCCATAGTACGGAAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCATCATAAATGCCATAG

     10410      10420      10430      10440      10450      10460      10470      10480      10490      10500
CTTGAGTAAACTATGCAGCCTGTAGCTCCACCTGAGAAGGTGTAAAAAATCCGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGC

     10510      10520      10530      10540      10550      10560      10570      10580      10590      10600
GGTTAGAGGAGACCCCTCCCTTACAAATCGCAGCAACAATGGGGGCCCAAGGCGAGATGAAGCTGTAGTCTCGCTGGAAGGACTAGAGGTTAGAGGAGAC

     10610      10620      10630      10640      10650      10660      10670      10680      10690      10700
CCCCCCGAAACAAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACGCCAGAAAATGGAATG

     10710      10720
GTGCTGTTGAATCAACAGGTTCT
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DENvax-2 Master Virus Seed (MVS)

[000158] Nucleotide sequence of the recombinant viral genome and deduced amino acid sequence of the translated protein are provided herein. The engineered virus is based on D2 PDK-53 virus. All engineered substitutions that are different from wild-type DEN-2 16681 virus (also the parental virus for PDK-53), as well as extra mutations (changes from engineered cDNA clone) detected in the MVS are marked.

Substitutions Included in the Genome and Protein:

[000159] D2 PDK-53 virus backbone (change from wt D2 16681): all in bold

a. 5'-noncoding region (NCR)-57 (nt-57 C-to-T): major attenuation locus (in red)
b. prM-29 Asp-to-Val (nt-524 A-to-T)
c. nt-2055 C-to-T (E gene) silent mutation
d. NS1-53 Gly-to-Asp (nt-2579 G-to-A): major attenuation locus (in red)
e. NS2A-181 Leu-to-Phe (nt-4018 C-to-T)
f. NS3-250 Glu-to-Val (nt-5270 A-to-T): major attenuation locus (in red)
g. nt-5547 (NS3 gene) T-to-C silent mutation
h. NS4A-75 Gly-to-Ala (nt-6599 G-to-C)

* nt-8571 C-to-T silent mutation of PDK-53 is not engineered in the vaccine virus

[000160] Engineered clone marker (silent mutation):

a. nt-900 T-to-C silent mutation: infectious clone marker

[000161] Additional substitutions found in vaccine seed (0.02% nt different from original clone)

a. prM-52 Lys-to-Glu (nt-592 A-to-G), in bold
b. NS5-412 Ile-to-Val (nt-8803 A-to-G), in bold

```
                                  NCR-57-T, D2 PDK-53 attenuation locus (wt D2 16681: C)
>5'-NC                                          !                                        >C
     10        20        30        40        50        60        70        80        90       100       110       120
AGTTGCTAGTGTACGTGGAACCGACAAAGAGAAGACTCTTTCGAGGAGGCTAAGCTCAAGCCAAGCGTCGTAACAGTTTCTTCAATTACAGCAGACATCTCTGATTGAATAACCAACGCAAAAAGGCC
                                                                                    M  N  N  Q  R  K  K  A
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      130       140       150       160       170       180       190       200       210       220       230       240
AAAAACACGCCTTTCAATATGCTGAAACGCGAGAGAAACCGCGTGTCGACTGTGCAACAGCTGACAAAGAGATTCTCACTTGGAATGCTGCAGGGACGAGGACCATTAAAACTGTTCATG
K  N  T  P  F  N  M  L  K  R  E  R  N  R  V  S  T  V  Q  Q  L  T  K  R  F  S  L  G  M  L  Q  G  R  G  P  L  K  L  F  M

      250       260       270       280       290       300       310       320       330       340       350       360
GCCCTGGTGGCGTTCCTTCGTTTCCTAACAATCCCACCAACAGCAGGGATATTGAAGAGATGGGGAACAATTAAAAAATCAAAAGCTATTAATGTTTTGAGAGGGTTCAGGAAAGAGATT
A  L  V  A  F  L  R  F  L  T  I  P  P  T  A  G  I  L  K  R  W  G  T  I  K  K  S  K  A  I  N  V  L  R  G  F  R  K  E  I

                                                                                                  >prM
      370       380       390       400       410       420       430       440       450       460       470       480
GGAAGGATGCTGAACATCTTGAATAGGAGACGCAGATCTGCAGGCATGATCATTATGCTGATTCCAACAGTGATGGCGTTCCATTTAACCACACGTAACGGAGAACCACACATGATCGTC
G  R  M  L  N  I  L  N  R  R  R  R  S  A  G  M  I  I  M  L  I  P  T  V  M  A  F  H  L  T  T  R  N  G  E  P  H  M  I  V

      490       500       510       520       530       540       550       560       570       580       590       600
AGCAGACAAGAGAAAGGGAAAAGTCTTCTGTTTAAAACAGAGGTTGGCGTGAACATGTGTACCCTCATGGCCATGGACCTTGGTGAATTGTGTGAAGACACAATCACGTACGAGTGTCCC
S  R  Q  E  K  G  K  S  L  L  F  K  T  E  V  G  V  N  M  C  T  L  M  A  M  D  L  G  E  L  C  E  D  T  I  T  Y  E  C  P
                                   |                                                                                 |
D2 PDK-53 specific prM-29 Val (wt D2 16681 Asp, nt-524-A)   Additional prM-52 Lys-to-Glu mutation (nt-592 A-to-G)

                                                                                                                  >M
      610       620       630       640       650       660       670       680       690       700       710       720
CTTCTCAGGCAGAATGAGCCAGAAGACATAGACTGTTGGTGCAACTCTACGTCCACGTGGGTAACTTATGGGACGTGTACCACCATGGGAGAACATAGAAGAGAAAAAAGATCAGTGGCA
L  L  R  Q  N  E  P  E  D  I  D  C  W  C  N  S  T  S  T  W  V  T  Y  G  T  C  T  T  M  G  E  H  R  R  E  K  R  S  V  A

      730       740       750       760       770       780       790       800       810       820       830       840
CTCGTTCCACATGTGGGAATGGGACTGGAGACACGAACTGAAACATGGATGTCATCAGAAGGGGCCTGGAAACATGTCCAGAGAATTGAAACTTGGATCTTGAGACATCCAGGCTTCACC
L  V  P  H  V  G  M  G  L  E  T  R  T  E  T  W  M  S  S  E  G  A  W  K  H  V  Q  R  I  E  T  W  I  L  R  H  P  G  F  T

                                                                                                  >E
      850       860       870       880       890       900       910       920       930       940       950       960
ATGATGGCAGCAATCCTGGCATACACCATAGGAACGACACATTTCCAAAGAGCCCTGATCTTCATCTTACTGACAGCTGTCACTCCTTCAATGACAATGCGTTGCATAGGAATGTCAAAT
M  M  A  A  I  L  A  Y  T  I  G  T  T  H  F  Q  R  A  L  I  F  I  L  L  T  A  V  T  P  S  M  T  M  R  C  I  G  M  S  N
                                                      |
                                                      Engineered silent clone marker: nt-900 T-to-C silent mutation

      970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
AGAGACTTTGTGGAAGGGGTTTCAGGAGGAAGCTGGGTTGACATAGTCTTAGAACATGGAAGCTGTGTGACGACGATGGCAAAAAACAAACCAACATTGGATTTTGAACTGATAAAAACA
R  D  F  V  E  G  V  S  G  G  S  W  V  D  I  V  L  E  H  G  S  C  V  T  T  M  A  K  N  K  P  T  L  D  F  E  L  I  K  T

     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
GAAGCCAAACAGCCTGCCACCCTAAGGAAGTACTGTATAGAGGCAAAGCTAACCAACACAACAACAGAATCTCGCTGCCCAACACAAGGGGAACCCAGCCTAAATGAAGAGCAGGACAAA
E  A  K  Q  P  A  T  L  R  K  Y  C  I  E  A  K  L  T  N  T  T  T  E  S  R  C  P  T  Q  G  E  P  S  L  N  E  E  Q  D  K

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
AGGTTCGTCTGCAAACACTCCATGGTAGACAGAGGATGGGGAAATGGATGTGGACTATTTGGAAAGGGAGGCATTGTGACCTGTGCTATGTTCAGATGCAAAAAGAACATGGAAGGAAAA
R  F  V  C  K  H  S  M  V  D  R  G  W  G  N  G  C  G  L  F  G  K  G  G  I  V  T  C  A  M  F  R  C  K  K  N  M  E  G  K

     1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
GTTGTGCAACCAGAAAACTTGGAATACACCATTGTGATAACACCTCACTCAGGGGAAGAGCATGCAGTCGGAAATGACACAGGAAAACATGGCAAGGAAATCAAAATAACACCACAGAGT
V  V  Q  P  E  N  L  E  Y  T  I  V  I  T  P  H  S  G  E  E  H  A  V  G  N  D  T  G  K  H  G  K  E  I  K  I  T  P  Q  S

     1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
TCCATCACAGAAGCAGAATTGACAGGTTATGGCACTGTCACAATGGAGTGCTCTCCAAGAACGGGCCTCGACTTCAATGAGATGGTGTTGCTGCAGATGGAAAATAAAGCTTGGCTGGTG
S  I  T  E  A  E  L  T  G  Y  G  T  V  T  M  E  C  S  P  R  T  G  L  D  F  N  E  M  V  L  L  Q  M  E  N  K  A  W  L  V

     1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
CACAGGCAATGGTTCCTAGACCTGCCGTTACCATGGTTGCCCGGAGCGGACACACAAGGGTCAAATTGGATACAGAAAGAGACATTGGTCACTTTCAAAAATCCCCATGCGAAGAAACAG
H  R  Q  W  F  L  D  L  P  L  P  W  L  P  G  A  D  T  Q  G  S  N  W  I  Q  K  E  T  L  V  T  F  K  N  P  H  A  K  K  Q

     1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
GATGTTGTTGTTTTAGGATCCCAAGAAGGGGCCATGCACACAGCACTTACAGGGGCCACAGAAATCCAAATGTCATCAGGAAACTTACTCTTCACAGGACATCTCAAGTGCAGGCTGAGA
D  V  V  V  L  G  S  Q  E  G  A  M  H  T  A  L  T  G  A  T  E  I  Q  M  S  S  G  N  L  L  F  T  G  H  L  K  C  R  L  R

     1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920
ATGGACAAGCTACAGCTCAAAGGAATGTCATACTCTATGTGCACAGGAAAGTTTAAAGTTGTGAAGGAAATAGCAGAAACACAACATGGAACAATAGTTATCAGAGTGCAATATGAAGGG
M  D  K  L  Q  L  K  G  M  S  Y  S  M  C  T  G  K  F  K  V  V  K  E  I  A  E  T  Q  H  G  T  I  V  I  R  V  Q  Y  E  G

     1930      1940      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040
GACGGCTCTCCATGCAAGATCCCTTTTGAGATAATGGATTTGGAAAAAAGACATGTCTTAGGTCGCCTGATTACAGTCAACCCAATTGTGACAGAAAAAGATAGCCCAGTCAACATAGAA
D  G  S  P  C  K  I  P  F  E  I  M  D  L  E  K  R  H  V  L  G  R  L  I  T  V  N  P  I  V  T  E  K  D  S  P  V  N  I  E

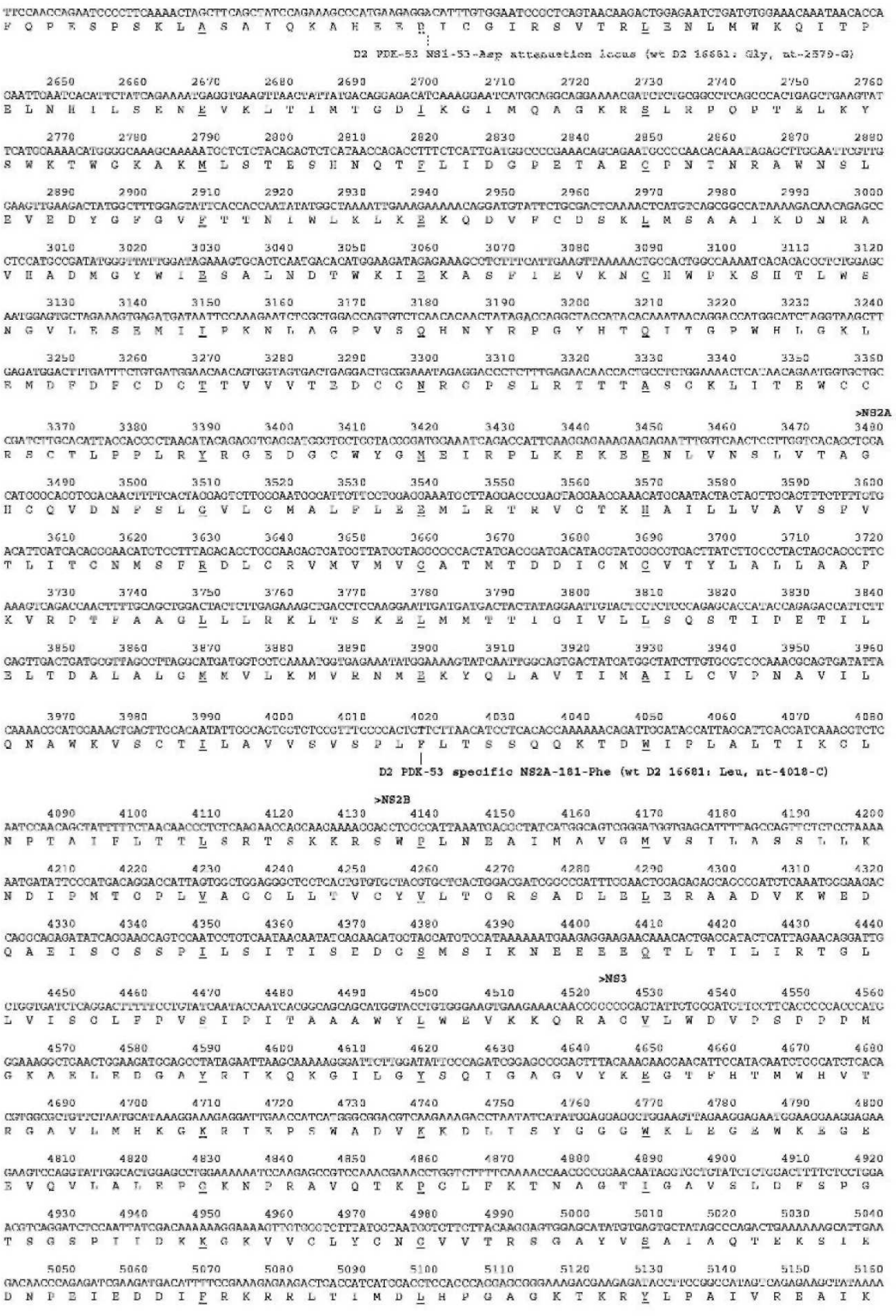
     2050      2060      2070      2080      2090      2100      2110      2120      2130      2140      2150      2160
GCAGAACCTCCATTTGGAGACAGCTACATCATCATAGGAGTAGAGCCGGGACAACTGAAGCTCAACTGGTTTAAGAAAGGAAGTTCTATCGGCCAAATGTTTGAGACAACAATGAGGGGG
A  E  P  P  F  G  D  S  Y  I  I  I  G  V  E  P  G  Q  L  K  L  N  W  F  K  K  G  S  S  I  G  Q  M  F  E  T  T  M  R  G
          |
D2 PDK-53 nt-2055-T silent mutation (D2 16681: C)

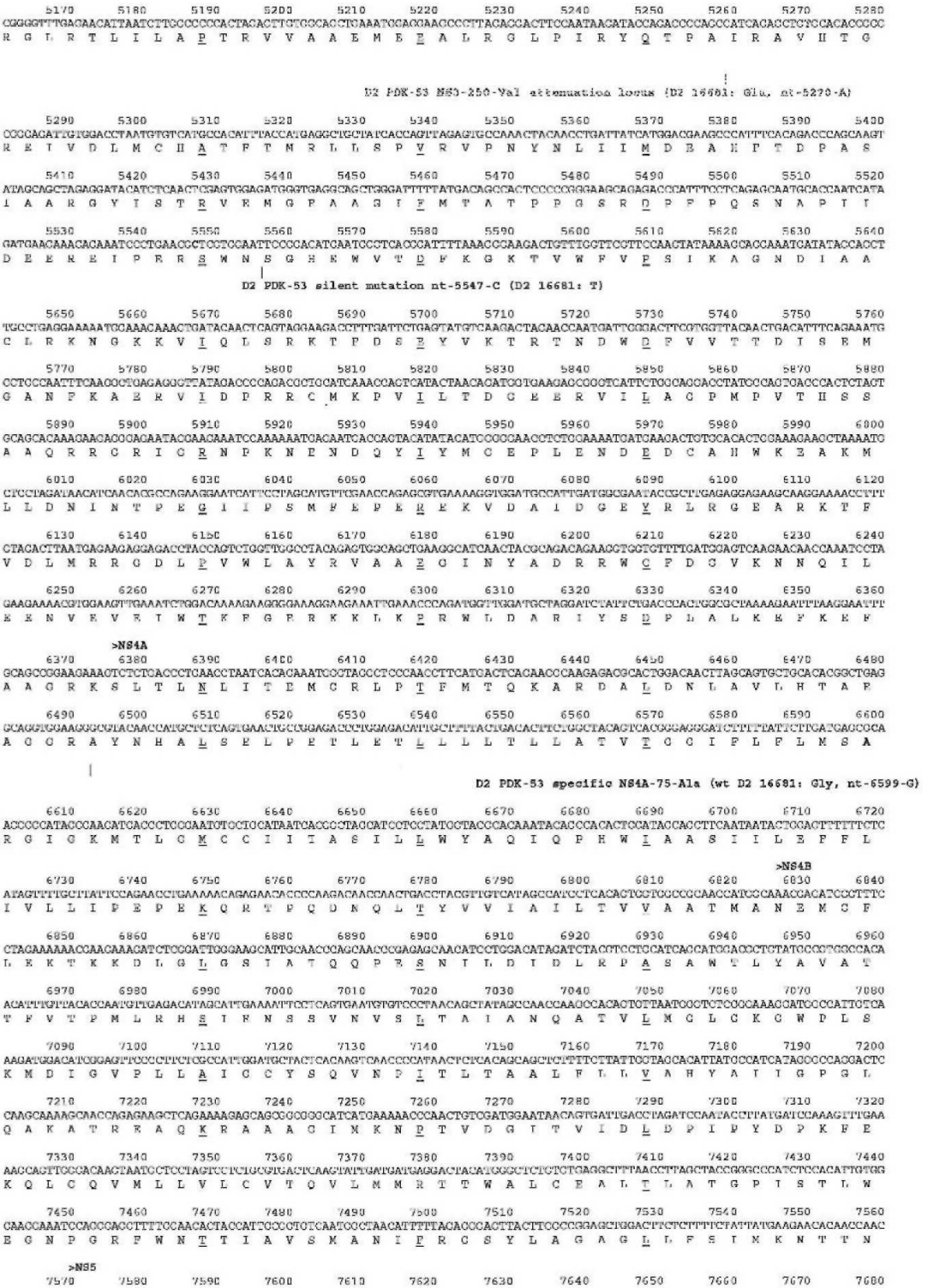
     2170      2180      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280
GCGAAGAGAATGGCCATTTTAGGTGACACAGCCTGGGATTTTGGATCCTTGGGAGGAGTGTTTACATCTATAGGAAAGGCTCTCCACCAAGTCTTTGGAGCAATCTATGGAGCTGCCTTC
A  K  R  M  A  I  L  G  D  T  A  W  D  F  G  S  L  G  G  V  F  T  S  I  G  K  A  L  H  Q  V  F  G  A  I  Y  G  A  A  F

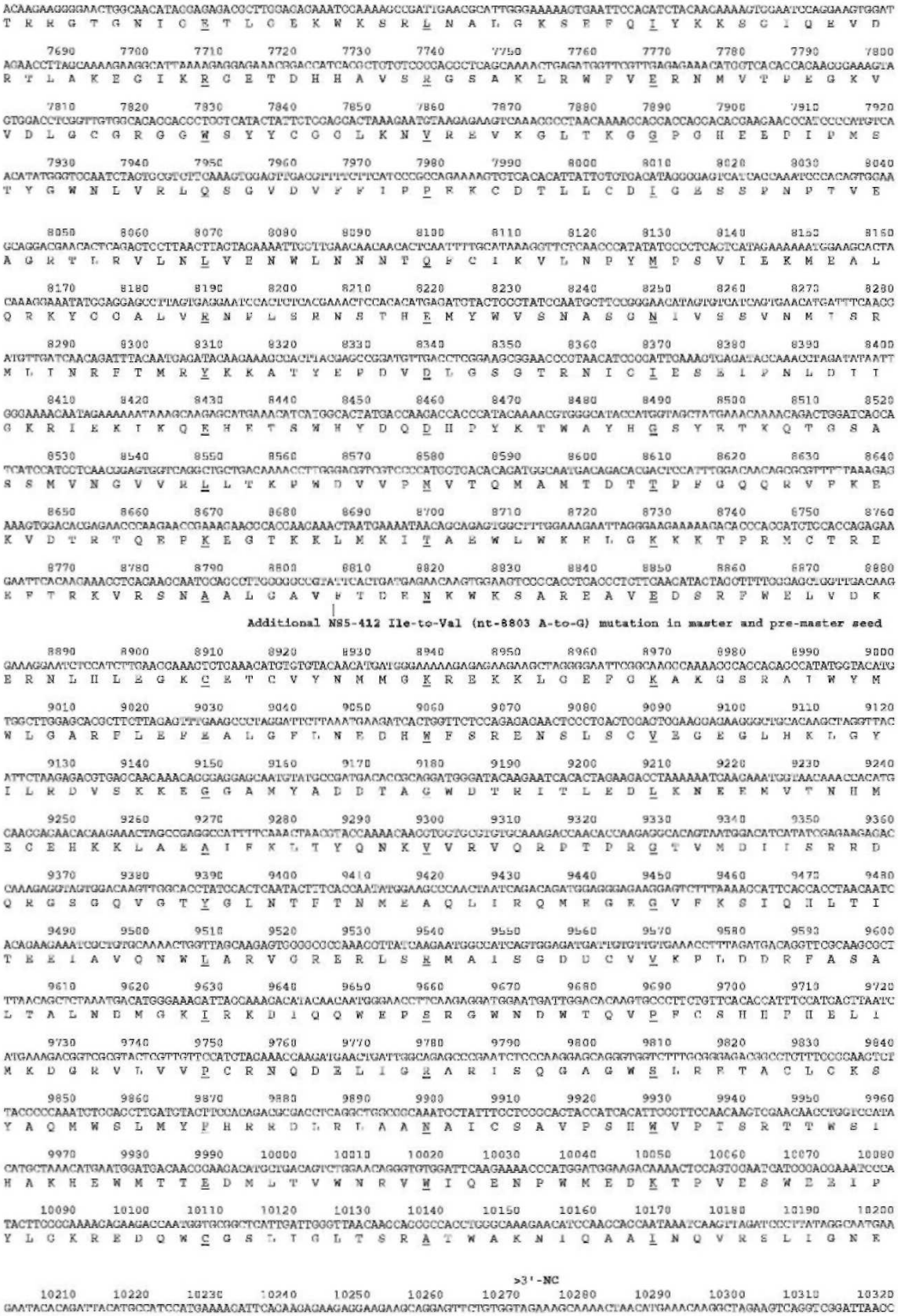
     2290      2300      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
AGTGGGGTTTCATGGACTATGAAAATCCTCATAGGAGTCATTATCACATGGATAGGAATGAATTCACGCAGCACCTCACTGTCTGTGACACTAGTATTGGTGGGAATTGTGACACTGTAT
S  G  V  S  W  T  M  K  I  L  I  G  V  I  I  T  W  I  G  M  N  S  R  S  T  S  L  S  V  T  L  V  L  V  G  I  V  T  L  Y

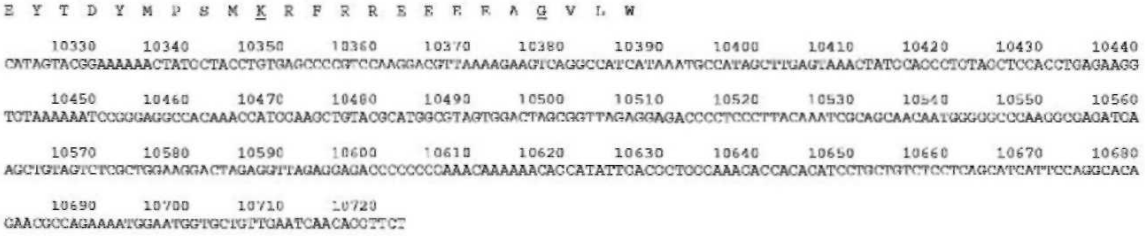
                >NS1
     2410      2420      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520
TTGGGAGTCATGGTGCAGGCCGATAGTGGTTGCGTTGTGAGCTGGAAAAACAAAGAACTGAAATGTGGCAGTGGGATTTTCATCACAGACAACGTGCACACATGGACAGAACAATACAAG
L  G  V  M  V  Q  A  D  S  G  C  V  V  S  W  K  N  K  E  L  K  C  G  S  G  I  F  I  T  D  N  V  H  T  W  T  E  Q  Y  K

     2530      2540      2550      2560      2570      2580      2590      2600      2610      2620      2630      2640
```


D2 PDK-53 NS1-53-Asp attenuation locus (wt D2 16681: Gly, nt-2579-G)
>NS2A
D2 PDK-53 specific NS2A-181-Phe (wt D2 16681: Leu, nt-4018-C)
>NS2B
>NS3


D2 PDK-53 NS5-250-Val attenuation locus (D2 16681: Glu, nt-5270-A)
D2 PDK-53 silent mutation nt-5547-C (D2 16681: T)
>NS4A
D2 PDK-53 specific NS4A-75-Ala (wt D2 16681: Gly, nt-6599-G)
>NS4B
>NS5


Additional NS5-412 Ile-to-Val (nt-8803 A-to-G) mutation in master and pre-master seed
>3'-NC



DENvax-3 Master Virus Seed (MVS)

[000162] Nucleotide sequence of the chimeric viral genome and deduced amino acid sequence of the translated protein are provided herein. Most of the prM-E gene (nt-457 to -2373, underlined) is wild-type (wt) DEN-3 16562 virus-specific; the remaining nucleotide sequence is DEN-2 PDK-53 virus-specific. The E protein of DEN-3 virus has two fewer amino acids than the E protein of DEN-2. Therefore, nt position starting from NgoMIV is 6 nt less than the original DEN-2 PDK-53 nt position. All engineered substitutions differ from wt virus (DEN-3 16562 or DEN-2 16681), as well as extra mutations (changes from engineered cDNA clone) are marked.

Substitutions Included in the Genome and Proein:

[000163] Junction sites:

a. MluI (nt 451-456): engineered silent mutation, nt-453 A-to-G
b. NgoMIV (nt 2374-2379): engineered mutations, nt-2375/2376 TG-to-CC (resulted in E-480 Val-to-Ala change)

[000164] D2 PDK-53 virus backbone (change from wt D2 16681): in bold

a. 5'-noncoding region(NCR)-57 (nt-57 C-to-T): major attenuation locus (in red)
b. NS1-53 Gly-to-Asp (nt-2573 G-to-A): major attenuation locus (in red)
c. NS2A-181 Leu-to-Phe (nt-4012 C-to-T)
d. NS3-250 Glu-to-Val (nt-5264 A-to-T): major attenuation locus (in red)
e. nt-5541 (NS3 gene) T-to-C silent mutation
f. NS4A-75 Gly-to-Ala (nt-6593 G-to-C)

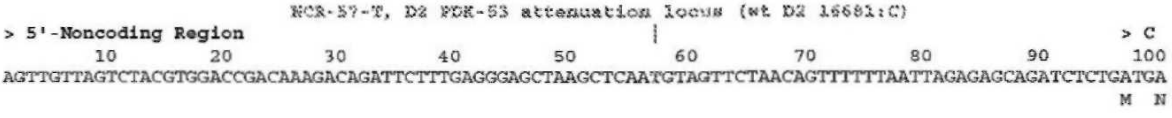
* nt-8565 C-to-T silent mutation of PDK-53 is not engineered in the vaccine virus

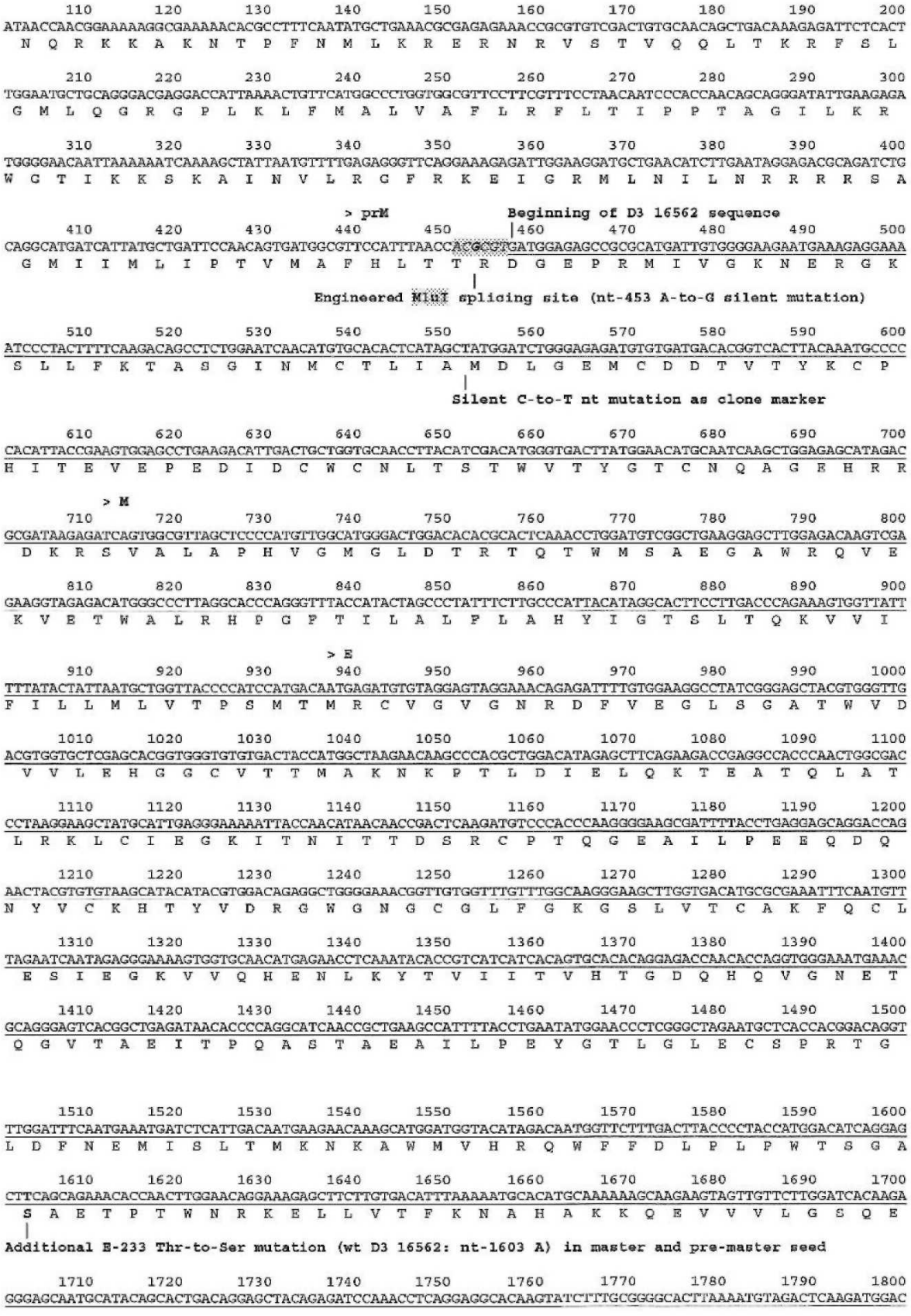
[000165] Engineered mutation in DEN-3 prM-E (change from wt D3 16562)

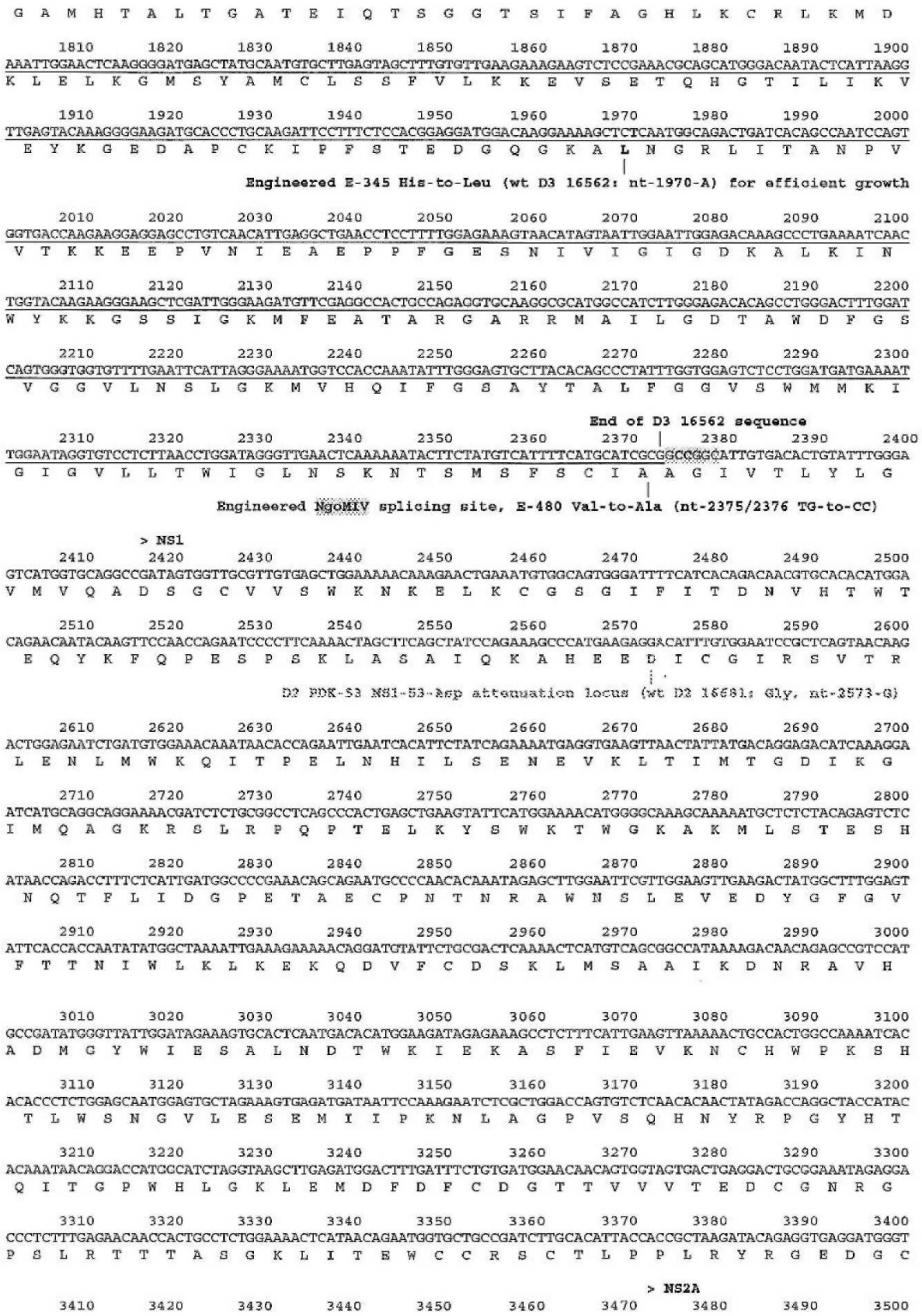
a. Engineered nt-552 C-to-T silent mutation: clone marker
b. Engineered E-345 His-to-Leu (nt-1970 A-to-T) for efficient replication in cultures

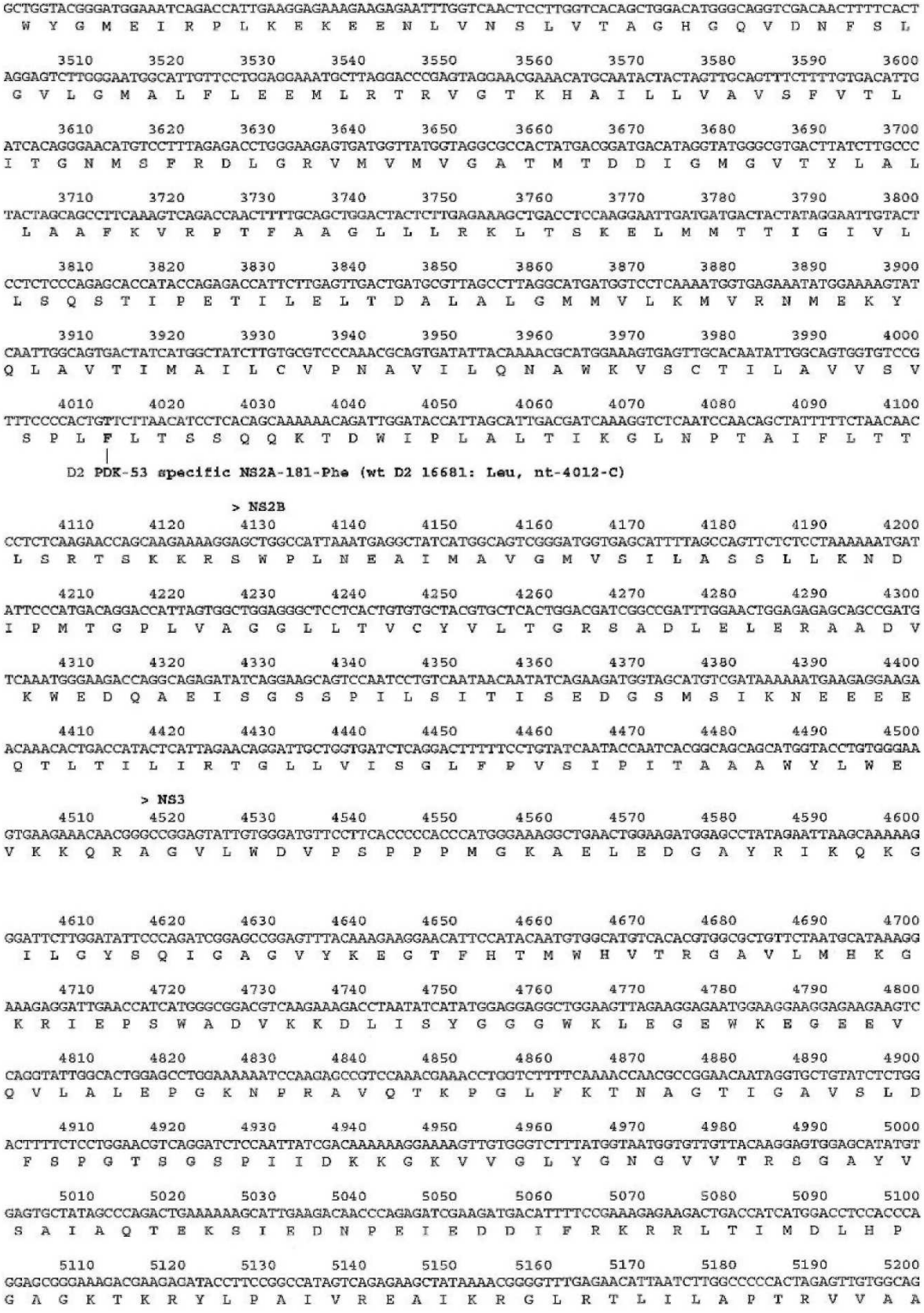
[000166] Additional substitutions found in vaccine seed (0.02% nt different from original clone)

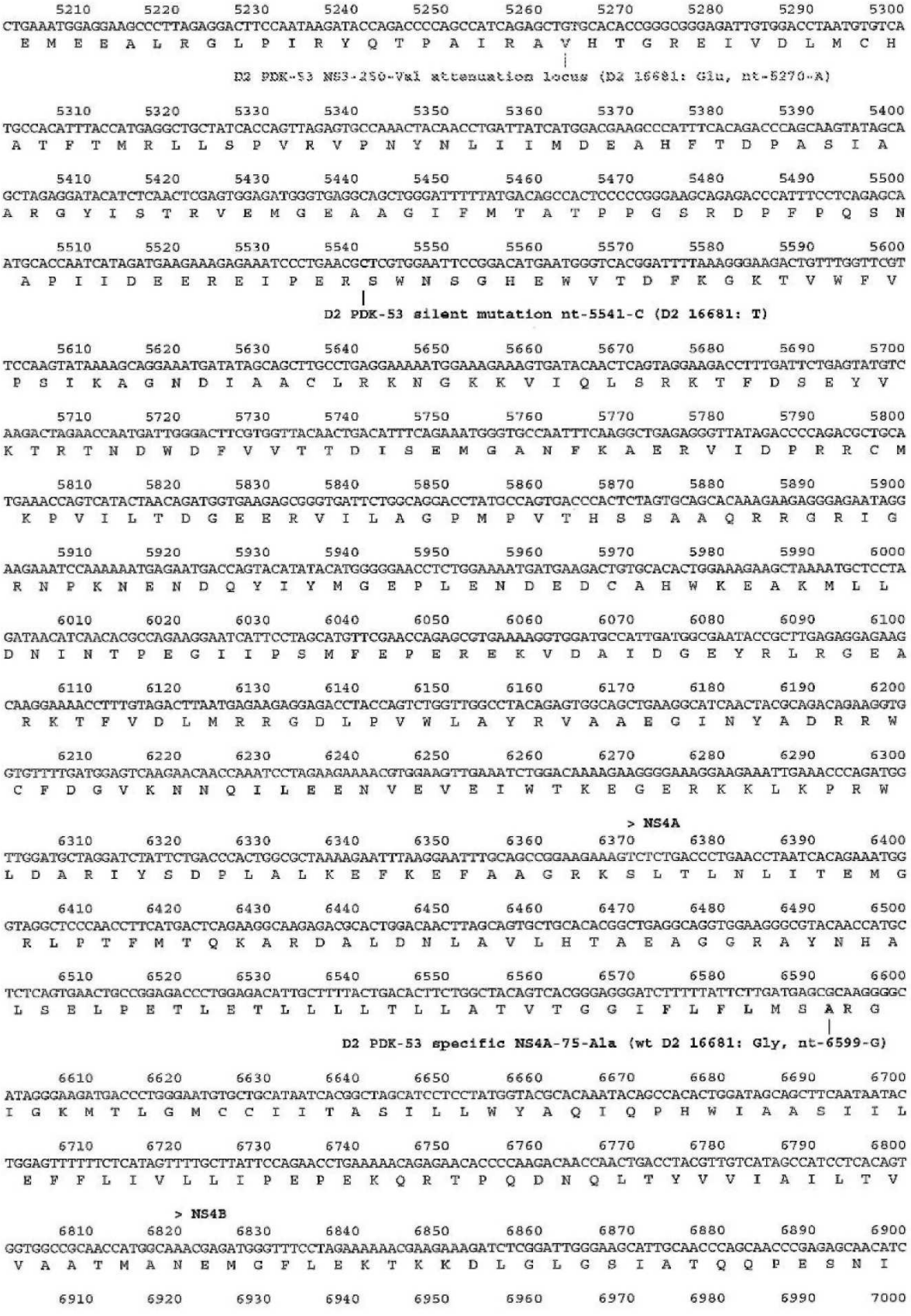
a. E-223 Thr-to-Ser mutation (nt-1603 A-to-T, in bold)
b. nt-7620 A-to-G silent mutation (in bold)




> prM
Beginning of D3 16562 sequence
Engineered MluI splicing site (nt-453 A-to-G silent mutation)
Silent C-to-T nt mutation as clone marker
> M
> E
Additional E-233 Thr-to-Ser mutation (wt D3 16562: nt-1603 A) in master and pre-master seed


G A M H T A L T G A T E I Q T S G G T S I F A G H L K C R L K M D
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
AAATTGGAACTCAAGGGGATGAGCTATGCAATGTGCTTGAGTAGCTTTGTGTTGAAGAAAGAAGTCTCCGAAACGCAGCATGGGACAATACTCATTAAGG
K L E L K G M S Y A M C L S S F V L K K E V S E T Q H G T I L I K V
1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
TTGAGTACAAAGGGGAAGATGCACCCTGCAAGATTCCTTTCTCCACGGAGGATGGACAAGGAAAAGCTCTCAATGGCAGACTGATCACAGCCAATCCAGT
E Y K G E D A P C K I P F S T E D G Q G K A L N G R L I T A N P V
Engineered E-345 His-to-Leu (wt D3 16562: nt-1970-A) for efficient growth
2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
GGTGACCAAGAAGGAGGAGCCTGTCAACATTGAGGCTGAACCTCCTTTTGGAGAAAGTAACATAGTAATTGGAATTGGAGACAAAGCCCTGAAAATCAAC
V T K K E E P V N I E A E P P F G E S N I V I G I G D K A L K I N
2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
TGGTACAAGAAGGGAAGCTCGATTGGGAAGATGTTCGAGGCCACTGCCAGAGGTGCAAGGCGCATGGCCATCTTGGGAGACACAGCCTGGGACTTTGGAT
W Y K K G S S I G K M F E A T A R G A R R M A I L G D T A W D F G S
2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
CAGTGGGTGGTGTTTTGAATTCATTAGGGAAAATGGTCCACCAAATATTTGGGAGTGCTTACACAGCCCTATTTGGTGGAGTCTCCTGGATGATGAAAAT
V G G V L N S L G K M V H Q I F G S A Y T A L F G G V S W M M K I
End of D3 16562 sequence
2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
TGGAATAGGTGTCCTCTTAACCTGGATAGGGTTGAACTCAAAAAATACTTCTATGTCATTTTCATGCATCGCGGCCGGCATTGTGACACTGTATTTGGGA
G I G V L L T W I G L N S K N T S M S F S C I A A G I V T L Y L G
Engineered NgoMIV splicing site, E-480 Val-to-Ala (nt-2375/2376 TG-to-CC)
> NS1
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
GTCATGGTGCAGGCCGATAGTGGTTGCGTTGTGAGCTGGAAAAACAAAGAACTGAAATGTGGCAGTGGGATTTTCATCACAGACAACGTGCACACATGGA
V M V Q A D S G C V V S W K N K E L K C G S G I F I T D N V H T W T
2510 2520 2530 2540 2550 2560 2570 2580 2590 2600
CAGAACAATACAAGTTCCAACCAGAATCCCCTTCAAAACTAGCTTCAGCTATCCAGAAAGCCCATGAAGAGGACATTTGTGGAATCCGCTCAGTAACAAG
E Q Y K F Q P E S P S K L A S A I Q K A H E E D I C G I R S V T R
D2 PDK-53 NS1-53-Asp attenuation locus (wt D2 16681: Gly, nt-2573-G)
2610 2620 2630 2640 2650 2660 2670 2680 2690 2700
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L E N L M W K Q I T P E L N H I L S E N E V K L T I M T G D I K G
2710 2720 2730 2740 2750 2760 2770 2780 2790 2800
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I M Q A G K R S L R P Q P T E L K Y S W K T W G K A K M L S T E S H
2810 2820 2830 2840 2850 2860 2870 2880 2890 2900
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N Q T F L I D G P E T A E C P N T N R A W N S L E V E D Y G F G V
2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
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F T T N I W L K L K E K Q D V F C D S K L M S A A I K D N R A V H
3010 3020 3030 3040 3050 3060 3070 3080 3090 3100
GCCGATATGGGTTATTGGATAGAAAGTGCACTCAATGACACATGGAAGATAGAGAAAGCCTCTTTCATTGAAGTTAAAAACTGCCACTGGCCAAAATCAC
A D M G Y W I E S A L N D T W K I E K A S F I E V K N C H W P K S H
3110 3120 3130 3140 3150 3160 3170 3180 3190 3200
ACACCCTCTGGAGCAATGGAGTGCTAGAAAGTGAGATGATAATTCCAAAGAATCTCGCTGGACCAGTGTCTCAACACAACTATAGACCAGGCTACCATAC
T L W S N G V L E S E M I I P K N L A G P V S Q H N Y R P G Y H T
3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
ACAAATAACAGGACCATGCCATCTAGGTAAGCTTGAGATGGACTTTGATTTCTGTGATGGAACAACAGTGGTAGTGACTGAGGACTGCGGAAATAGAGGA
Q I T G P W H L G K L E M D F D F C D G T T V V V T E D C G N R G
3310 3320 3330 3340 3350 3360 3370 3380 3390 3400
CCCTCTTTGAGAACAACCACTGCCTCTGGAAAACTCATAACAGAATGGTGCTGCCGATCTTGCACATTACCACCGCTAAGATACAGAGGTGAGGATGGGT
P S L R T T T A S G K L I T E W C C R S C T L P P L R Y R G E D G C
> NS2A
3410 3420 3430 3440 3450 3460 3470 3480 3490 3500


D2 PDK-53 specific NS2A-181-Phe (wt D2 16681: Leu, nt-4012-C)
> NS2B
> NS3


5210 5220 5230 5240 5250 5260 5270 5280 5290 5300
CTGAAATGGAGGAAGCCCTTAGAGGACTTCCAATAAGATACCAGACCCCAGCCATCAGAGCTGTGCACACCGGGCGGGAGATTGTGGACCTAATGTGTCA
E M E E A L R G L P I R Y Q T P A I R A V H T G R E I V D L M C H
D2 PDK-53 NS3-250-Val attenuation locus (D2 16681: Glu, nt-5270-A)
5310 5320 5330 5340 5350 5360 5370 5380 5390 5400
TGCCACATTTACCATGAGGCTGCTATCACCAGTTAGAGTGCCAAACTACAACCTGATTATCATGGACGAAGCCCATTTCACAGACCCAGCAAGTATAGCA
A T F T M R L L S P V R V P N Y N L I I M D E A H F T D P A S I A
5410 5420 5430 5440 5450 5460 5470 5480 5490 5500
GCTAGAGGATACATCTCAACTCGAGTGGAGATGGGTGAGGCAGCTGGGATTTTTATGACAGCCACTCCCCCGGGAAGCAGAGACCCATTTCCTCAGAGCA
A R G Y I S T R V E M G E A A G I F M T A T P P G S R D P F P Q S N
5510 5520 5530 5540 5550 5560 5570 5580 5590 5600
ATGCACCAATCATAGATGAAGAAAGAGAAATCCCTGAACGCTCGTGGAATTCCGGACATGAATGGGTCACGGATTTTAAAGGGAAGACTGTTTGGTTCGT
A P I I D E E R E I P E R S W N S G H E W V T D F K G K T V W F V
D2 PDK-53 silent mutation nt-5541-C (D2 16681: T)
5610 5620 5630 5640 5650 5660 5670 5680 5690 5700
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P S I K A G N D I A A C L R K N G K K V I Q L S R K T F D S E Y V
5710 5720 5730 5740 5750 5760 5770 5780 5790 5800
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K T R T N D W D F V V T T D I S E M G A N F K A E R V I D P R R C M
5810 5820 5830 5840 5850 5860 5870 5880 5890 5900
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K P V I L T D G E E R V I L A G P M P V T H S S A A Q R R G R I G
5910 5920 5930 5940 5950 5960 5970 5980 5990 6000
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R N P K N E N D Q Y I Y M G E P L E N D E D C A H W K E A K M L L
6010 6020 6030 6040 6050 6060 6070 6080 6090 6100
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D N I N T P E G I I P S M F E P E R E K V D A I D G E Y R L R G E A
6110 6120 6130 6140 6150 6160 6170 6180 6190 6200
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R K T F V D L M R R G D L P V W L A Y R V A A E G I N Y A D R R W
6210 6220 6230 6240 6250 6260 6270 6280 6290 6300
GTGTTTTGATGGAGTCAAGAACAACCAAATCCTAGAAGAAAACGTGGAAGTTGAAATCTGGACAAAAGAAGGGGAAAGGAAGAAATTGAAACCCAGATGG
C F D G V K N N Q I L E E N V E V E I W T K E G E R K K L K P R W
> NS4A
6310 6320 6330 6340 6350 6360 6370 6380 6390 6400
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L D A R I Y S D P L A L K E F K E F A A G R K S L T L N L I T E M G
6410 6420 6430 6440 6450 6460 6470 6480 6490 6500
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R L P T F M T Q K A R D A L D N L A V L H T A E A G G R A Y N H A
6510 6520 6530 6540 6550 6560 6570 6580 6590 6600
TCTCAGTGAACTGCCGGAGACCCTGGAGACATTGCTTTTACTGACACTTCTGGCTACAGTCACGGGAGGGATCTTTTTATTCTTGATGAGCGCAAGGGGC
L S E L P E T L E T L L L L T L L A T V T G G I F L F L M S A R G
D2 PDK-53 specific NS4A-75-Ala (wt D2 16681: Gly, nt-6599-G)
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700
ATAGGGAAGATGACCCTGGGAATGTGCTGCATAATCACGGCTAGCATCCTCCTATGGTACGCACAAATACAGCCACACTGGATAGCAGCTTCAATAATAC
I G K M T L G M C C I I T A S I L L W Y A Q I Q P H W I A A S I I L
6710 6720 6730 6740 6750 6760 6770 6780 6790 6800
TGGAGTTTTTTCTCATAGTTTTGCTTATTCCAGAACCTGAAAAACAGAGAACACCCCAAGACAACCAACTGACCTACGTTGTCATAGCCATCCTCACAGT
E F F L I V L L I P E P E K Q R T P Q D N Q L T Y V V I A I L T V
> NS4B
6810 6820 6830 6840 6850 6860 6870 6880 6890 6900
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V A A T M A N E M G F L E K T K K D L G L G S I A T Q Q P E S N I
6910 6920 6930 6940 6950 6960 6970 6980 6990 7000

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CTGGACATAGATCTACGTCCTGCATCAGCATGGACGCTGTATGCCGTGGCCACAACATTTGTTACACCAATGTTGAGACATAGCATTGAAAATTCCTCAG
L  D  I  D  L  R  P  A  S  A  W  T  L  Y  A  V  A  T  T  F  V  T  P  M  L  R  H  S  I  E  N  S  S  V

      7010       7020       7030       7040       7050       7060       7070       7080       7090       7100
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  N  V  S  L  T  A  I  A  N  Q  A  T  V  L  M  G  L  G  K  G  W  P  L  S  K  M  D  I  G  V  P  L  L

      7110       7120       7130       7140       7150       7160       7170       7180       7190       7200
CGCCATTGGATGCTACTCACAAGTCAACCCCATAACTCTCACAGCAGCTCTTTTCTTATTGGTAGCACATTATGCCATCATAGGGCCAGGACTCCAAGCA
 A  I  G  C  Y  S  Q  V  N  P  I  T  L  T  A  A  L  F  L  L  V  A  H  Y  A  I  I  G  P  G  L  Q  A

      7210       7220       7230       7240       7250       7260       7270       7280       7290       7300
AAAGCAACCAGAGAAGCTCAGAAAAGAGCAGCGGCGGGCATCATGAAAAACCCAACTGTCGATGGAATAACAGTGATTGACCTAGATCCAATACCTTATG
K  A  T  R  E  A  Q  K  R  A  A  A  G  I  M  K  N  P  T  V  D  G  I  T  V  I  D  L  D  P  I  P  Y  D

      7310       7320       7330       7340       7350       7360       7370       7380       7390       7400
ATCCAAAGTTTGAAAAGCAGTTGGGACAAGTAATGCTCCTAGTCCTCTGCGTGACTCAAGTATTGATGATGAGGACTACATGGGCTCTGTGTGAGGCTTT
  P  K  F  E  K  Q  L  G  Q  V  M  L  L  V  L  C  V  T  Q  V  L  M  M  R  T  T  W  A  L  C  E  A  L

      7410       7420       7430       7440       7450       7460       7470       7480       7490       7500
AACCTTAGCTACCGGGCCCATCTCCACATTGTGGGAAGGAAATCCAGGGAGGTTTTGGAACACTACCATTGCGGTGTCAATGGCTAACATTTTTAGAGGG
 T  L  A  T  G  P  I  S  T  L  W  E  G  N  P  G  R  F  W  N  T  T  I  A  V  S  M  A  N  I  F  R  G

                                                                                  > NS5
      7510       7520       7530       7540       7550       7560       7570       7580       7590       7600
AGTTACTTGGCCGGAGCTGGACTTCTCTTTTCTATTATGAAGAACACAACCAACACAAGAAGGGGAACTGGCAACATAGGAGAGACGCTTGGAGAGAAAT
S  Y  L  A  G  A  G  L  L  F  S  I  M  K  N  T  T  N  T  R  R  G  T  G  N  I  G  E  T  L  G  E  K  W

      7610       7620       7630       7640       7650       7660       7670       7680       7690       7700
GGAAAAGCCGATTGAACGCGTTGGGAAAAAGTGAATTCCAGATCTACAAGAAAAGTGGAATCCAGGAAGTGGATAGAACCTTAGCAAAAGAAGGCATTAA
  K  S  R  L  N  A  L  G  K  S  E  F  Q  I  Y  K  K  S  G  I  Q  E  V  D  R  T  L  A  K  E  G  I  K
                  |
          Additional nt-7260 A-to-G silent mutation in master and pre-master seeds

      7710       7720       7730       7740       7750       7760       7770       7780       7790       7800
AAGAGGAGAAACGGACCATCACGCTGTGTCGCGAGGCTCAGCAAAACTGAGATGGTTCGTTGAGAGAAACATGGTCACACCAGAAGGGAAAGTAGTGGAC
 R  G  E  T  D  H  H  A  V  S  R  G  S  A  K  L  R  W  F  V  E  R  N  M  V  T  P  E  G  K  V  V  D

      7810       7820       7830       7840       7850       7860       7870       7880       7890       7900
CTCGGTTGTGGCAGAGGAGGCTGGTCATACTATTGTGGAGGACTAAAGAATGTAAGAGAAGTCAAAGGCCTAACAAAAGGAGGACCAGGACACGAAGAAC
L  G  C  G  R  G  G  W  S  Y  Y  C  G  G  L  K  N  V  R  E  V  K  G  L  T  K  G  G  P  G  H  E  E  P

      7910       7920       7930       7940       7950       7960       7970       7980       7990       8000
CCATCCCCATGTCAACATATGGGTGGAATCTAGTGCGTCTTCAAAGTGGAGTTGACGTTTTCTTCATCCCGCCAGAAAAGTGTGACACATTATTGTGTGA
  I  P  M  S  T  Y  G  W  N  L  V  R  L  Q  S  G  V  D  V  F  F  I  P  P  E  K  C  D  T  L  L  C  D

      8010       8020       8030       8040       8050       8060       8070       8080       8090       8100
CATAGGGGAGTCATCACCAAATCCCACAGTGGAAGCAGGACGAACACTCAGAGTCCTTAACTTAGTAGAAAATTGGTTGAACAACAACACTCAATTTTGC
 I  G  E  S  S  P  N  P  T  V  E  A  G  R  T  L  R  V  L  N  L  V  E  N  W  L  N  N  N  T  Q  F  C

      8110       8120       8130       8140       8150       8160       8170       8180       8190       8200
ATAAAGGTTCTCAACCCATATATGCCCTCAGTCATAGAAAAAATGGAAGCACTACAAAGGAAATATGGAGGAGCCTTAGTGAGGAATCCACTCTCACGAA
I  K  V  L  N  P  Y  M  P  S  V  I  E  K  M  E  A  L  Q  R  K  Y  G  G  A  L  V  R  N  P  L  S  R  N

      8210       8220       8230       8240       8250       8260       8270       8280       8290       8300
ACTCCACACATGAGATGTACTGGGTATCCAATGCTTCCCGGAACATAGTGTCATCAGTGAACATGATTTCAAGGATGTTGATCAACAGATTTACAATGAG
  S  T  H  E  M  Y  W  V  S  N  A  S  G  N  I  V  S  S  V  N  M  I  S  R  M  L  I  N  R  F  T  M  R

      8310       8320       8330       8340       8350       8360       8370       8380       8390       8400
ATACAAGAAAGCCACTTACGAGCCGGATGTTGACCTCGGAAGCGGAACCCGTAACATCGGGATTGAAAGTGAGATACCAAACCTAGATATAATTGGGAAA
 Y  K  K  A  T  Y  E  P  D  V  D  L  G  S  G  T  R  N  I  G  I  E  S  E  I  P  N  L  D  I  I  G  K

      8410       8420       8430       8440       8450       8460       8470       8480       8490       8500
AGAATAGAAAAAATAAAGCAAGAGCATGAAACATCATGGCACTATGACCAAGACCACCCATACAAAACGTGGGCATACCATGGTAGCTATGAAACAAAAC
R  I  E  K  I  K  Q  E  H  E  T  S  W  H  Y  D  Q  D  H  P  Y  K  T  W  A  Y  H  G  S  Y  E  T  K  Q

      8510       8520       8530       8540       8550       8560       8570       8580       8590       8600
AGACTGGATCAGCATCATCCATGGTCAACGGAGTGGTCAGGCTGCTGACAAAACCTTGGGACGTCGTCCCCATGGTGACACAGATGGCAATGACAGACAC
  T  G  S  A  S  S  M  V  N  G  V  V  R  L  L  T  K  P  W  D  V  V  P  M  V  T  Q  M  A  M  T  D  T

      8610       8620       8630       8640       8650       8660       8670       8680       8690       8700
GACTCCATTTGGACAACAGCGCGTTTTTAAAGAGAAAGTGGACACGAGAACCCAAGAACCGAAAGAAGGCACGAAGAAACTAATGAAAATAACAGCAGAG
 T  P  F  G  Q  Q  R  V  F  K  E  K  V  D  T  R  T  Q  E  P  K  E  G  T  K  K  L  M  K  I  T  A  E

      8710       8720       8730       8740       8750       8760       8770       8780       8790       8800
TGGCTTTGGAAAGAATTAGGGAAGAAAAAGACACCCAGGATGTGCACCAGAGAAGAATTCACAAGAAAGGTGAGAAGCAATGCAGCCTTGGGGGCCATAT
W  L  W  K  E  L  G  K  K  K  T  P  R  M  C  T  R  E  E  F  T  R  K  V  R  S  N  A  A  L  G  A  I  F
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      8810       8820       8830       8840       8850       8860       8870       8880       8890       8900
TCACTGATGAGAACAAGTGGAAGTCGGCACGTGAGGCTGTTGAAGATAGTAGGTTTTGGGAGCTGGTTGACAAGGAAAGGAATCTCCATCTTGAAGGAAA
  T  D  E  N  K  W  K  S  A  R  E  A  V  E  D  S  R  F  W  E  L  V  D  K  E  R  N  L  H  L  E  G  K

      8910       8920       8930       8940       8950       8960       8970       8980       8990       9000
GTGTGAAACATGTGTGTACAACATGATGGGAAAAAGAGAGAAGAAGCTAGGGGAATTCGGCAAGGCAAAAGGCAGCAGAGCCATATGGTACATGTGGCTT
  C  E  T  C  V  Y  N  M  M  G  K  R  E  K  K  L  G  E  F  G  K  A  K  G  S  R  A  I  W  Y  M  W  L

      9010       9020       9030       9040       9050       9060       9070       9080       9090       9100
GGAGCACGCTTCTTAGAGTTTGAAGCCCTAGGATTCTTAAATGAAGATCACTGGTTCTCCAGAGAGAACTCCCTGAGTGGAGTGGAAGGAGAAGGGCTGC
  G  A  R  F  L  E  F  E  A  L  G  F  L  N  E  D  H  W  F  S  R  E  N  S  L  S  G  V  E  G  E  G  L  H

      9110       9120       9130       9140       9150       9160       9170       9180       9190       9200
ACAAGCTAGGTTACATTCTAAGAGACGTGAGCAAGAAAGAGGGAGGAGCAATGTATGCCGATGACACCGCAGGATGGGATACAAGAATCACACTAGAAGA
  K  L  G  Y  I  L  R  D  V  S  K  K  E  G  G  A  M  Y  A  D  D  T  A  G  W  D  T  R  I  T  L  E  D

      9210       9220       9230       9240       9250       9260       9270       9280       9290       9300
CCTAAAAAATGAAGAAATGGTAACAAACCACATGGAAGGAGAACACAAGAAACTAGCCGAGGCCATTTTCAAACTAACGTACCAAAACAAGGTGGTGCGT
  L  K  N  E  E  M  V  T  N  H  M  E  G  E  H  K  K  L  A  E  A  I  F  K  L  T  Y  Q  N  K  V  V  R

      9310       9320       9330       9340       9350       9360       9370       9380       9390       9400
GTGCAAAGACCAACACCAAGAGGCACAGTAATGGACATCATATCGAGAAGAGACCAAAGAGGTAGTGGACAAGTTGGCACCTATGGACTCAATACTTTCA
  V  Q  R  P  T  P  R  G  T  V  M  D  I  I  S  R  R  D  Q  R  G  S  G  Q  V  G  T  Y  G  L  N  T  F  T

      9410       9420       9430       9440       9450       9460       9470       9480       9490       9500
CCAATATGGAAGCCCAACTAATCAGACAGATGGAGGGAGAAGGAGTCTTTAAAAGCATTCAGCACCTAACAATCACAGAAGAAATCGCTGTGCAAAACTG
  N  M  E  A  Q  L  I  R  Q  M  E  G  E  G  V  F  K  S  I  Q  H  L  T  I  T  E  E  I  A  V  Q  N  W

      9510       9520       9530       9540       9550       9560       9570       9580       9590       9600
GTTAGCAAGAGTGGGGCGCGAAAGGTTATCAAGAATGGCCATCAGTGGAGATGATTGTGTTGTGAAACCTTTAGATGACAGGTTCGCAAGCGCTTTAACA
  L  A  R  V  G  R  E  R  L  S  R  M  A  I  S  G  D  D  C  V  V  K  P  L  D  D  R  F  A  S  A  L  T

      9610       9620       9630       9640       9650       9660       9670       9680       9690       9700
GCTCTAAATGACATGGGAAAGATTAGGAAAGACATACAACAATGGGAACCTTCAAGAGGATGGAATGATTGGACACAAGTGCCCTTCTGTTCACACCATT
  A  L  N  D  M  G  K  I  R  K  D  I  Q  Q  W  E  P  S  R  G  W  N  D  W  T  Q  V  P  F  C  S  H  H  F

      9710       9720       9730       9740       9750       9760       9770       9780       9790       9800
TCCATGAGTTAATCATGAAAGACGGTCGCGTACTCGTTGTTCCATGTAGAAACCAAGATGAACTGATTGGCAGAGCCCGAATCTCCCAAGGAGCAGGGTG
  H  E  L  I  M  K  D  G  R  V  L  V  V  P  C  R  N  Q  D  E  L  I  G  R  A  R  I  S  Q  G  A  G  W

      9810       9820       9830       9840       9850       9860       9870       9880       9890       9900
GTCTTTGCGGGAGACGGCCTGTTTGGGGAAGTCTTACGCCCAAATGTGGAGCTTGATGTACTTCCACAGACGCGACCTCAGGCTGGCGGCAAATGCTATT
  S  L  R  E  T  A  C  L  G  K  S  Y  A  Q  M  W  S  L  M  Y  F  H  R  R  D  L  R  L  A  A  N  A  I

      9910       9920       9930       9940       9950       9960       9970       9980       9990      10000
TGCTCGGCAGTACCATCACATTGGGTTCCAACAAGTCGAACAACCTGGTCCATACATGCTAAACATGAATGGATGACAACGGAAGACATGCTGACAGTCT
  C  S  A  V  P  S  H  W  V  P  T  S  R  T  T  W  S  I  H  A  K  H  E  W  M  T  T  E  D  M  L  T  V  W

     10010      10020      10030      10040      10050      10060      10070      10080      10090      10100
GGAACAGGGTGTGGATTCAAGAAAACCCATGGATGGAAGACAAAACTCCAGTGGAATCATGGGAGGAAATCCCATACTTGGGGAAAAGAGAAGACCAATG
  N  R  V  W  I  Q  E  N  P  W  M  E  D  K  T  P  V  E  S  W  E  E  I  P  Y  L  G  K  R  E  D  Q  W

     10110      10120      10130      10140      10150      10160      10170      10180      10190      10200
GTGCGGCTCATTGATTGGGTTAACAAGCAGGGCCACCTGGGCAAAGAACATCCAAGCAGCAATAAATCAAGTTAGATCCCTTATAGGCAATGAAGAATAC
  C  G  S  L  I  G  L  T  S  R  A  T  W  A  K  N  I  Q  A  A  I  N  Q  V  R  S  L  I  G  N  E  E  Y

                                                                                  > 3'-Noncoding Region
     10210      10220      10230      10240      10250      10260      10270      10280      10290      10300
ACAGATTACATGCCATCCATGAAAAGATTCAGAAGAGAAGAGGAAGAAGCAGGAGTTCTGTGGTAGAAAGCAAAACTAACATGAAACAAGGCTAGAAGTC
  T  D  Y  M  P  S  M  K  R  F  R  R  E  E  E  E  A  G  V  L  W  *

     10310      10320      10330      10340      10350      10360      10370      10380      10390      10400
AGGTCGGATTAAGCCATAGTACGGAAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCATCATAAATGCCATAGCTTGAG

     10410      10420      10430      10440      10450      10460      10470      10480      10490      10500
TAAACTATGCAGCCTGTAGCTCCACCTGAGAAGGTGTAAAAAATCCGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAG

     10510      10520      10530      10540      10550      10560      10570      10580      10590      10600
AGGAGACCCCTCCCTTACAAATCGCAGCAACAATGGGGGCCCAAGGCGAGATGAAGCTGTAGTCTCGCTGGAAGGACTAGAGGTTAGAGGAGACCCCCCC

     10610      10620      10630      10640      10650      10660      10670      10680      10690      10700
GAAACAAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACGCCAGAAAATGGAATGGTGCTG

     10710
```

TTGAATCAACAGGTTCT

DENvax-4 Master Virus Seed (MVS)

[000167] Nucleotide sequence of the chimeric viral genome and deduced amino acid sequence of the translated protein. Most of the prM-E gene (nt-457 to -2379, underlined) is wild-type (wt) DEN-4 1036 virus-specific; the remaining nucleotide sequence is DEN-2 PDK-53 virus-specific. All engineered substitutions differ from wt virus (DEN-3 16562 or DEN-2 16681), as well as extra mutations (changes from engineered cDNA clone) are marked.

Substitutions Included in the Genome and Protein:

[000168] Junction sites:

a. MluI (nt 451-456): engineered silent mutation, nt-453 A-to-G
b. NgoMIV (nt 2380-2385): engineered mutations, nt-2381/2382 TG-to-CC (resulted in E-482 Val-to-Ala change)

[000169] D2 PDK-53 virus backbone (change from wt D2 16681)

a. 5'-noncoding region(NCR)-57 (nt-57 C-to-T): major attenuation locus (in red)
b. NS1-53 Gly-to-Asp (nt-2579 G-to-A): major attenuation locus (in red)
c. NS2A-181 Leu-to-Phe (nt-4018 C-to-T, in bold)
d. NS3-250 Glu-to-Val (nt-5270 A-to-T): major attenuation locus (in red)
e. nt-5547 (NS3 gene) T-to-C silent mutation (in bold)
f. NS4A-75 Gly-to-Ala (nt-6599 G-to-C, in bold)

* nt-8571 C-to-T silent mutation of PDK-53 is not engineered in the vaccine virus

[000170] Engineered substitutions in cDNA clone

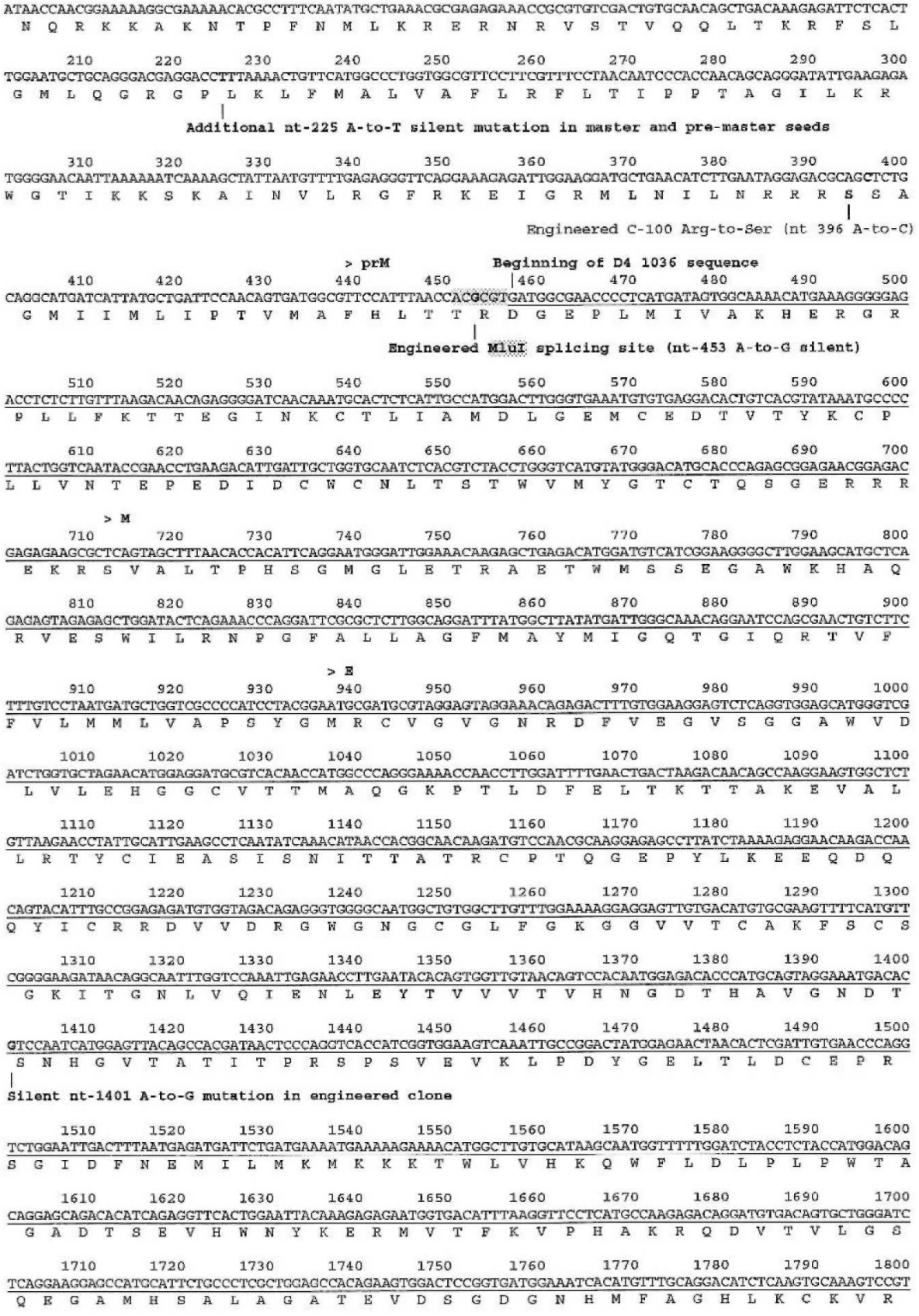
a. Engineered C-100 Arg-to-Ser (nt-396 A-to-C): may improve viral replication in culture
b. Engineered nt-1401 A-to-G silent mutation
c. Engineered E-364 Ala-to-Val (nt-2027 C-to-T): may improve viral replication in culture
d. Engineered E-447 Met-to-Leu (nt-2275 A-to-C): may improve viral replication in culture

[000171] Additional substitutions found in vaccine seed (0.06% nt different from original clone)

a. nt-225 (C gene) A-to-T silent mutation (in bold)
b. NS2A-66 Asp-to-Gly (nt-3674 A-to-G) mutation (in bold)
c. NS2A-99 Lys-to-Lys/Arg mix (nt-3773 A-to-A/G mix, in bold)
d. nt-5391 C-to-T (NS3 gene) silent mutation (in bold)
e. NS4A-21 Ala-to-Val (nt-6437 C-to-T, in bold)
f. nt-7026 T-to-C/T mix silent mutation (in bold)
g. nt-9750 A-to-C silent mutation (in bold)

```
                                        NCR-57-T, D2 PDK-53 attenuation locus (wt D2 16681: C)
> 5'-Noncoding Region                                                                        >C
        10        20        30        40        50        60        70        80        90       100
AGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAATGTAGTTCTAACAGTTTTTTAATTAGAGAGCAGATCTCTGATGA
                                                                                          M  N

       110       120       130       140       150       160       170       180       190       200
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Additional nt-225 A-to-T silent mutation in master and pre-master seeds
Engineered C-100 Arg-to-Ser (nt 396 A-to-C)
> prM
Beginning of D4 1036 sequence
Engineered MluI splicing site (nt-453 A-to-G silent)
> M
> E
Silent nt-1401 A-to-G mutation in engineered clone

```
      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900
ATGGAGAAATTGAGAATCAAGGGAATGTCATACACGATGTGTTCAGGAAAGTTCTCAATTGACAAAGAGATGGCAGAAACACAGCATGGGACAACAGTGG
M  E  K  L  R  I  K  G  M  S  Y  T  M  C  S  G  K  F  S  I  D  K  E  M  A  E  T  Q  H  G  T  T  V  V

      1910       1920       1930       1940       1950       1960       1970       1980       1990       2000
TGAAAGTCAAGTATGAAGGTGCTGGAGCTCCGTGTAAAGTCCCCATAGAGATAAGAGATGTGAACAAGGAAAAAGTGGTTGGGCGTATCATCTCATCCAC
  K  V  K  Y  E  G  A  G  A  P  C  K  V  P  I  E  I  R  D  V  N  K  E  K  V  V  G  R  I  I  S  S  T

      2010       2020       2030       2040       2050       2060       2070       2080       2090       2100
CCCTTTGGCTGAGAATACCAACAGTGTAACCAACATAGAGTTAGAACCCCCCTTTGGGGACAGCTACATAGTGATAGGTGTTGGAAACAGTGCATTAACA
 P  L  A  E  N  T  N  S  V  T  N  I  E  L  E  P  P  F  G  D  S  Y  I  V  I  G  V  G  N  S  A  L  T
                        |
              Engineered E-364 Ala-to-Val (nt-2027 C-to-T) to improve viral growth in culture

      2110       2120       2130       2140       2150       2160       2170       2180       2190       2200
CTCCATTGGTTCAGGAAAGGGAGTTCCATTGGCAAGATGTTTGAGTCCACATACAGAGGTGCAAAACGAATGGCCATTCTAGGTGAAACAGCTTGGGATT
L  H  W  F  R  K  G  S  S  I  G  K  M  F  E  S  T  Y  R  G  A  K  R  M  A  I  L  G  E  T  A  W  D  F

      2210       2220       2230       2240       2250       2260       2270       2280       2290       2300
TTGGTTCCGTTGGTGGACTGTTCACATCATTGGGAAAGGCTGTGCACCAGGTTTTTGGAAGTGTGTATACAACCCTGTTTGGAGGAGTCTCATGGATGAT
  G  S  V  G  G  L  F  T  S  L  G  K  A  V  H  Q  V  F  G  S  V  Y  T  T  L  F  G  G  V  S  W  M  I
                                                                          |
                                                  Engineered E-447 Met-to-Leu (nt-2275 A-to-C) mutation

                                                                End of D4 1036 sequence
      2310       2320       2330       2340       2350       2360       2370       |       2390       2400
TAGAATCCTAATTGGGTTCCTAGTGTTGTGGATTGGCACGAACTCAAGGAACACTTCAATGGCTATGACGTGCATAGCTGCCGGCATTGTGACACTGTAT
 R  I  L  I  G  F  L  V  L  W  I  G  T  N  S  R  N  T  S  M  A  M  T  C  I  A  A  G  I  V  T  L  Y
                                                                                 |
                                                  Engineered NgoMIV splicing site, E-482 Val-to-Ala (nt-2381/2382 TG-to-CC)

                        > NS1
      2410       2420       2430       2440       2450       2460       2470       2480       2490       2500
TTGGGAGTCATGGTGCAGGCCGATAGTGGTTGCGTTGTGAGCTGGAAAAACAAAGAACTGAAATGTGGCAGTGGGATTTTCATCACAGACAACGTGCACA
L  G  V  M  V  Q  A  D  S  G  C  V  V  S  W  K  N  K  E  L  K  C  G  S  G  I  F  I  T  D  N  V  H  T

      2510       2520       2530       2540       2550       2560       2570       2580       2590       2600
CATGGACAGAACAATACAAGTTCCAACCAGAATCCCCTTCAAAACTAGCTTCAGCTATCCAGAAAGCCCATGAAGAGGACATTTGTGGAATCCGCTCAGT
  W  T  E  Q  Y  K  F  Q  P  E  S  P  S  K  L  A  S  A  I  Q  K  A  H  E  E  D  I  C  G  I  R  S  V
                                                                          |
                                                  D2 PDK-53 NS1-53-Asp attenuation locus (wt D2 16681: Gly, nt-2579-G)

      2610       2620       2630       2640       2650       2660       2670       2680       2690       2700
AACAAGACTGGAGAATCTGATGTGGAAACAAATAACACCAGAATTGAATCACATTCTATCAGAAAATGAGGTGAAGTTAACTATTATGACAGGAGACATC
 T  R  L  E  N  L  M  W  K  Q  I  T  P  E  L  N  H  I  L  S  E  N  E  V  K  L  T  I  M  T  G  D  I

      2710       2720       2730       2740       2750       2760       2770       2780       2790       2800
AAAGGAATCATGCAGGCAGGAAAACGATCTCTGCGGCCTCAGCCCACTGAGCTGAAGTATTCATGGAAAACATGGGGCAAAGCAAAAATGCTCTCTACAG
K  G  I  M  Q  A  G  K  R  S  L  R  P  Q  P  T  E  L  K  Y  S  W  K  T  W  G  K  A  K  M  L  S  T  E

      2810       2820       2830       2840       2850       2860       2870       2880       2890       2900
AGTCTCATAACCAGACCTTTCTCATTGATGGCCCCGAAACAGCAGAATGCCCCAACACAAATAGAGCTTGGAATTCGTTGGAAGTTGAAGACTATGGCTT
  S  H  N  Q  T  F  L  I  D  G  P  E  T  A  E  C  P  N  T  N  R  A  W  N  S  L  E  V  E  D  Y  G  F

      2910       2920       2930       2940       2950       2960       2970       2980       2990       3000
TGGAGTATTCACCACCAATATATGGCTAAAATTGAAAGAAAAACAGGATGTATTCTGCGACTCAAAACTCATGTCAGCGGCCATAAAAGACAACAGAGCC
 G  V  F  T  T  N  I  W  L  K  L  K  E  K  Q  D  V  F  C  D  S  K  L  M  S  A  A  I  K  D  N  R  A

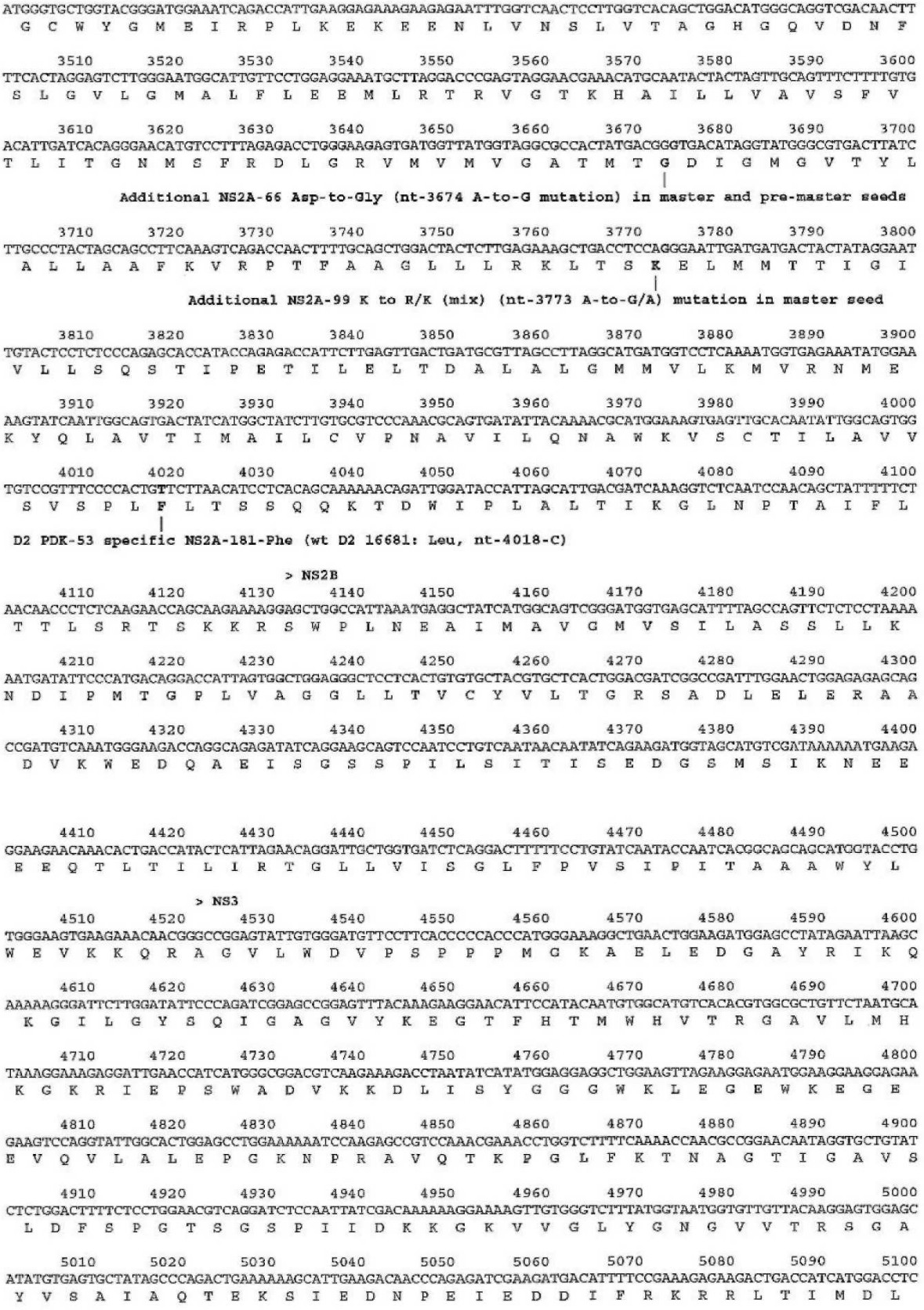
      3010       3020       3030       3040       3050       3060       3070       3080       3090       3100
GTCCATGCCGATATGGGTTATTGGATAGAAAGTGCACTCAATGACACATGGAAGATAGAGAAAGCCTCTTTCATTGAAGTTAAAAACTGCCACTGGCCAA
V  H  A  D  M  G  Y  W  I  E  S  A  L  N  D  T  W  K  I  E  K  A  S  F  I  E  V  K  N  C  H  W  P  K

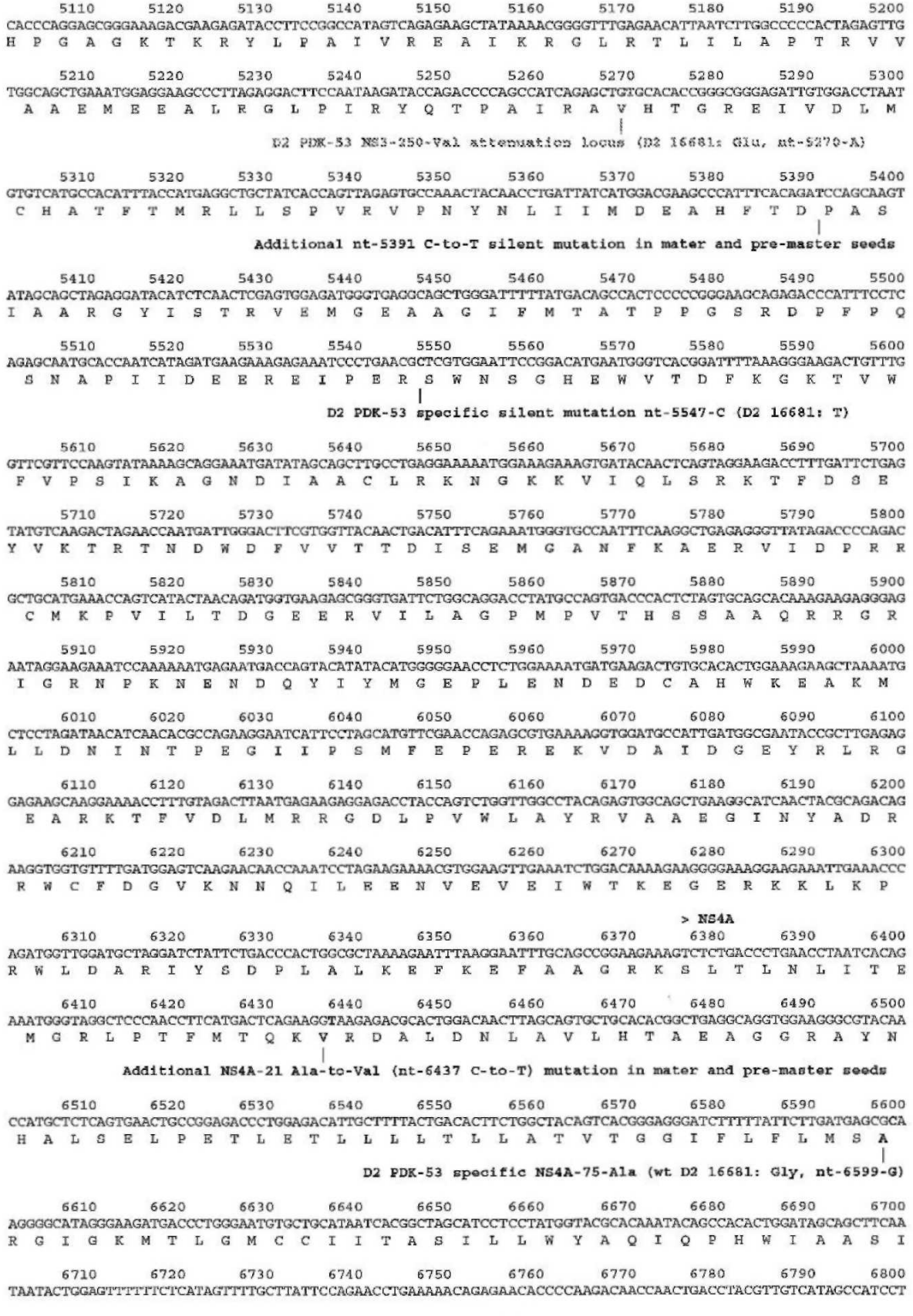
      3110       3120       3130       3140       3150       3160       3170       3180       3190       3200
AATCACACACCCTCTGGAGCAATGGAGTGCTAGAAAGTGAGATGATAATTCCAAAGAATCTCGCTGGACCAGTGTCTCAACACAACTATAGACCAGGCTA
  S  H  T  L  W  S  N  G  V  L  E  S  E  M  I  I  P  K  N  L  A  G  P  V  S  Q  H  N  Y  R  P  G  Y

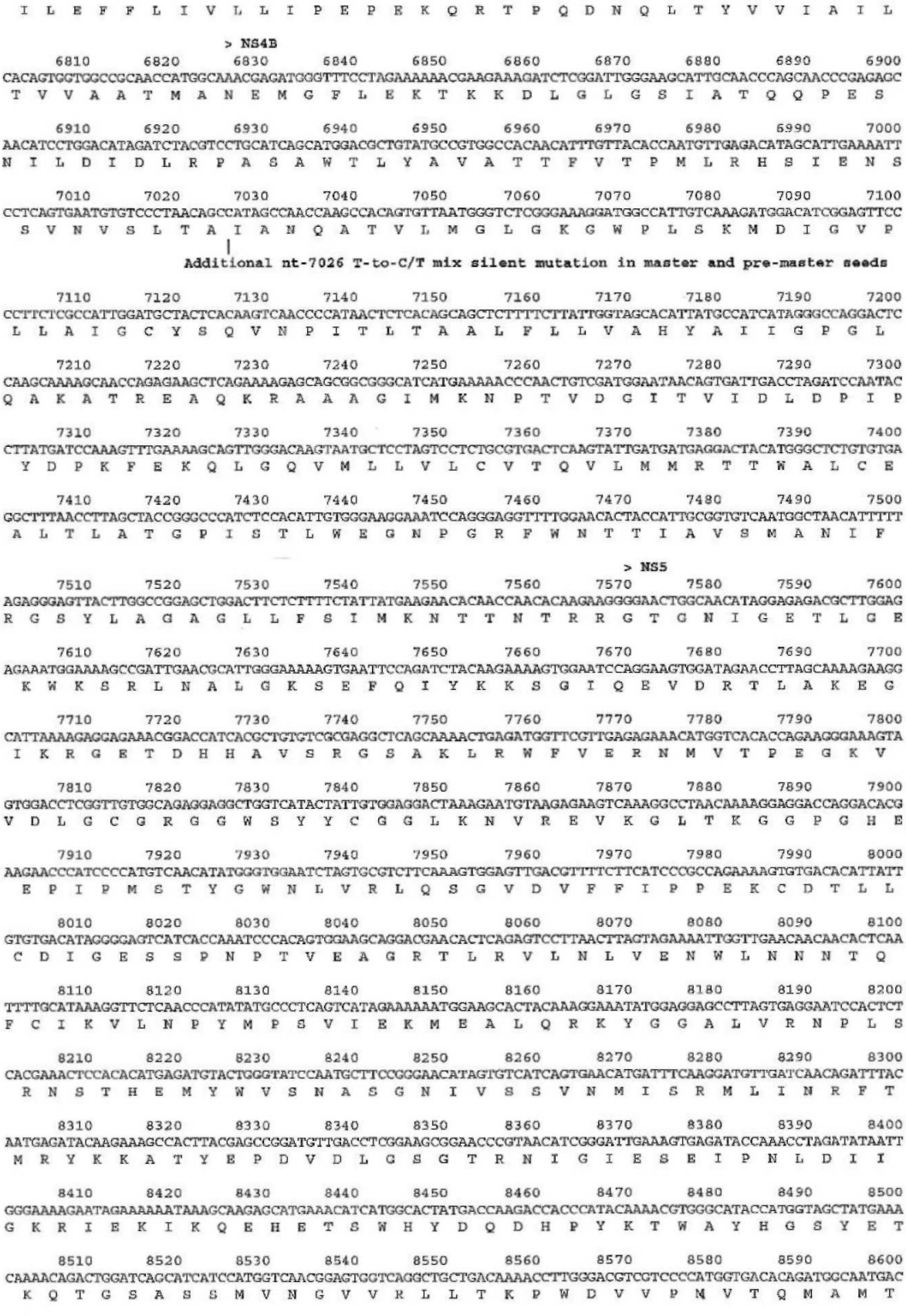
      3210       3220       3230       3240       3250       3260       3270       3280       3290       3300
CCATACACAAATAACAGGACCATGGCATCTAGGTAAGCTTGAGATGGACTTTGATTTCTGTGATGGAACAACAGTGGTAGTGACTGAGGACTGCGGAAAT
 H  T  Q  I  T  G  P  W  H  L  G  K  L  E  M  D  F  D  F  C  D  G  T  T  V  V  V  T  E  D  C  G  N

      3310       3320       3330       3340       3350       3360       3370       3380       3390       3400
AGAGGACCCTCTTTGAGAACAACCACTGCCTCTGGAAAACTCATAACAGAATGGTGCTGCCGATCTTGCACATTACCACCGCTAAGATACAGAGGTGAGG
R  G  P  S  L  R  T  T  T  A  S  G  K  L  I  T  E  W  C  C  R  S  C  T  L  P  P  L  R  Y  R  G  E  D

                                                                                    > NS2A
      3410       3420       3430       3440       3450       3460       3470       3480       3490       3500
```


Additional NS2A-66 Asp-to-Gly (nt-3674 A-to-G mutation) in master and pre-master seeds
Additional NS2A-99 K to R/K (mix) (nt-3773 A-to-G/A) mutation in master seed
D2 PDK-53 specific NS2A-181-Phe (wt D2 16681: Leu, nt-4018-C)
> NS2B
> NS3


5110 5120 5130 5140 5150 5160 5170 5180 5190 5200
CACCCAGGAGCGGGAAAGACGAAGAGATACCTTCCGGCCATAGTCAGAGAAGCTATAAAACGGGGTTTGAGAACATTAATCTTGGCCCCCACTAGAGTTG
H P G A G K T K R Y L P A I V R E A I K R G L R T L I L A P T R V V
5210 5220 5230 5240 5250 5260 5270 5280 5290 5300
TGGCAGCTGAAATGGAGGAAGCCCTTAGAGGACTTCCAATAAGATACCAGACCCCAGCCATCAGAGCTGTGCACACCGGGCGGGAGATTGTGGACCTAAT
A A E M E E A L R G L P I R Y Q T P A I R A V H T G R E I V D L M
D2 PDK-53 NS3-250-Val attenuation locus (D2 16681: Glu, nt-5270-A)
5310 5320 5330 5340 5350 5360 5370 5380 5390 5400
GTGTCATGCCACATTTACCATGAGGCTGCTATCACCAGTTAGAGTGCCAAACTACAACCTGATTATCATGGACGAAGCCCATTTCACAGATCCAGCAAGT
C H A T F T M R L L S P V R V P N Y N L I I M D E A H F T D P A S
Additional nt-5391 C-to-T silent mutation in mater and pre-master seeds
5410 5420 5430 5440 5450 5460 5470 5480 5490 5500
ATAGCAGCTAGAGGATACATCTCAACTCGAGTGGAGATGGGTGAGGCAGCTGGGATTTTTATGACAGCCACTCCCCCGGGAAGCAGAGACCCATTTCCTC
I A A R G Y I S T R V E M G E A A G I F M T A T P P G S R D P F P Q
5510 5520 5530 5540 5550 5560 5570 5580 5590 5600
AGAGCAATGCACCAATCATAGATGAAGAAAGAGAAATCCCTGAACGCTCGTGGAATTCCGGACATGAATGGGTCACGGATTTTAAAGGGAAGACTGTTTG
S N A P I I D E E R E I P E R S W N S G H E W V T D F K G K T V W
D2 PDK-53 specific silent mutation nt-5547-C (D2 16681: T)
5610 5620 5630 5640 5650 5660 5670 5680 5690 5700
GTTCGTTCCAAGTATAAAAGCAGGAAATGATATAGCAGCTTGCCTGAGGAAAAATGGAAAGAAAGTGATACAACTCAGTAGGAAGACCTTTGATTCTGAG
F V P S I K A G N D I A A C L R K N G K K V I Q L S R K T F D S E
5710 5720 5730 5740 5750 5760 5770 5780 5790 5800
TATGTCAAGACTAGAACCAATGATTGGGACTTCGTGGTTACAACTGACATTTCAGAAATGGGTGCCAATTTCAAGGCTGAGAGGGTTATAGACCCCAGAC
Y V K T R T N D W D F V V T T D I S E M G A N F K A E R V I D P R R
5810 5820 5830 5840 5850 5860 5870 5880 5890 5900
GCTGCATGAAACCAGTCATACTAACAGATGGTGAAGAGCGGGTGATTCTGGCAGGACCTATGCCAGTGACCCACTCTAGTGCAGCACAAAGAAGAGGGAG
C M K P V I L T D G E E R V I L A G P M P V T H S S A A Q R R G R
5910 5920 5930 5940 5950 5960 5970 5980 5990 6000
AATAGGAAGAAATCCAAAAAATGAGAATGACCAGTACATATACATGGGGGAACCTCTGGAAAATGATGAAGACTGTGCACACTGGAAAGAAGCTAAAATG
I G R N P K N E N D Q Y I Y M G E P L E N D E D C A H W K E A K M
6010 6020 6030 6040 6050 6060 6070 6080 6090 6100
CTCCTAGATAACATCAACACGCCAGAAGGAATCATTCCTAGCATGTTCGAACCAGAGCGTGAAAAGGTGGATGCCATTGATGGCGAATACCGCTTGAGAG
L L D N I N T P E G I I P S M F E P E R E K V D A I D G E Y R L R G
6110 6120 6130 6140 6150 6160 6170 6180 6190 6200
GAGAAGCAAGGAAAACCTTTGTAGACTTAATGAGAAGAGGAGACCTACCAGTCTGGTTGGCCTACAGAGTGGCAGCTGAAGGCATCAACTACGCAGACAG
E A R K T F V D L M R R G D L P V W L A Y R V A A E G I N Y A D R
6210 6220 6230 6240 6250 6260 6270 6280 6290 6300
AAGGTGGTGTTTTGATGGAGTCAAGAACAACCAAATCCTAGAAGAAAACGTGGAAGTTGAAATCTGGACAAAAGAAGGGGAAAGGAAGAAATTGAAACCC
R W C F D G V K N N Q I L E E N V E V E I W T K E G E R K K L K P
> NS4A
6310 6320 6330 6340 6350 6360 6370 6380 6390 6400
AGATGGTTGGATGCTAGGATCTATTCTGACCCACTGGCGCTAAAAGAATTTAAGGAATTTGCAGCCGGAAGAAAGTCTCTGACCCTGAACCTAATCACAG
R W L D A R I Y S D P L A L K E F K E F A A G R K S L T L N L I T E
6410 6420 6430 6440 6450 6460 6470 6480 6490 6500
AAATGGGTAGGCTCCCAACCTTCATGACTCAGAAGGTAAGAGACGCACTGGACAACTTAGCAGTGCTGCACACGGCTGAGGCAGGTGGAAGGGCGTACAA
M G R L P T F M T Q K V R D A L D N L A V L H T A E A G G R A Y N
Additional NS4A-21 Ala-to-Val (nt-6437 C-to-T) mutation in mater and pre-master seeds
6510 6520 6530 6540 6550 6560 6570 6580 6590 6600
CCATGCTCTCAGTGAACTGCCGGAGACCCTGGAGACATTGCTTTTACTGACACTTCTGGCTACAGTCACGGGAGGGATCTTTTTATTCTTGATGAGCGCA
H A L S E L P E T L E T L L L L T L L A T V T G G I F L F L M S A
D2 PDK-53 specific NS4A-75-Ala (wt D2 16681: Gly, nt-6599-G)
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700
AGGGGCATAGGGAAGATGACCCTGGGAATGTGCTGCATAATCACGGCTAGCATCCTCCTATGGTACGCACAAATACAGCCACACTGGATAGCAGCTTCAA
R G I G K M T L G M C C I I T A S I L L W Y A Q I Q P H W I A A S I
6710 6720 6730 6740 6750 6760 6770 6780 6790 6800
TAATACTGGAGTTTTTTCTCATAGTTTTGCTTATTCCAGAACCTGAAAAACAGAGAACACCCCAAGACAACCAACTGACCTACGTTGTCATAGCCATCCT


I L E F F L I V L L I P E P E K Q R T P Q D N Q L T Y V V I A I L
> NS4B
6810 6820 6830 6840 6850 6860 6870 6880 6890 6900
CACAGTGGTGGCCGCAACCATGGCAAACGAGATGGGTTTCCTAGAAAAAACGAAGAAAGATCTCGGATTGGGAAGCATTGCAACCCAGCAACCCGAGAGC
T V V A A T M A N E M G F L E K T K K D L G L G S I A T Q Q P E S
6910 6920 6930 6940 6950 6960 6970 6980 6990 7000
AACATCCTGGACATAGATCTACGTCCTGCATCAGCATGGACGCTGTATGCCGTGGCCACAACATTTGTTACACCAATGTTGAGACATAGCATTGAAAATT
N I L D I D L R P A S A W T L Y A V A T T F V T P M L R H S I E N S
7010 7020 7030 7040 7050 7060 7070 7080 7090 7100
CCTCAGTGAATGTGTCCCTAACAGCCATAGCCAACCAAGCCACAGTGTTAATGGGTCTCGGGAAAGGATGGCCATTGTCAAAGATGGACATCGGAGTTCC
S V N V S L T A I A N Q A T V L M G L G K G W P L S K M D I G V P
Additional nt-7026 T-to-C/T mix silent mutation in master and pre-master seeds
7110 7120 7130 7140 7150 7160 7170 7180 7190 7200
CCTTCTCGCCATTGGATGCTACTCACAAGTCAACCCCATAACTCTCACAGCAGCTCTTTTCTTATTGGTAGCACATTATGCCATCATAGGGCCAGGACTC
L L A I G C Y S Q V N P I T L T A A L F L L V A H Y A I I G P G L
7210 7220 7230 7240 7250 7260 7270 7280 7290 7300
CAAGCAAAAGCAACCAGAGAAGCTCAGAAAAGAGCAGCGGCGGGCATCATGAAAAACCCAACTGTCGATGGAATAACAGTGATTGACCTAGATCCAATAC
Q A K A T R E A Q K R A A A G I M K N P T V D G I T V I D L D P I P
7310 7320 7330 7340 7350 7360 7370 7380 7390 7400
CTTATGATCCAAAGTTTGAAAAGCAGTTGGGACAAGTAATGCTCCTAGTCCTCTGCGTGACTCAAGTATTGATGATGAGGACTACATGGGCTCTGTGTGA
Y D P K F E K Q L G Q V M L L V L C V T Q V L M M R T T W A L C E
7410 7420 7430 7440 7450 7460 7470 7480 7490 7500
GGCTTTAACCTTAGCTACCGGGCCCATCTCCACATTGTGGGAAGGAAATCCAGGGAGGTTTTGGAACACTACCATTGCGGTGTCAATGGCTAACATTTTT
A L T L A T G P I S T L W E G N P G R F W N T T I A V S M A N I F
> NS5
7510 7520 7530 7540 7550 7560 7570 7580 7590 7600
AGAGGGAGTTACTTGGCCGGAGCTGGACTTCTCTTTTCTATTATGAAGAACACAACCAACACAAGAAGGGGAACTGGCAACATAGGAGAGACGCTTGGAG
R G S Y L A G A G L L F S I M K N T T N T R R G T G N I G E T L G E
7610 7620 7630 7640 7650 7660 7670 7680 7690 7700
AGAAATGGAAAAGCCGATTGAACGCATTGGGAAAAAGTGAATTCCAGATCTACAAGAAAAGTGGAATCCAGGAAGTGGATAGAACCTTAGCAAAAGAAGG
K W K S R L N A L G K S E F Q I Y K K S G I Q E V D R T L A K E G
7710 7720 7730 7740 7750 7760 7770 7780 7790 7800
CATTAAAAGAGGAGAAACGGACCATCACGCTGTGTCGCGAGGCTCAGCAAAACTGAGATGGTTCGTTGAGAGAAACATGGTCACACCAGAAGGGAAAGTA
I K R G E T D H H A V S R G S A K L R W F V E R N M V T P E G K V
7810 7820 7830 7840 7850 7860 7870 7880 7890 7900
GTGGACCTCGGTTGTGGCAGAGGAGGCTGGTCATACTATTGTGGAGGACTAAAGAATGTAAGAGAAGTCAAAGGCCTAACAAAAGGAGGACCAGGACACG
V D L G C G R G G W S Y Y C G G L K N V R E V K G L T K G G P G H E
7910 7920 7930 7940 7950 7960 7970 7980 7990 8000
AAGAACCCATCCCCATGTCAACATATGGGTGGAATCTAGTGCGTCTTCAAAGTGGAGTTGACGTTTTCTTCATCCCGCCAGAAAAGTGTGACACATTATT
E P I P M S T Y G W N L V R L Q S G V D V F F I P P E K C D T L L
8010 8020 8030 8040 8050 8060 8070 8080 8090 8100
GTGTGACATAGGGGAGTCATCACCAAATCCCACAGTGGAAGCAGGACGAACACTCAGAGTCCTTAACTTAGTAGAAAATTGGTTGAACAACAACACTCAA
C D I G E S S P N P T V E A G R T L R V L N L V E N W L N N N T Q
8110 8120 8130 8140 8150 8160 8170 8180 8190 8200
TTTTGCATAAAGGTTCTCAACCCATATATGCCCTCAGTCATAGAAAAAATGGAAGCACTACAAAGGAAATATGGAGGAGCCTTAGTGAGGAATCCACTCT
F C I K V L N P Y M P S V I E K M E A L Q R K Y G G A L V R N P L S
8210 8220 8230 8240 8250 8260 8270 8280 8290 8300
CACGAAACTCCACACATGAGATGTACTGGGTATCCAATGCTTCCGGGAACATAGTGTCATCAGTGAACATGATTTCAAGGATGTTGATCAACAGATTTAC
R N S T H E M Y W V S N A S G N I V S S V N M I S R M L I N R F T
8310 8320 8330 8340 8350 8360 8370 8380 8390 8400
AATGAGATACAAGAAAGCCACTTACGAGCCGGATGTTGACCTCGGAAGCGGAACCCGTAACATCGGGATTGAAAGTGAGATACCAAACCTAGATATAATT
M R Y K K A T Y E P D V D L G S G T R N I G I E S E I P N L D I I
8410 8420 8430 8440 8450 8460 8470 8480 8490 8500
GGGAAAAGAATAGAAAAAATAAAGCAAGAGCATGAAACATCATGGCACTATGACCAAGACCACCCATACAAAACGTGGGCATACCATGGTAGCTATGAAA
G K R I E K I K Q E H E T S W H Y D Q D H P Y K T W A Y H G S Y E T
8510 8520 8530 8540 8550 8560 8570 8580 8590 8600
CAAAACAGACTGGATCAGCATCATCCATGGTCAACGGAGTGGTCAGGCTGCTGACAAAACCTTGGGACGTCGTCCCCATGGTGACACAGATGGCAATGAC
K Q T G S A S S M V N G V V R L L T K P W D V V P M V T Q M A M T

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      8610       8620       8630       8640       8650       8660       8670       8680       8690       8700
AGACACGACTCCATTTGGACAACAGCGCGTTTTTAAAGAGAAAGTGGACACGAGAACCCAAGAACCGAAAGAAGGCACGAAGAAACTAATGAAAATAACA
 D  T  T  P  F  G  Q  Q  R  V  F  K  E  K  V  D  T  R  T  Q  E  P  K  E  G  T  K  K  L  M  K  I  T

      8710       8720       8730       8740       8750       8760       8770       8780       8790       8800
GCAGAGTGGCTTTGGAAAGAATTAGGGAAGAAAAAGACACCCAGGATGTGCACCAGAGAAGAATTCACAAGAAAGGTGAGAAGCAATGCAGCCTTGGGGG
 A  E  W  L  W  K  E  L  G  K  K  K  T  P  R  M  C  T  R  E  E  F  T  R  K  V  R  S  N  A  A  L  G  A

      8810       8820       8830       8840       8850       8860       8870       8880       8890       8900
CCATATTCACTGATGAGAACAAGTGGAAGTCGGCACGTGAGGCTGTTGAAGATAGTAGGTTTTGGGAGCTGGTTGACAAGGAAAGGAATCTCCATCTTGA
  I  F  T  D  E  N  K  W  K  S  A  R  E  A  V  E  D  S  R  F  W  E  L  V  D  K  E  R  N  L  H  L  E

      8910       8920       8930       8940       8950       8960       8970       8980       8990       9000
AGGAAAGTGTGAAACATGTGTGTACAACATGATGGGAAAAAGAGAGAAGAAGCTAGGGGAATTCGGCAAGGCAAAAGGCAGCAGAGCCATATGGTACATG
 G  K  C  E  T  C  V  Y  N  M  M  G  K  R  E  K  K  L  G  E  F  G  K  A  K  G  S  R  A  I  W  Y  M


   9010       9020       9030       9040       9050       9060       9070       9080       9090       9100
TGGCTTGGAGCACGCTTCTTAGAGTTTGAAGCCCTAGGATTCTTAAATGAAGATCACTGGTTCTCCAGAGAGAACTCCCTGAGTGGAGTGGAAGGAGAAG
 W  L  G  A  R  F  L  E  F  E  A  L  G  F  L  N  E  D  H  W  F  S  R  E  N  S  L  S  G  V  E  G  E  G

      9110       9120       9130       9140       9150       9160       9170       9180       9190       9200
GGCTGCACAAGCTAGGTTACATTCTAAGAGACGTGAGCAAGAAAGAGGGAGGAGCAATGTATGCCGATGACACCGCAGGATGGGATACAAGAATCACACT
  L  H  K  L  G  Y  I  L  R  D  V  S  K  K  E  G  G  A  M  Y  A  D  D  T  A  G  W  D  T  R  I  T  L

      9210       9220       9230       9240       9250       9260       9270       9280       9290       9300
AGAAGACCTAAAAAATGAAGAAATGGTAACAAACCACATGGAAGGAGAACACAAGAAACTAGCCGAGGCCATTTTCAAACTAACGTACCAAAACAAGGTG
 E  D  L  K  N  E  E  M  V  T  N  H  M  E  G  E  H  K  K  L  A  E  A  I  F  K  L  T  Y  Q  N  K  V

      9310       9320       9330       9340       9350       9360       9370       9380       9390       9400
GTGCGTGTGCAAAGACCAACACCAAGAGGCACAGTAATGGACATCATATCGAGAAGAGACCAAAGAGGTAGTGGACAAGTTGGCACCTATGGACTCAATA
 V  R  V  Q  R  P  T  P  R  G  T  V  M  D  I  I  S  R  R  D  Q  R  G  S  G  Q  V  G  T  Y  G  L  N  T

      9410       9420       9430       9440       9450       9460       9470       9480       9490       9500
CTTTCACCAATATGGAAGCCCAACTAATCAGACAGATGGAGGGAGAAGGAGTCTTTAAAAGCATTCAGCACCTAACAATCACAGAAGAAATCGCTGTGCA
  F  T  N  M  E  A  Q  L  I  R  Q  M  E  G  E  G  V  F  K  S  I  Q  H  L  T  I  T  E  E  I  A  V  Q

      9510       9520       9530       9540       9550       9560       9570       9580       9590       9600
AAACTGGTTAGCAAGAGTGGGGCGCGAAAGGTTATCAAGAATGGCCATCAGTGGAGATGATTGTGTTGTGAAACCTTTAGATGACAGGTTCGCAAGCGCT
 N  W  L  A  R  V  G  R  E  R  L  S  R  M  A  I  S  G  D  D  C  V  V  K  P  L  D  D  R  F  A  S  A

      9610       9620       9630       9640       9650       9660       9670       9680       9690       9700
TTAACAGCTCTAAATGACATGGGAAAGATTAGGAAAGACATACAACAATGGGAACCTTCAAGAGGATGGAATGATTGGACACAAGTGCCCTTCTGTTCAC
L  T  A  L  N  D  M  G  K  I  R  K  D  I  Q  Q  W  E  P  S  R  G  W  N  D  W  T  Q  V  P  F  C  S  H

      9710       9720       9730       9740       9750       9760       9770       9780       9790       9800
ACCATTTCCATGAGTTAATCATGAAAGACGGTCGCGTACTCGTTGTTCCCTGTAGAAACCAAGATGAACTGATTGGCAGAGCCCGAATCTCCCAAGGAGC
  H  F  H  E  L  I  M  K  D  G  R  V  L  V  V  P  C  R  N  Q  D  E  L  I  G  R  A  R  I  S  Q  G  A
                                                 |
                                  Additional nt-9750 A-to-C silent mutation in master and pre-master seeds

      9810       9820       9830       9840       9850       9860       9870       9880       9890       9900
AGGGTGGTCTTTGCGGGAGACGGCCTGTTTGGGGAAGTCTTACGCCCAAATGTGGAGCTTGATGTACTTCCACAGACGCGACCTCAGGCTGGCGGCAAAT
 G  W  S  L  R  E  T  A  C  L  G  K  S  Y  A  Q  M  W  S  L  M  Y  F  H  R  R  D  L  R  L  A  A  N

      9910       9920       9930       9940       9950       9960       9970       9980       9990      10000
GCTATTTGCTCGGCAGTACCATCACATTGGGTTCCAACAAGTCGAACAACCTGGTCCATACATGCTAAACATGAATGGATGACAACGGAAGACATGCTGA
A  I  C  S  A  V  P  S  H  W  V  P  T  S  R  T  T  W  S  I  H  A  K  H  E  W  M  T  T  E  D  M  L  T

     10010      10020      10030      10040      10050      10060      10070      10080      10090      10100
CAGTCTGGAACAGGGTGTGGATTCAAGAAAACCCATGGATGGAAGACAAAACTCCAGTGGAATCATGGGAGGAAATCCCATACTTGGGGAAAAGAGAAGA
  V  W  N  R  V  W  I  Q  E  N  P  W  M  E  D  K  T  P  V  E  S  W  E  E  I  P  Y  L  G  K  R  E  D

     10110      10120      10130      10140      10150      10160      10170      10180      10190      10200
CCAATGGTGCGGCTCATTGATTGGGTTAACAAGCAGGGCCACCTGGGCAAAGAACATCCAAGCAGCAATAAATCAAGTTAGATCCCTTATAGGCAATGAA
 Q  W  C  G  S  L  I  G  L  T  S  R  A  T  W  A  K  N  I  Q  A  A  I  N  Q  V  R  S  L  I  G  N  E

                                                                                  > 3'-Noncoding Region
     10210      10220      10230      10240      10250      10260      10270      10280      10290      10300
GAATACACAGATTACATGCCATCCATGAAAAGATTCAGAAGAGAAGAGGAAGAAGCAGGAGTTCTGTGGTAGAAAGCAAAACTAACATGAAACAAGGCTA
E  Y  T  D  Y  M  P  S  M  K  R  F  R  R  E  E  E  E  A  G  V  L  W  *

     10310      10320      10330      10340      10350      10360      10370      10380      10390      10400
GAAGTCAGGTCGGATTAAGCCATAGTACGGAAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCATCATAAATGCCATAG
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     10410      10420      10430      10440      10450      10460      10470      10480      10490      10500
CTTGAGTAAACTATGCAGCCTGTAGCTCCACCTGAGAAGGTGTAAAAAATCCGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGC

     10510      10520      10530      10540      10550      10560      10570      10580      10590      10600
GGTTAGAGGAGACCCCTCCCTTACAAATCGCAGCAACAATGGGGGCCCAAGGCGAGATGAAGCTGTAGTCTCGCTGGAAGGACTAGAGGTTAGAGGAGAC

     10610      10620      10630      10640      10650      10660      10670      10680      10690      10700
CCCCCCGAAACAAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACGCCAGAAAATGGAATG

     10710      10720
GTGCTGTTGAATCAACAGGTTCT
```

What is claimed:

1. A polynucleotide molecule encoding a modified live, attenuated dengue-2 virus strain PDK-53-V polypeptide molecule, wherein the polynucleotide molecule comprises:

an adenine to guanine mutation at position 592 encoding a glutamic acid instead of a lysine in the polypeptide molecule at amino acid position 166 corresponding to prM-52; and

an adenine to guanine mutation at position 8803 encoding a valine instead of an isoleucine in the polypeptide molecule at amino acid position 2903 corresponding to NS5-412.

2. The polynucleotide molecule according to claim 1 comprising the polynucleotide represented by SEQ ID NO: 14.

3. The polynucleotide molecule according to claim 1 or 2 encoding a modified, live attenuated dengue-2 virus strain PDK-53-V polypeptide molecule, wherein the polypeptide molecule is represented by SEQ ID NO: 6.

4. A polypeptide molecule encoded by a polynucleotide molecule according to any one of claims 1-3.

5. A modified live, attenuated dengue-2 strain PDK-53-V virus,
comprising the polypeptide molecule according to claim 4, or
represented by a polynucleotide molecule according to any one of claims 1-3.

6. A pharmaceutical composition comprising a polynucleotide molecule according to any one of claims 1-3, a polypeptide molecule according to claim 4, or a dengue-2 virus according to claim 5, and a pharmaceutically acceptable excipient.

7. The pharmaceutical composition according to claim 6 for use as a medicament.

8. Use of the pharmaceutical composition according to claim 6 in the manufacture of a medicament for inducing an immune response in a subject.

9. A vector encoding a polynucleotide molecule according to any one of claims 1-3.

10. A cell comprising the polynucleotide molecule according to any one of claims 1-3 or the vector according to claim 9.

11. An immunogenic composition comprising a modified live, attenuated dengue-2 strain

PDK-53-V virus represented by a polynucleotide molecule according to any of claims 1-3 and a pharmaceutically acceptable carrier.

12. The immunogenic composition according to claim 11, further comprising a dengue-1/dengue-2 chimera represented by a polynucleotide molecule encoding a dengue-1/dengue-2 polypeptide chimera, the polynucleotide molecule comprising a first nucleotide sequence encoding nonstructural proteins from a modified live, attenuated dengue-2 virus strain PDK 53-V, and a second nucleotide sequence encoding at least one structural protein from dengue-1, wherein the polynucleotide molecule comprises:

an adenine to a cytosine mutation at position 3823 encoding a leucine instead of an isoleucine in the dengue-1/dengue-2 polypeptide chimera at amino acid position 1243 corresponding to NS2A-116;

an adenine to thymine mutation at position 4407 encoding an aspartic acid instead of a glutamic acid in the dengue-1/dengue-2 polypeptide chimera at amino acid position 1437 corresponding to NS2B-92; and

an adenine to guanine mutation at position 7311.

13. The immunogenic composition according to claim 12, wherein the polynucleotide molecule encoding the dengue-1/dengue-2 polypeptide chimera further comprises:

a cytosine to thymine mutation at position 7148 encoding an isoleucine instead of a threonine in the dengue-1/dengue-2 polypeptide chimera at amino acid position 2351 corresponding to NS4B-108; and

a guanine to cytosine mutation at position 2384 encoding an alanine instead of glycine in the dengue-1/dengue-2 polypeptide chimera at amino acid position 763 corresponding to E-483.

14. The immunogenic composition according to claim 12, wherein the polynucleotide molecule encoding the dengue-1/dengue-2 polypeptide chimera comprises the polynucleotide represented by SEQ ID NO: 13.

15. The immunogenic composition according to claim 12 or 14, wherein the polynucleotide molecule encodes the dengue-1/dengue-2 polypeptide chimera represented by SEQ ID NO: 3.

16. The immunogenic composition according to claim 11, further comprising a dengue-3/dengue-2 chimera represented by a polynucleotide molecule encoding a dengue-3/dengue-2 polypeptide chimera, the polynucleotide molecule comprising a first nucleotide sequence encoding nonstructural proteins from a modified live, attenuated dengue-2 virus strain PDK-53-V, and a second nucleotide sequence encoding at least one structural protein from dengue-3, wherein the polynucleotide molecule comprises:

an adenine to thymine mutation at position 1603 encoding a serine instead of a threonine in the dengue-3/dengue-2 polypeptide chimera at amino acid position 503 corresponding to E-223; and

an adenine to guanine mutation at position 7620.

17. The immunogenic composition according to claim 16, wherein the polynucleotide molecule encoding the dengue-3/dengue-2 polypeptide chimera further comprises a guanine to adenine mutation at position 6436 encoding an asparagine instead of an aspartic acid in the dengue-3/dengue-2 polypeptide chimera at amino acid position 2114 corresponding to NS4A-23.

18. The immunogenic composition according to claim 16, wherein the polynucleotide molecule encoding the dengue-3/dengue-2 polypeptide chimera comprises the polynucleotide represented by SEQ ID NO: 15.

19. The immunogenic composition according to claim 16 or 18, wherein the polynucleotide molecule encodes the dengue-3/dengue-2 polypeptide chimera represented by SEQ ID NO: 9.

20. The immunogenic composition according to claim 11, further comprising a dengue-4/dengue-2 chimera represented by a polynucleotide molecule encoding a dengue-4/dengue-2 polypeptide chimera, the polynucleotide molecule comprising a first nucleotide sequence encoding nonstructural proteins from a modified live, attenuated dengue-2 virus strain PDK-53-V, and a second nucleotide sequence encoding at least one structural protein from dengue-4, wherein the polynucleotide molecule comprises:

an adenine to thymine mutation at position 225;

an adenine to guanine mutation at position 3674 encoding a glycine instead of an aspartic acid in the dengue-4/dengue-2 polypeptide chimera at amino acid position 1193 corresponding to NS2A-66;

a cytosine to thymine mutation at position 5391;

a cytosine to thymine mutation at position 6437 encoding a valine instead of an alanine in the dengue-4/dengue-2 polypeptide chimera at amino acid position 2114 corresponding to NS4A-21,

an adenine to cytosine mutation at position 9750, and

optionally a thymine to cytosine mutation at position 7026, and optionally an adenine to guanine mutation at position 3773 encoding an arginine instead of a lysine in the dengue-4/dengue-2 polypeptide chimera at amino acid position 1226 corresponding to NS2A-99.

21. The immunogenic composition according to claim 20, wherein the polynucleotide molecule encoding the dengue-4/dengue-2 polypeptide chimera further comprises a cytosine to

thymine mutation at position 7538 encoding a phenylalanine instead of a serine in the dengue-4/dengue-2 polypeptide chimera at amino acid position 2481 corresponding to NS4B-238.

22. The immunogenic composition according to claim 20, wherein the polynucleotide molecule encoding the dengue-4/dengue-2 polypeptide chimera comprises the polynucleotide represented by SEQ ID NO: 16.

23. The immunogenic composition according to any one of claims 20 or 22, wherein the polynucleotide molecule encodes the dengue-4/dengue-2 polypeptide chimera represented by SEQ ID NO: 12.

24. The immunogenic composition according to any of claims 11 to 23, wherein the composition contains all four dengue virus serotypes, which is a tetravalent composition.

25. The immunogenic composition according to any of claims 11 to 24, further comprising an immunogenic composition against other Flaviviruses such as West Nile virus, Japanese encephalitis.

26. A composition comprising one or more live attenuated viruses according to claim 5 and a pharmaceutically acceptable carrier.

27. The composition according to claim 26, wherein the composition comprises a tetravalent dengue virus composition capable of inducing an immune response in a subject against all four dengue virus serotypes.

28. The vector according to claim 9, wherein the vector is a plasmid vector.

29. The vector according to claim 9, wherein the vector is a cDNA infectious clone.