VACCINES AGAINST JAPANESE ENCEPHALITIS VIRUS AND WEST NILE VIRUS

Field of the Invention

This invention relates to vaccines against Japanese encephalitis virus and West Nile virus.

Background of the Invention

The *Flavivirus* genus of the *Flaviviridae* family includes approximately 70 viruses, mostly arboviruses, many of which, such as yellow fever (YF), dengue (DEN), Japanese encephalitis (JE), and tick-borne encephalitis (TBE) viruses, are major human pathogens (rev. in Burke and Monath, Fields Virology, 4th Ed.:1043-1126, 2001). For example, Japanese encephalitis is the leading cause of viral encephalitis in Asia, where 30,000 to 50,000 new cases are reported each year. As another example, since the first cases were diagnosed in the New York area in 1999, West Nile virus has continued to spread rapidly across North America. The risks of this virus migrating into South America, as well as an epidemic in underdeveloped countries, are extremely high. Effective methods for preventing infection by these viruses are needed, with vaccination being the most cost effective measure.

The Flavivirus particle contains a nucleocapsid composed of viral RNA and capsid protein C. The nucleocapsid is surrounded by an envelope containing the envelope glycoprotein E (50-60 kDa) and a small membrane protein M (7-8 kDa). Translation of the genomic RNA results in a polyprotein precursor that is cleaved by cellular and viral proteases into viral proteins, in the order: C, prM/M, E, NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5, where C through E are the structural components of the virion and NS1 through NS5 are nonstructural proteins required for replication (Lindenbach and Rice, Fields Virology, 4th Ed.:991-1041, 2001). The prM protein (~25 kDa) is the intracellular precursor for M. Immature virions containing prM are produced by budding into the lumen of the endoplasmic reticulum (ER) and are transported to the cell surface through the exocytosis pathway. Cleavage of prM

occurs shortly prior to particle release in post-Golgi vesicles. Mature extracellular virus contains predominantly M protein, although a small fraction of uncleaved prM can also be present.

The E protein is the main functional and antigenic surface component of the virion. The molecular structure of the ectodomain of E, which forms a homodimer on the surface of mature viral particles at neutral pH, has been resolved by cryoelectron microscopy (Rey et al., Nature 375:291-298, 1995) and fitted into the electron density map of viral particles (Kuhn et al., Cell 108:717-725, 2002). During infection, the E protein functions as a class II fusion protein (Modis et al., Nature 427:313-319, 2004). Following virus binding to a cellular receptor and internalization, the acidic pH in the resulting endosomes triggers dissociation of the dimers such that the previously hidden hydrophobic fusion loop of each monomer is exposed outwardly. Concurrently, the loops insert into the cell (endosome) membrane and monomers rearrange into elongated trimers. Further refolding of the trimers brings the cell and viral membranes into close proximity and forces them to fuse, releasing the contents of the viral particle into the cytoplasm. Previous studies showed that some substitutions in the E protein of DEN and JE, which are selected during serial passages in mouse brain and in cultured monkey kidney and mosquito cells, have been localized in particular regions of the 3D structure of the protein, and were reported to be associated with changes in the fusion function of the viruses. The studies showed that the fusion pH threshold for some attenuated vaccines decreased by 0.6 to 1 pH unit by comparison with the corresponding parental virus isolate. Some changes in six residues in the DEN3 protein E (residues 54, 191, 202, 266, 268, and 277) map to the region in domain II. This region is proposed as a focus for the low-pH mediated conformational change required for the surface exposure of the conserved hydrophobic *cd* fusion loop (Lee et al., Virology 232:281-290, 1997).

There is no evidence that the small (mature) M protein plays a role in the events leading to virus internalization from the endosome or has any other appreciable function, while its intracellular precursor, prM, is known to be important for morphogenesis and transport of progeny viral particles. The prM protein also facilitates proper folding of E (Lorenz et al., J. Virol. 76:5480-5491, 2002) and functions to protect the E protein dimer from premature conformational

rearrangement during passage of new particles towards the cell surface through acidic secretory compartments (Guirakhoo et al., J. Gen. Virol. 72:1323-1329, 1991; Guirakhoo et al., Virology 191:921-931, 1992).

ChimeriVax™ technology has been used to create live, attenuated vaccine candidates against medically important Flaviviruses. It employs the YF 17D vaccine virus as a vector in which the prM-E genes are replaced with the prM-E genes from a heterologous Flavivirus, such as JE, dengue, West Nile, or St. Louis encephalitis viruses (Monath et al., Vaccine 17:1869-1882, 1999; Monath et al., Curr. Drug Targets - Inf. Disorders 1:37-50, 2001; Monath et al., Vaccine 20:1004-1018, 2002; Guirakhoo et al., Virology 257:363-372, 1999; Guirakhoo et al., J. Virol. 75:7290-7304, 2001; Guirakhoo et al., Virology 298:146-159, 2002; Pugachev et al., Int. J. Parasitol. 33:567-582, 2003; Guirakhoo et al., J. Virol. 78:4761-4775, 2004). Previously, the ChimeriVax™-JE vaccine virus, containing the prM-E genes from the SA14-14-2 virus (live attenuated JE vaccine used in China), was propagated to high titers in Vero cells cultured in media supplemented with fetal bovine serum (FBS) (Monath et al., Biologicals 33:131-144, 2005). It was successfully tested in preclinical and Phase I and II clinical trials (Monath et al., Vaccine 20:1004-1018, 2002; Monath et al., J. Infect. Dis. 188:1213-1230, 2003). Similarly, successful Phase I clinical trials have been conducted with a ChimeriVax™-WN vaccine candidate, which contains the prM-E sequence from a West Nile virus (NY99 strain), with three specific amino acid changes incorporated into the E protein to increase attenuation (Arroyo et al., J. Virol. 78:12497-12507, 2004).

Summary of the Invention

The invention provides recombinant Flaviviruses that include one or more membrane (M) protein mutations (e.g., substitutions, deletions, or insertions), such as mutations that attenuate the Flavivirus (e.g., mutations that decrease the viscerotropism/viremia of the Flavivirus), increase genetic stability of the Flavivirus during propagation in cell culture (e.g., manufacturing in serum free cultures), and/or increase vaccine virus yields. The Flaviviruses of the invention can be chimeric Flaviviruses, such as Flaviviruses that include capsid and non-structural proteins of a first Flavivirus (e.g., a yellow fever virus, such as YF 17D) and membrane and/or envelope proteins of a second Flavivirus (e.g., Japanese encephalitis virus, West Nile

virus, a dengue virus (dengue-1, dengue-2, dengue-3, or dengue-4 virus), St. Louis encephalitis virus, Murray Valley encephalitis virus, tick-borne encephalitis virus, as well as any other Flavivirus that is a human/animal pathogen from the YF, JE, DEN, and TBE serocomplexes).

In the Flaviviruses of the invention, the mutation (e.g., substitution) can be in the transmembrane or ectodomain of membrane protein M. For example, the mutation can be in the region of amino acids 40-75 of the predicted membrane helix of the membrane protein M of the Flavivirus. As an example, the mutation can be a substitution of amino acid 60 of the membrane protein of a Flavivirus such as Japanese encephalitis virus (e.g., arginine to cysteine in the Japanese encephalitis virus M protein), or in a corresponding amino acid of another Flavivirus. Determination of which amino acid in a given Flavivirus "corresponds" to that of another Flavivirus can be carried out by standard amino acid sequence alignment, as is well known to those of skill in this art. As another example, the mutation can be a substitution of amino acid 66 of the membrane protein of a Flavivirus such as West Nile virus (e.g., a substitution of leucine with proline in the M protein of West Nile virus), or in a corresponding amino acid of another Flavivirus. In other examples, the mutation is at another membrane anchor amino acid, e.g., one or more amino acids selected from the group flanking the M66 residue, including positions 60, 61, 62, 63, 64, 65, and 66 of Japanese encephalitis virus or West Nile virus (or corresponding amino acids in other Flaviviruses) or other amino acid residues of the transmembrane domain.

We also provide for the first time evidence that the ectodomain of the M protein is of important functional significance, because a glutamine to proline change at the M5 residue increased the pH threshold of infection. Therefore, it can now be expected that Flavivirus attenuation can be achieved through amino acid changes or introduction of various deletions or insertions in the amino-terminal ectodomain, or surface part of the M protein, not only its C-terminal hydrophobic anchor. Thus, in other examples, the viruses of the invention include one or more mutations in the M protein ectodomain (residues 1-40) as described herein. This result is quite unexpected, given the lack of any known function of the mature M protein of Flaviviruses.

In addition to the membrane protein mutations noted above, in the case of chimeric Flaviviruses that include membrane and envelope proteins of a West Nile virus, the viruses of the invention can include one or more envelope protein mutations in amino acids selected from the group consisting of amino acids 107, 138, 176, 177, 224, 264, 280, 316, and 440. In other Flaviviruses, the mutations can be present in amino acids that correspond to these amino acids. As a specific example, the Flavivirus can include a mutation corresponding to mutation(s) in West Nile M protein amino acid 66 and E protein mutations at amino acids corresponding to West Nile virus amino acids 107, 316, and 440. In addition to the mutations described above, the Flaviviruses of the invention can also include one or more mutations in the hydrophobic pocket of the hinge region of the envelope protein, as described elsewhere herein. Further mutations that can be included in the viruses of the invention are mutations in the 3'UTR, the capsid protein, or other envelope protein regions, as described further below.

The invention also provides vaccine compositions that include the Flaviviruses described above and elsewhere herein and pharmaceutically acceptable carriers or diluents, as well as methods of inducing immune responses to Flaviviruses in patients by administration of such vaccine compositions. The patients treated according to such methods include those that do not have, but are at risk of developing, infection by the Flavivirus, as well as patients that are infected by the Flavivirus. Further, the invention includes the use of the Flaviviruses described herein in the prophylactic and therapeutic methods described herein, as well as in the manufacture of medicaments for these purposes.

The invention further provides methods of producing vaccines that include a Flavivirus as described herein, which involve introducing into the membrane protein of the Flavivirus a mutation that results in decreased viscerotropism/viremia, and/or increased genetic stability/yields. Further, the invention provides nucleic acid molecules (RNA or DNA) corresponding to the genomes of the Flaviviruses described herein (or the complements thereof), and methods of using such nucleic acid molecules to make the viruses of the invention.

The Flaviviruses of the invention are advantageous because, in having decreased virulence (shown, e.g., by decreased viscerotropism/viremia), they provide an additional level of safety, as compared to their non-mutated counterparts, when

administered to patients. An additional advantage is that some mutations, such as the M-60 mutation in ChimeriVax™-JE, preclude accumulation of undesirable mutations during vaccine manufacture that otherwise could compromise safety, and increase manufacturing yields. Additional advantages of these viruses are provided by the fact that they can include sequences of yellow fever virus strain YF17D (e.g., sequences encoding capsid and non-structural proteins), which (i) has had its safety established for >60 years, during which over 350 million doses have been administered to humans, (ii) induces a long duration of immunity after a single dose, and (iii) induces immunity rapidly, within a few days of inoculation. In addition, the vaccine viruses of the invention cause an active infection in the treated patients. As the cytokine milieu and innate immune response of immunized individuals are similar to those in natural infection, the antigenic mass expands in the host, properly folded conformational epitopes are processed efficiently, the adaptive immune response is robust, and memory is established.

The beneficial aspects of mutations in the M protein on vaccine safety and manufacture in cell culture are novel and unexpected, given the lack of any known function of the mature M protein of Flaviviruses.

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

Brief Description of the Drawings

Fig. 1A is a schematic representation of the 3' untranslated region of yellow fever virus, which shows domains within this region (repeat sequences (RS), conserved sequences CS2, CS1, and the 3'-extreme stem-loop structure), as well as examples of mutations that can be included in the viruses of the invention (e.g., deletions dA, dB, dC, dD, d7, d14, CS2 d5, and CS2 d16).

Fig. 1B is a schematic representation of the sequence and published secondary structure prediction of the 3' untranslated region of yellow fever 17D virus, from the middle of the 3rd RS element to the end of the UTR (Proutski et al., J. Gen. Virol. 78:1543-1549, 1999).

Fig. 1C is an illustration of the optimal YF 17D 3'UTR secondary structure prediction produced using the Zuker RNA folding algorithm.

Fig. 1D is an illustration of the effects of 3'UTR deletions (shown for the dC deletion; Zuker method) on the optimal YF 17D structure (compare with Fig. 1C).

Fig. 2A is a schematic representation of the sequence of the capsid protein of tick-borne encephalitis virus, as well as deletions in this protein reported by Kofler et al., J. Virol. 76:3534-3543, 2002.

Fig. 2B is a schematic representation of the sequence of the capsid protein of YF 17D virus. Regions predicted by computer analysis to have α-helical secondary structure (α-helices I-IV), as well as hydrophobic regions (solid bars) and deletions introduced in this protein in certain ChimeriVax™-WN viruses (e.g., deletions C1 and C2; boxed) are indicated.

Fig. 3 is a graph showing growth of the indicated viruses (WN01, WN02 P5, Large Plaque, Small Plaque, and YF/17D) in HepG2 cells.

Fig. 4 is a graph showing growth of the indicated viruses (WN01, WN02 P5, Large Plaque, Small Plaque, and YF/17D) in THLE-3 cells.

Fig. 5 is a graph showing the viremia in hamsters induced by the indicated viruses (WN02 P5; mixed plaque), Small Plaque (PMS, P10), and Large Plaque (PMS, P10)).

Fig. 6 is a schematic representation of the passage of SF ChimeriVax™-JE virus samples (g.s., experimental passages to study genetic stability).

Fig. 7 is a graph showing growth curves of SF ChimeriVax™-JE viruses of the invention (uncloned P2, P3 MS (E-107), P4 PS (E-107), P5 g.s. (M-60), and P5 VB (E-107)) at the indicated times post-infection, which shows higher growth rates in SF culture of virus samples containing the M-60 [arginine (R) →cysteine (C) and E-107 phenylalanine (F)→leucine (L)] mutants as compared to nonmutant virus (P2).

Fig. 8A is a graph showing infectivities of the M-5 ChimeriVax™-JE mutant (Clone E) compared to P5 uncloned vaccine bulk and Clone I (E-107 mutant), non-mutant (Clone A), and M-60 mutant (Clone C) after treatment with a range of acidic pH. Of significance is the appearance of the slopes and at which pH the viruses lost infectivity, but not initial titers in diluted samples (e.g., at pH 6.8).

Fig. 8B is a Survival Plot of ChimeriVax™-JE vaccine (1.9 $\log_{10}$ PFU/dose, as determined by back titration of inocula) in comparison to ChimeriVax™-JE M5 mutant (1.4 $\log_{10}$ PFU/dose, as determined by back titration of inocula) in 3-4 day old suckling mice inoculated by the intracerebral route.

Fig. 8C is a Survival Plot of ChimeriVax™-JE M5 mutant virus (1.4 $\log_{10}$ PFU/dose as determined by back titration of inocula) in comparison to YF-VAX® (0.9 $\log_{10}$ PFU/dose as determined by back titration of inocula) in 3-4 day old suckling mice inoculated by the intracerebral route.

Fig. 8D shows the results of an Indirect Fusion assay, which provides a comparison of P7 and P10 of ChimeriVax™-DEN1-4 viruses. The virus output for each experiment was determined by standard plaque assay. A, ChimeriVax™-DEN1 PMS P7 (triangles) and P10 (diamonds); B, ChimeriVax™-DEN2 PMS P7 (triangles) and P10 (diamonds); C, ChimeriVax™-DEN3 PMS P7 (triangles) and P10 (diamonds); D, ChimeriVax™-DEN4 PMS P7 (triangles) and P10 (diamonds).

Fig. 8E shows the results of an Indirect Fusion assay with the ChimeriVax™-DEN3, comparing the PMS (P7) vaccine with the Vaccine lot (P10) and the P15 virus. The virus output for each experiment was determined by standard plaque assay. ChimeriVax™-DEN3 PMS P7 (triangles), P10 (diamonds), and P15 (squares).

Fig. 8F shows the structure of a DEN1 E-protein dimer (amino acids 1 to 394) of ChimeriVax™-DEN1 virus (Guirakhoo et al., J. Virol. 78:9998-10008, 2004). (A) The position of the positively charged lysine (K) at residue 204 of the P7 (PMS, 204K) virus is shown by CPK (displays spheres sized to van der Waal radii) representation. Three structural domains are shown in black (domain I), light grey (domain II), and dark grey (domain III). (B) Close up of marked area in panel A. (C) The same area as in panel B from the E protein model of the mutant DEN1 virus (P10, 204R shown in black). Selected amino acids in panel B and C are shown in stick representation. Medium grey, carbon; dark grey, nitrogen; black, oxygen; light grey, sulfur.

Fig. 9A is a graph showing the penetration efficiency of ChimeriVax™-JE viruses M60 mutant (Clone C), E107 mutant (Clone I), and non-mutant (Clone A) at the indicated times. These results indicate that the M60 mutation facilitates penetration in SF Vero cells apparent at the 5 and 10 minute time points. SF Vero cells were infected with appropriately diluted viruses (Clones A, C, and I in serum

free medium) for 5, 10, 20, or 60 minutes, and then were treated for 3 minutes with a solution of 0.1 M glycine, 0.1 M NaCl, pH 3.0, to inactivate extracellular virus. Wells were washed twice with PBS, and then monolayers were overlaid with methyl-cellulose followed by staining plaques on day 5 with crystal violet. Efficiency of penetration is shown as percentages of observed plaque numbers after glycine treatment as compared to control infected wells that were treated with PBS instead of glycine.

Fig. 9B is a schematic representation of the locations of the E-107, M-5, and M-60 amino acid residues in the envelope proteins E and M, illustrating the hypothetical effect of the M-5 residue on fusion. The dashed stretch at the tip of domain II of the E protein containing the E-107 residue represents the fusion peptide (c-d loop), which inserts into cell membrane (Rey et al., Nature 375:291-298, 1995). The M-5 residue is in the N terminal part of the ectodomain of the M protein. The E protein monomers rearrange into trimeric complexes, which fold to force the cell and virus membranes to fuse (Modis et al., Nature 427(6972):313-319, 2004). The M protein may be a functional component of the complexes, e.g., facilitating fusion of the viral membrane with the cell membrane via its interaction with the E protein. The M-60 residue is between the two C-terminal transmembrane stretches of M and may participate in the interaction of the cell and viral membranes during fusion.

Detailed Description

The invention provides vaccines and methods for use in preventing and treating Flavivirus (e.g., Japanese encephalitis (JE) or West Nile (WN) virus) infection. The methods of the invention generally involve vaccination of subjects with a live, attenuated chimeric Flavivirus that consists of a first Flavivirus (e.g., yellow fever virus) in which one or more structural proteins (e.g., membrane and/or envelope proteins) have been replaced with those of a second Flavivirus (e.g., Japanese encephalitis (JE) and/or West Nile (WN) virus; also see below). The membrane proteins of the chimeras of the invention include one or more mutations, as is described further below. Also as is described below, structural proteins such as membrane and/or envelope proteins of other Flaviviruses can be used in place of those of Japanese encephalitis virus or West Nile virus in the chimeric viruses of the present invention. Further, the membrane protein mutations of the invention can also

be used in intact, non-chimeric Flaviviruses (e.g., any of those listed herein), not including any replacements of structural proteins, and optionally with one or more additional mutations, such as those described herein.

A specific example of a chimeric virus that can be included in the vaccines of the invention is the human yellow fever vaccine strain, YF 17D (e.g., YF17D-204 (YF-VAX®, Sanofi-Pasteur, Swiftwater, PA, USA; Stamaril®, Sanofi-Pasteur, Marcy-L'Etoile, France; ARILVAX™, Chiron, Speke, Liverpool, UK; FLAVIMUN®, Berna Biotech, Bern, Switzerland); YF17D-204 France (X15067, X15062); YF17D-204, 234 US (Rice et al., Science 229:726-733, 1985)), in which the membrane and envelope proteins have been replaced with the membrane and envelope proteins (including an M protein mutation, such as a substitution in M60, as described herein) of Japanese encephalitis virus. In another example, the YF 17D membrane and envelope proteins are replaced with those of a West Nile virus (including an M protein mutation, such as a substitution in M66, as described herein).

In other examples, another Flavivirus, such as a dengue virus (serotype 1, 2, 3, or 4), St. Louis encephalitis virus, Murray Valley encephalitis virus, yellow fever virus, including YF 17D strains, or any other Flavivirus, can provide the membrane and/or envelope proteins in such a chimeric virus. Additional Flaviviruses that can be attenuated according to the invention, whether as intact, non-chimeric viruses or as the source of membrane and/or envelope proteins in chimeras, include other mosquito-borne Flaviviruses, such as Kunjin, Rocio encephalitis, and Ilheus viruses; tick-borne Flaviviruses, such as Central European encephalitis, Siberian encephalitis, Russian Spring-Summer encephalitis, Kyasanur Forest Disease, Omsk Hemorrhagic fever, Louping ill, Powassan, Negishi, Absettarov, Hansalova, Apoi, and Hypr viruses; as well as viruses from the Hepacivirus genus (e.g., Hepatitis C virus). Other yellow fever virus strains, e.g., YF17DD (GenBank Accession No. U 17066), YF17D-213 (GenBank Accession No. U17067; dos Santos et al., Virus Res. 35:35-41, 1995), and yellow fever virus 17DD strains described by Galler et al., Vaccines 16(9/10):1024-1028, 1998, can also be used as the backbone viruses into which heterologous structural proteins can be inserted according to the invention.

The viruses listed above each have some propensity to infect visceral organs. The viscerotropism of these viruses may cause dysfunction of vital visceral organs, such as observed in YF vaccine-associated adverse disease events, albeit very

infrequently. The replication of virus in these organs can also cause viremia and thus contribute to invasion of the central nervous system. Decreasing the viscerotropism of these viruses by mutagenesis according to the present invention can thus reduce the abilities of the viruses to cause adverse viscerotropic disease and/or to invade the brain and cause encephalitis.

The mutations of the invention result in beneficial effects to the viruses, which can include, for example, increased attenuation, stability, and/or replication. The mutations are present in the membrane protein, e.g., in the transmembrane region or in the ectodomain of the membrane protein. For example, the mutations can be in amino acid 60 or 66 of the membrane protein and/or in other amino acids within the predicted transmembrane domain (e.g., in any one or more of amino acids 40-75), or in the N-terminal ectodomain of the M protein (e.g., M-5). As a specific example, membrane protein amino acid 60 (arginine in wild type Japanese Encephalitis virus) can be replaced with another amino acid, such as cysteine. A substitution from arginine to cysteine at position M-60 in the ChimeriVax™-JE virus significantly reduced the viremia (viscerotropism) of the virus for humans in clinical trials in which variants of the vaccine with and without the M-60 mutation were tested (Tables 11A and 11B). In addition to cysteine, other amino acids, such as serine, threonine, glycine, methionine, etc., can substitute the wild type amino acid at position 60 of the membrane protein. In another example, membrane protein amino acid 66 (leucine in wild type West Nile virus) can be replaced with another amino acid, such as proline. In addition to proline, other hydrophobic amino acids, such as isoleucine, methionine, or valine, or small amino acids, such as alanine or glycine, can substitute the wild type amino acid at position 66 of the membrane protein. These mutations can also be present in corresponding amino acids of other Flaviviruses, as described herein.

As other examples of substitutions that can be made in membrane protein sequences, amino acids at positions 61, 62, 63, and/or 64 can be substituted, alone or in combination with each other, a mutation at position 60, a mutation at position 66, and/or another mutation(s). Examples of substitutions at these positions in the West Nile virus membrane protein sequence include: valine to alanine at position 61, valine to glutamic acid or methionine at position 62, phenylalanine to serine at position 63, and valine to isoleucine at position 64. These mutations can also be present in corresponding amino acids of other Flaviviruses, as described herein.

Examples of substitutions at these or surrounding positions in the JE virus membrane protein sequence include any of the remaining 20 amino acids with the expectation that a desired effect on viscerotropism and/or vaccine virus replication/stability in cell culture during manufacturing will be achieved. Other examples in chimeric or non-chimeric Flaviviruses include any amino acid substitutions, alone or in combinations, in the N-terminal ectodomain of the M protein composed of residues 1 - ~40 of the protein, as well as deletion(s) of various sizes (e.g., 1, 2, 3, 4, 5, etc., amino acids long) introduced into the ectodomain and/or the transmembrane domain of the M protein.

In addition to one or more of the membrane protein mutations noted above, the viruses of the invention can also include one or more additional mutations. For example, in the case of West Nile virus, such an additional mutation(s) can be in the region of position 107 (e.g., L to F), 316 (e.g., A to V), or 440 (e.g., K to R) (or a combination thereof) of the West Nile virus envelope protein. The mutations can thus be, for example, in one or more of amino acids 102-112, 138 (e.g., E to K), 176 (e.g., Y to V), 177 (e.g., T to A), 244 (e.g., E to G), 264 (e.g., Q to H), 280 (e.g., K to M), 311-321, and/or 435-445 of the West Nile envelope protein. As a specific example, using the sequence of West Nile virus strain NY99-flamingo 382-99 (GenBank Accession Number AF196835) as a reference, the lysine at position 107 can be replaced with phenylalanine, the alanine at position 316 can be replaced with valine, and/or the lysine at position 440 can be replaced with arginine. Examples of additional combinations of amino acids that can be mutated include are as follows: 176, 177, and 280; 176, 177, 244, 264, and 280; and 138, 176, 177, and 280. Further, these mutations can also be present in corresponding amino acids of other Flaviviruses, as described herein.

The ChimeriVax™-JE vaccine already includes all of the above-noted SA14-14-2 specific mutations as it contains the SA14-14-2-specific JE envelope. Additional amino acid changes in the E protein can also be selected and introduced based on the knowledge of the structure/function of the E protein for additional attenuation (e.g., as described below). These mutations can also be present in corresponding amino acids of other Flaviviruses, as described herein.

In addition to the amino acids noted above, the substitutions can be made with other amino acids, such as amino acids that would result in conservative changes from those noted above. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, and leucine; aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

The viruses of the invention (e.g., Japanese encephalitis and West Nile viruses, and chimeric Flaviviruses including membrane and envelope proteins from these or other flaviviruses) can also include in addition to the mutation(s) (e.g., membrane protein mutations) discussed above, one or more mutations in the hinge region or the hydrophobic pocket of the envelope protein, as such mutations have been shown to result in decreased viscerotropism (Monath et al., J. Virol. 76:1932-1943, 2002; WO 03/103571 A2; WO 05/082020; Guirakhoo et al., J. Virol. 78(18):9998-10008, 2004). The polypeptide chain of the envelope protein folds into three distinct domains: a central domain (domain I), a dimerization domain (domain II), and an immunoglobulin-like module domain (domain III). The hinge region is present between domains I and II and, upon exposure to acidic pH, undergoes a conformational change (hence the designation "hinge") that results in the formation of envelope protein trimers that are involved in the fusion of viral and endosomal membranes, after virus uptake by receptor-mediated endocytosis. Prior to the conformational change, the proteins are present in the form of dimers.

Numerous envelope amino acids are present in the hinge region including, for example, amino acids 48-61, 127-131, and 196-283 of yellow fever virus (Rey et al., Nature 375:291-298, 1995). Any of these amino acids, or closely surrounding amino acids (and corresponding amino acids in other Flavivirus envelope proteins), can be mutated according to the invention, and tested for attenuation. Of particular interest are amino acids within the hydrophobic pocket of the hinge region. As a specific example, it has been shown that substituting envelope protein amino acid 204 (K to R), which is in the hydrophobic pocket of the hinge region, in a chimeric Flavivirus including dengue 1 envelope protein sequences inserted into a yellow fever virus vector results in attenuation (Guirakhoo et al., J. Virol. 78:9998-10008, 2004). This substitution leads to an alteration in the structure of the envelope protein, such that intermolecular hydrogen bonding between one envelope monomer and another in the

wild type protein is disrupted and replaced with new intramolecular interactions within monomers. This observation led to a proposal that the attenuation resulting from this substitution is due to these new interactions, which change the structure of the protein in the pre-fusion conformation, most likely by altering the pH threshold that is required for fusion of viral membrane with the host cell, and provides a basis for the design of further attenuated mutants in which additional substitutions are used to increase intramolecular interactions in the hydrophobic pocket, leading to attenuation. Examples of such mutations/substitutions that can be made in the hydrophobic pocket, and included in the viruses of the invention, include substitutions in E202K, E204K, E252V, E253L, E257E, E258G, and E261H (and corresponding substitutions in other Flaviviruses). Any amino acid changes in the corresponding region of the E protein of JE and WN viruses can be designed and incorporated based on the knowledge of homologous protein structure.

The E gene contains functional domains within which amino acid changes may affect function and thereby reduce virulence, as described by Hurrelbrink and McMinn (Adv. Virus Dis. 60:1-42, 2003). The functional regions of the E protein in which mutations may be inserted that, together with the membrane deletions/mutations described in the present application, may result in an appropriately attenuated vaccine include: a) the putative *receptor binding region* on the external surface of domain III, b) the *molecular hinge region* between domains I and II, which determines the acid-dependent conformational changes of the E protein in the endosome and reduce the efficiency of virus internalization; c) *the interface of prM and E proteins*, a region of the E protein that interfaces with prM following the rearrangement from dimer to trimer after exposure to low pH in the endosome; d) the *tip of the fusion domain* of domain II, which is involved in fusion to the membrane of the endosome during internalization events; and e) the *stem-anchor region*, which is also functionally is involved in conformational changes of the E protein during acid-induced fusion events.

Additional attenuating mutations that can be included with one or more membrane protein mutations in the viruses of the invention include mutations in the 3'untranslated region of the yellow fever virus backbone. The organization of the 3'UTR of a yellow fever virus vaccine strain, YF 17D, which is shared by all ChimeriVax™ viruses, is shown in Fig. 1A. It includes in order from the 3' end (i) a 3'-extreme stem-and-loop structure that has been hypothesized to function as a

promoter for minus-strand RNA synthesis and is conserved for all Flaviviruses, (ii) two conserved sequence elements, CS1 and CS2, which share a high degree of nucleotide sequence homology with all mosquito-borne Flaviviruses, and (iii) unique for West African yellow fever virus strains, including the YF17D vaccine virus, three copies of a repeat sequence element (RS) located in the upstream portion of the 3'UTR (Chambers et al., Annu. Rev. Microbiol. 44:649-688, 1990). The 3'UTR also includes numerous stem-loop structures, such as those in the non-conserved region downstream from the RS elements, as depicted in Fig. 1B.

3'UTR mutations that can be included in the viruses of the invention generally are short, attenuating deletions of, for example, less than 30 nucleotides (e.g., 1, 2, 3, etc., and up to 29 (e.g., 2-25, 3-20, 4-15, 5-10, or 6-8 nucleotides in length); U.S. Patent Application Nos. 60/674,546 and 60/674,415). In some examples, the short 3'UTR deletions are designed to destabilize the secondary structure of one or more of the stem structures in the 3'UTR. In addition to deletions, mutations in such structures can also include substitutions that similarly result in stem structure destabilization. In certain examples, the stem-loop structures that are subject to the mutations are present in non-conserved regions of the 3'UTR or in conserved regions that can tolerate such mutations (e.g., in CS2). For example, the stem destabilizing mutations can be present in any one or more of the predicted stem structures shown in Fig. 1B, which shows four examples of such deletions (dA, dB, dC, and dD). Thus, in addition to these specific examples, other examples of 3'UTR mutations in yellow fever virus include mutations that comprise, e.g., 1-2, 3-8, 4-7, or 5-6 nucleotides of the following stem sequences, which are shown in Fig. 1B as read from 5' to 3': TGGAG, CTCCA, GACAG, TTGTC, AGTTT, GGCTG, CAGCC, AACCTGG, TTCTGGG, CTACCACC, GGTGGTAG, GGGGTCT, AGACCCT, AGTGG, and TTGACG. These mutations can also be present in corresponding amino acids of other Flaviviruses, as described herein.

In addition to stem destabilizing mutations, other short deletions in the 3'UTR can also be included with one or more membrane (and possibly other) mutations in the viruses of the invention. For example, the previously described Δ30 mutation (Men et al., J. Virol. 70:3930-3937, 1996; U.S. Patent No. 6,184,024 B1) or mutations that fall within this sequence can be used. Thus, for example, the invention includes any viable deletions that are 1, 2, 3, etc., and up to 29 (e.g., 1-25, 2-20, 3-15, 4-14, 5-13, 6-12, 7-11, 8-10, or 9) nucleotides in length within this region. As a specific example, viruses of the

invention can include deletion d7, in which the following nucleotides from this region in YF17D are deleted: nucleotides 345-351 (AAGACGG; numbering from the 1st nucleotide of the 3'UTR, after the UGA termination codon of the viral ORF; Fig. 1A). Mutations that include deletion of, for example, 1, 2, 3, 4, or 5 additional nucleotides from the 3' or 5' end of this sequence are also included in the invention. In other examples, short deletions in conserved sequences CS1 and CS2 are included in the invention. These mutations can include deletion of, e.g., 1-29, 2-25, 3-20, 4-15, 5-10, or 6-8 nucleotides of these sequences. As two specific examples, nucleotides 360-364 (GGTTA; CS2d5; Fig. 1A) and/or nucleotides 360-375 (GGTTAGAGGAGACCCT; CS2d16; Fig. 1A) are deleted from CS2 of the YF17D-specific 3'UTR. Mutations that include deletion of, for example, 1, 2, 3, 4, or 5 additional nucleotides from the 3' or 5' end of this sequence can also be used. For other flavivirus 3'UTRs, similar mutations can be made, based on the secondary structures of the 3'UTR's. Predictions of secondary structures of 3'UTR of other flaviviruses have been published, e.g., for dengue, Kunjin, and TBE (see, e.g., Proutski et al., Virus Res. 64:107-123, 1999) and HCV (see, e.g., Kolykhalov et al., J. Virol. 70:3363-3371, 1996). Further, numerous 3'UTR nucleotide sequences for many strains of flaviviruses representing all four major serocomplexes (YF, JE, dengue, and TBE) are available from GenBank. Sequences of additional strains can be determined by virus sequencing. The secondary structures of these sequences can be easily predicted using standard software (e.g., mfold or RNAfold programs) to reveal potential stem-loop structures that can be subject to mutagenesis.

It should be noted that the true secondary structures of the 3'UTRs of Flaviviruses, including YF 17D virus, are unknown because there are no available methods to experimentally prove their existence in the context of whole viruses, and therefore published predictions, e.g., the one predicted for YF 17D by Proutski and co-workers (Fig. 1B), may be incorrect. Many alternative structures can be predicted to form in a relatively long RNA molecule (Zuker et al., N.A.R. 19:2707-2714, 2001), and it is possible that different structures (in plus or minus strands) form and function at different steps of the viral life cycle. True structures can be influenced by the formation of various pseudoknots (Olsthoorn et al., RNA 7:1370-1377, 2001) and long range RNA interactions (e.g., RNA cyclization and other interactions (Alvarez et al., J. Virol. 79:6631-6643, 2005)), as well as possible RNA interactions with host and viral proteins. To further complicate interpretation of published results of theoretical computer predictions, manual

operations are often used, such as initial folding of partial sequences with subsequent forcing of initially predicted structures into structures of longer RNA sequences, the artificial use of N's during initial folding steps, and subjective selection of preferred structure elements (e.g., Mutebi et al., J. Virol. 78:9652-9665, 2004). To this end, we folded the 3'UTR RNA sequence of YF 17D using the commonly used Zuker's prediction algorithm. The predicted optimal structure is shown in Fig. 1C, which differs from the Proutsky prediction shown in Fig. 1B. It is important that the small deletions dA, dB, dC, dD, d7, and d14 in Figs. 1A and 1B generally destabilized the predicted native YF 17D optimal (Fig. 1C) and suboptimal structures. An example of one such altered optimal structure (for the dC mutant) is shown in Fig. 1D. In contrast, the CS2d5 and CS2d16 deletions (Figs. 1A and 1B) did not noticeably change the optimal native structure, indicating that these deletions may attenuate the virus (attenuation was demonstrated in the hamster model for ChimeriVax™-WN) by virtue of altering the sequence of CS2 *per se* rather than the 3'UTR structure, or alternatively by altering some suboptimal structures. Thus, even though some of the deletions were designed based on the Proutski structure prediction (Fig. 1B), their true effect may be due to destabilizing different structure elements than the predicted stem-loops in Fig. 1B.

Additional mutations that can be included with membrane protein (and possibly other) mutations in the viruses of the invention are short deletion (e.g., deletions of 1, 2, 3, or 4 amino acids) mutations within the capsid protein. Examples of such mutations, provided in reference to the YF 17D virus capsid protein, include viable deletions affecting Helix I of the protein (see Fig. 2A). A specific example of such a mutation is mutation C2, which includes a deletion of amino acids PSR from Helix I (Fig. 2A). Other short mutations in this region (as well as corresponding mutations in other Flavivirus sequences) can be tested for viability and attenuation, and can also be used in the invention. Capsid protein sequences of other flaviviruses have been published, e.g., for TBE, WN, Kunjin, JE, and dengue viruses (e.g., Pletnev et al., Virology 174:250-263, 1990).

The following are specific examples of chimeric Flaviviruses, which were deposited with the American Type Culture Collection (ATCC) in Manassas, Virginia, U.S.A. under the terms of the Budapest Treaty and granted a deposit date of January 6, 1998, that can be used to make viruses of the invention: Chimeric Yellow Fever

17D/Dengue Type 2 Virus (YF/DEN-2; ATCC accession number ATCC VR-2593) and Chimeric Yellow Fever 17D/Japanese Encephalitis SA14-14-2 Virus (YF/JE A1.3; ATCC accession number ATCC VR-2594). Details of making chimeric viruses that can be used in the invention are provided, for example, in U.S. Patent No. 6,696,281 B1; international applications PCT/US98/03894 (WO 98/37911) and PCT/US00/32821 (WO 01/39802); and Chambers et al., J. Virol. 73:3095-3101, 1999, and are also provided below. These methods can be modified for use in the present invention by including a step of introducing one or more mutations as described herein into inserted sequences (e.g., Japanese encephalitis virus or West Nile virus membrane protein or other sequences). Methods that can be used for producing viruses in the invention are also described in PCT/US03/01319 (WO 03/060088 A2; also see below).

Mutations can be made in the viruses of the invention using standard methods, such as site-directed mutagenesis. One example of the type of mutation present in the viruses of the invention is substitutions, but other types of mutations, such as deletions and insertions, can be used as well. In addition, as is noted above, the mutations can be present singly or in the context of one or more additional mutations, whether within the membrane protein itself or in any combination of, e.g., 3'UTR, capsid, or envelope sequences.

The viruses (including chimeras) of the present invention can be made using standard methods in the art. For example, an RNA molecule corresponding to the genome of a virus can be introduced into primary cells, chick embryos, or diploid cell lines, from which (or the supernatants of which) progeny virus can then be purified. Another method that can be used to produce the viruses employs heteroploid cells, such as Vero cells (Yasumura et al., Nihon Rinsho 21:1201-1215, 1963). In this method, a nucleic acid molecule (e.g., an RNA molecule) corresponding to the genome of a virus is introduced into the heteroploid cells, virus is harvested from the medium in which the cells have been cultured, and harvested virus is treated with a nuclease (e.g., an endonuclease that degrades both DNA and RNA, such as Benzonase™; U.S. Patent No. 5,173,418). In the case of Benzonase™, 15 units/mL can be used, and the conditioned medium refrigerated at 2-8°C for about 16 or more hours to allow for digestion of nucleic acids. The nuclease-treated virus is then concentrated (e.g., by use of ultrafiltration using a filter having a molecular weight

cut-off of, e.g., 500 kDa (e.g., a Pellicon-2 Mini unltrafilter cassette)), diafiltered against MEME without phenol red or FBS, formulated by the addition of lactose, and filtered into a sterile container. Details of this method are provided in WO 03/060088 A2. Further, cells used for propagation of viruses of the invention can be grown in serum free medium, as described below.

The viruses of the invention can be administered as primary prophylactic agents in those at risk of infection, or can be used as secondary agents for treating infected patients. Because the viruses are attenuated, they are particularly well-suited for administration to "at risk individuals" such as the elderly, children, or HIV infected persons. The vaccines can also be used in veterinary contexts, e.g., in the vaccination of horses against West Nile virus infection, or in the vaccination of domestic pets (e.g., cats, dogs, and birds), livestock (e.g., sheep, cattle, pigs, birds, and goats), and valuable animals such as rare birds. Further, the vaccines of the invention can include a virus, such as a chimeric virus, including a particular mutation (e.g., the M5, M60, and/or M66 mutation), in a mixture with viruses lacking such mutations.

Formulation of the viruses of the invention can be carried out using methods that are standard in the art. Numerous pharmaceutically acceptable solutions for use in vaccine preparation are well known and can readily be adapted for use in the present invention by those of skill in this art (see, e.g., *Remington's Pharmaceutical Sciences* (18^{th} edition), ed. A. Gennaro, 1990, Mack Publishing Co., Easton, PA). In two specific examples, the viruses are formulated in Minimum Essential Medium Earle's Salt (MEME) containing 7.5% lactose and 2.5% human serum albumin or MEME containing 10% sorbitol. However, the viruses can simply be diluted in a physiologically acceptable solution, such as sterile saline or sterile buffered saline. In another example, the viruses can be administered and formulated, for example, in the same manner as the yellow fever 17D vaccine, e.g., as a clarified suspension of infected chicken embryo tissue, or a fluid harvested from cell cultures infected with the chimeric yellow fever virus.

The vaccines of the invention can be administered using methods that are well known in the art, and appropriate amounts of the vaccines to be administered can readily be determined by those of skill in the art. What is determined to be an

appropriate amount of virus to administer can be determined by consideration of factors such as, e.g., the size and general health of the subject to whom the virus is to be administered. For example, the viruses of the invention can be formulated as sterile aqueous solutions containing between 10^2 and 10^8, e.g., 10^3 to 10^7 or 10^4 to 10^6, infectious units (e.g., plaque-forming units or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml, to be administered by, for example, intramuscular, subcutaneous, or intradermal routes. In addition, because Flaviviruses may be capable of infecting the human host *via* mucosal routes, such as the oral route (Gresikova et al., "Tick-borne Encephalitis," In *The Arboviruses, Ecology and Epidemiology*, Monath (ed.), CRC Press, Boca Raton, Florida, 1988, Volume IV, 177-203), the viruses can be administered by mucosal (e.g., oral) routes as well. Further, the vaccines of the invention can be administered in a single dose or, optionally, administration can involve the use of a priming dose followed by one or more booster doses that are administered, e.g., 2-6 months later, as determined to be appropriate by those of skill in the art.

Optionally, adjuvants that are known to those skilled in the art can be used in the administration of the viruses of the invention. Adjuvants that can be used to enhance the immunogenicity of the viruses include, for example, liposomal formulations, synthetic adjuvants, such as (e.g., QS21), muramyl dipeptide, monophosphoryl lipid A, or polyphosphazine. Although these adjuvants are typically used to enhance immune responses to inactivated vaccines, they can also be used with live vaccines. In the case of a virus delivered via a mucosal route, for example, orally, mucosal adjuvants such as the heat-labile toxin of *E. coli* (LT) or mutant derivations of LT can be used as adjuvants. In addition, genes encoding cytokines that have adjuvant activities can be inserted into the viruses. Thus, genes encoding cytokines, such as GM-CSF, IL-2, IL-12, IL-13, or IL-5, can be inserted together with foreign antigen genes to produce a vaccine that results in enhanced immune responses, or to modulate immunity directed more specifically towards cellular, humoral, or mucosal responses. Additional adjuvants that can optionally be used in the invention include toll-like receptor (TLR) modulators.

In the case of dengue viruses and/or chimeric Flaviviruses including membrane and envelope proteins of a dengue virus, against which optimal vaccination can involve the induction of immunity against all four of the dengue serotypes, the viruses of the invention can be used in the formulation of tetravalent vaccines. Any or all of the viruses used in such tetravalent formulations can include one or more mutations that decrease viscerotropism, as is described herein. The viruses can be mixed to form tetravalent preparations at any point during formulation, or can be administered in series. In the case of a tetravalent vaccine, equivalent amounts of each virus may be used. Alternatively, the amounts of each of the different viruses present in the administered vaccines can vary (WO 03/101397 A2).

The invention also includes nucleic acid molecules (e.g., RNA or DNA (e.g., cDNA) molecules) that correspond to the genomes of the viruses of the invention as described herein, or the complements thereof. These nucleic acid molecules can be used, for example, in methods of manufacturing the viruses of the invention. In such methods, a nucleic acid molecule corresponding to the genome of a virus is introduced into cells in which the virus can be produced and replicate (e.g., primary cells, chick embryos, diploid cell lines, or heteroploid cell lines (e.g., Vero cells)), and from which (or the supernatants of which) progeny virus can then be purified. These methods can further include virus purification steps, as is known in the art.

As is noted above, details of making chimeric viruses that can be used in the invention are provided, for example, in U.S. Patent No. 6,696,281 B1; international applications PCT/US98/03894 (WO 98/37911) and PCT/US00/32821 (WO 01/39802); and Chambers et al., J. Virol. 73:3095-3101, 1999. Details of the construction of a chimeric Flavivirus including pre-membrane and envelope proteins of Japanese encephalitis virus (or West Nile virus), and capsid and non-structural proteins of yellow fever virus, are provided as follows. These methods can readily be adapted by those of skill in the art for use in constructing chimeras including the mutations described herein, as well as chimeras including other pre-membrane and envelope sequences.

Briefly, derivation of a YF/JE chimera can involve the following. YF genomic sequences are propagated in two plasmids (YF5'3'IV and YFM5.2), which encode the YF sequences from nucleotides 1-2,276 and 8,279-10,861 (YF5'3'IV) and

from 1,373-8,704 (YFM5.2) (Rice et al., The New Biologist 1:285-296, 1989). Full-length cDNA templates are generated by ligation of appropriate restriction fragments derived from these plasmids. YF sequences within the YF5'3'IV and YFM5.2 plasmids are then replaced by the corresponding JE sequences from the start of the prM protein (nucleotide 478, amino acid 128) through the E/NS1 cleavage site (nucleotide 2,452, amino acid 817).

Clones of authentic JE structural protein genes were generated from the JE SA14-14-2 strain (JE live, attenuated vaccine strain; JE SA14-14-2 virus is available from the Centers for Disease Control, Fort Collins, Colorado and the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut, which are World Health Organization-designated Reference Centers for Arboviruses in the United States). JE SA14-14-2 virus at passage level PDK-5 was obtained and passaged in LLC-MK_2 cells to obtain sufficient amounts of virus for cDNA cloning. The strategy used involved cloning the structural region in two pieces that overlap at an *Nhe*I site (JE nucleotide 1,125), which can then be used for *in vitro* ligation.

RNA was extracted from monolayers of infected LLC-MK_2 cells and first strand synthesis of negative sense cDNA was carried out using reverse transcriptase with a negative sense primer (JE nucleotide sequence 2,456-71) containing nested *Xba*I and *Nar*I restriction sites for cloning initially into pBluescript II KS(+), and subsequently into YFM5.2(*Nar*I), respectively. First strand cDNA synthesis was followed by PCR amplification of the JE sequence from nucleotides 1,108-2,471 using the same negative sense primer and a positive sense primer (JE nucleotides sequence 1,108-1,130) containing nested *Xba*I and *Nsi*I restriction sites for cloning into pBluescript and YFM5.2(*Nar*I), respectively. JE sequences were verified by restriction enzyme digestion and nucleotide sequencing. The JE nucleotide sequence from nucleotides 1 to 1,130 was derived by PCR amplification of negative strand JE cDNA using a negative sense primer corresponding to JE nucleotides 1,116 to 1,130 and a positive sense primer corresponding to JE nucleotides 1 to 18, both containing an *Eco*RI restriction site. PCR fragments were cloned into pBluescript and JE sequences were verified by nucleotide sequencing. Together, this represents cloning of the JE sequence from nucleotides 1-2,471 (amino acids 1-792).

To insert the C terminus of the JE envelope protein at the YF E/NS1 cleavage site, a unique *Nar*I restriction site was introduced into the YFM5.2 plasmid by oligonucleotide-directed mutagenesis of the signalase sequence at the E/NS1 cleavage site (YF nucleotides 2,447-2,452, amino acids 816-817) to create YFM5.2(*Nar*I). Transcripts derived from templates incorporating this change were checked for infectivity and yielded a specific infectivity similar to the parental templates (approximately 100 plaque-forming units/250 nanograms of transcript). The JE sequence from nucleotides 1,108 to 2,471 was subcloned from several independent PCR-derived clones of pBluescript/JE into YFM5.2(*Nar*I) using the unique *Nsi*I and *Nar*I restriction sites. YF5'3'IV/JE clones containing the YF 5' untranslated region (nucleotides 1-118) adjacent to the JE prM-E region were derived by PCR amplification.

To derive sequences containing the junction of the YF capsid and JE prM, a negative sense chimeric primer spanning this region was used with a positive sense primer corresponding to YF5'3'IV nucleotides 6,625-6,639 to generate PCR fragments that were then used as negative sense PCR primers in conjunction with a positive sense primer complementary to the pBluescript vector sequence upstream of the *Eco*RI site, to amplify the JE sequence (encoded in reverse orientation in the pBluescript vector) from nucleotide 477 (N-terminus of the prM protein) through the *Nhe*I site at nucleotide 1,125. The resulting PCR fragments were inserted into the YF5'3'IV plasmid using the *Not*I and *Eco*RI restriction sites. This construct contains the SP6 promoter preceding the YF 5'-untranslated region, followed by the sequence: YF (C) JE (prM-E), and contains the *Nhe*I site (JE nucleotide 1,125) required for *in vitro* ligation.

To use the *Nhe*I site within the JE envelope sequence as a 5' *in vitro* ligation site, a redundant *Nhe*I site in the YFM5.2 plasmid (nucleotide 5,459) was eliminated. This was accomplished by silent mutation of the YF sequence at nucleotide 5,461 (T C; alanine, amino acid 1820). This site was incorporated into YFM5.2 by ligation of appropriate restriction fragments and introduced into YFM5.2(*Nar*I)/JE by exchange of an *Nsi*I/*Nar*I fragment encoding the chimeric YF/JE sequence.

To create a unique 3' restriction site for *in vitro* ligation, a *Bsp*EI site was engineered downstream of the *Aat*II site normally used to generate full-length templates from YF5'3'IV and YFM5.2. (Multiple *Aat*II sites are present in the JE

structural sequence, precluding use of this site for *in vitro* ligation.) The *BspE*I site was created by silent mutation of YF nucleotide 8,581 (A C; serine, amino acid 2,860), and was introduced into YFM5.2 by exchange of appropriate restriction fragments. The unique site was incorporated into YFM5.2/JE by exchange of the *XbaI/SphI* fragment, and into the YF5'3'IV/JE(prM-E) plasmids by three-piece ligation of appropriate restriction fragments from these parent plasmids and from a derivative of YFM5.2 (*BspEI*) deleting the YF sequence between the *EcoR*I sites at nucleotides 1 and 6,912.

cDNA from a clone of the JE Nakayama strain, which has been extensively characterized in expression experiments and for its capacity to induce protective immunity (see, e.g., McIda et al., Virology 158:348-360, 1987; the JE Nakayama strain is available from the Centers for Disease Control, Fort Collins, Colorado, and the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut), was also used in the construction of chimeric flaviviruses. The Nakayama cDNA was inserted into the YF/JE chimeric plasmids using available restriction sites (*Hind*III to *Pvu*II and *Bpm*I to *Mun*I) to replace the entire prM-E region in the two plasmid system except for a single amino acid, serine, at position 49, which was left intact in order to utilize the *Nhe*I site for *in vitro* ligation.

Procedures for generating full-length cDNA templates are essentially as described in Rice et al. (The New Biologist 1:285-96, 1989). In the case of chimeric templates, the plasmids YF5'3'IV/JE (prM-E) and YFM5.2/JE are digested with *NheI/BspE*I and *in vitro* ligation is performed using 300 nanograms of purified fragments in the presence of T4 DNA ligase. The ligation products are linearized with *Xho*I to allow run-off transcription. SP6 transcripts are synthesized using 50 nanograms of purified template, quantitated by incorporation of ^{3}H-UTP, and integrity of the RNA is verified by non-denaturing agarose gel electrophoresis. Yields range from 5 to 10 micrograms of RNA per reaction using this procedure, most of which is present as full-length transcripts. Transfection of RNA transcripts in the presence of cationic liposomes is carried out as described by Rice et al. (supra) for YF 17D, to generate the chimeric viruses.

In the case of chimeric flaviviruses including West Nile virus and yellow fever virus sequences, the two-plasmid system described above can also be used. In one example, the West Nile (WN) virus prM and E genes used were cloned from WNV

flamingo isolate 383-99, sequence GenBank accession number AF196835. Virus prME cDNA was obtained by RT-PCR (XL-PCR Kit, Perkin Elmer). The 5' end of WN prM gene was cloned precisely at the 3'end of the YF 17D capsid gene by overlap-extension PCR using Pwo polymerase (Roche). The 3' end of the E gene was also cloned precisely at the 5'end of the YF NS1 coding sequence by overlap-extension PCR. Silent mutations were introduced into the sequence of prM and E to create unique restriction sites *Bsp* EI and *Eag* I. Digestion of the two plasmids with these enzymes generated DNA fragments that were gel purified and ligated *in vitro* to produce a full-length chimeric cDNA. The cDNA was linearized with *Xho* I to facilitate *in vitro* transcription by SP6 polymerase (Epicentre). The RNA product was introduced into eukaryotic cell lines permissive for viral RNA translation and replication of the virus. As with the YF/JE chimera, described above, mutations of the invention can be introduced into YF/WN chimeras as described herein, using standard methods.

Other Flavivirus chimeras can be engineered with a similar strategy, using natural or engineered restriction sites and, for example, oligonucleotide primers as shown in Table 14.

The invention is based, in part, on the experimental results described in the following Examples.

EXAMPLES

Example 1: ChimeriVax™-WN

Experimental Results

Background and Summary

A chimeric yellow fever-West Nile (YF-WN) virus, ChimeriVax™-WN, was produced by insertion of pre-membrane (prM) and envelope (E) genes of a WN virus (NY99) into the YF17D backbone. The virus was produced in Vero cells under serum free conditions (at Passage 5, P5), evaluated for safety, immunogenicity, and efficacy in preclinical models, and has been tested in a phase I study in humans. Additional attenuation of the vaccine virus (P5) is determined by three SA14-14-2-specific mutations in the E protein (residues 107, 316, and 440). The vaccine virus was less neurovirulent than YF-VAX® when tested in mouse and monkeys inoculated by the IC route and protected mice, hamsters, and monkeys upon a single inoculation

(Arroyo et al., J. Virol. 78:12497-12507, 2004; Tesh et al., Emer. Infect. Dis. 8:1392-1397, 2002). The vaccine virus contained a mixed population of viruses (exhibiting small, S, and large, L, plaque phenotypes), which differed by a single amino acid residue in the M protein at position 66 (M66). This mutation did not affect neurovirulence of the virus for 8 day old suckling mice (Arroyo et al., J. Virol. 78:12497-12507, 2004). In the current invention, we describe the discovery that the M66 mutation reduces viremia in the host and thus can be used to improve the safety of the current vaccine (ChimeriVax™-WN02, P5, mixed population of parent and mutant viruses) or the large plaque variant (non mutant) virus.

A nucleotide heterogeneity (~50%) of T and C (CTA/CCA) was observed in the consensus sequence of ChimeriVax™-West Nile vaccine virus at P5 produced in Vero cells under serum free conditions. This mutation would result in presence of viruses containing either amino acid Proline (mutant) or Leucine (parent wild type) in the membrane (M) protein at residue 66 (herein designated as M66 mutation). The sequences of ChimeriVax™WN02 and the ChimeriVax™WN02 M66 variant are provided in the enclosed sequence appendix, which also includes an alignment of the amino acid sequences of these proteins.

The M protein of West Nile virus contains 75 amino acids. The structure of the protein was predicted and compared to the structures of M proteins of JE SA14 (AAA67174), Kunjin (AAP78942), MVE (CAA27184), SLE MSI (AAP44973, and SLE CORAN (AAP44972) by submission of the protein sequence to the http://www.predictprotein.org website. In all predicted structures, the first 40 amino acids of the M protein (SLTVQTHGESTLANKKGAWMDSTKATRYLVKTESWILRN) are predicted to be a non-membrane region, whereas the remaining 35 amino acids (40-75) (PGYALVAAVIGWMLGSNTMQRVVFVVLLLLVAPAYS) are predicted to be within the viral membrane region. In addition, there are 9-10 charged amino acids (3-4 acidic, E or D) and 6 basic (R or K) within the first 40 amino acid residues, whereas there is only one charged amino acid (basic) at residue 60 of all 5 Flaviviruses (WNV, SLE, MVE, JE, and Kunjin) described here. Thus, it may be that the M60 residue plays a vital role in biology of the virus by interaction within its neighboring amino acids.

The plaque morphology of the vaccine virus at P5 revealed a mixed population of L and S plaque size phenotypes. The sequencing of the P2, P3, P4, and P5 viruses revealed that the mutation first appeared at P4 (10% of the total population) and reached ~50% in P5. The sequencing of the S and L plaque isolates of the vaccine virus showed that the mutation is responsible for a change in plaque size from L to S. Both S and L virus variants (prepared as research virus) did not significantly differ in their neurovirulence for 8 day old suckling mice (p=<0.0001).

Pre-Master Seed (PMS, P10) stocks of both L and S viruses were produced in Vero cells from ChimeriVax™-WN02 (p5) under "clean laboratory condition" by 3 rounds of direct plaque to plaque purifications and 2 rounds of virus amplification. The sequencing of P10 S and L viruses revealed a single amino acid difference in the M66 residue (S virus contained Proline at M66 residue, whereas L virus contained Leucine at this site). The M66 mutation seemed to be stable under large scale manufacturing conditions. When the S plaque virus (P10, PMS) was inoculated into hamsters by subcutaneous inoculation, it induced a very low level of viremia compared to the vaccine virus (P5) or the L plaque virus variant (P10, PMS). In sera of monkeys and humans inoculated with ChimeriVax™-WN P5 virus (contained ~50:50 S and L plaque variants), the majority of viruses were of L plaque size phenotype. In addition, it was shown that the S plaques grow to a lower titer than the L plaques in human hepatoma cell lines. These data indicated that the S plaque virus (ChimeriVax™-WN02 with M66 mutation) may induce a lower level of viremia in humans than ChimeriVax™-WN02 (without M66 mutation), and therefore could constitute a suitable (safe) WN vaccine candidate for "at risks individuals," such as the elderly, children, or HIV infected persons. Additional mutations in the M region were found by sequencing individual plaques isolated from large scale manufacturing passages (e.g., M62, M63, and M64) of PMS S plaque from P10 to P12 or monkeys inoculated with ChimeriVax™-WN02 vaccine (e.g., M60, M61, and M63). These mutations can also be used in the construction of viruses of the invention.

Production of PMS of S and L plaque viruses in Vero cells

ChimeriVax™-WN02 vaccine material (P5) was grown in Serum Free Vero cells; 10 plaques identified as "small" (S) and 10 plaques identified as "large" (L) were picked. Each isolate was then passaged on Serum Free Vero cells, and one

plaque was picked from each isolate. The procedure was repeated one final time, for a total of three rounds of plaque purification. The plaque purified isolates (P8) were amplified in T25 cm^2 flasks containing Serum Free Vero cells (and grown in serum free (SF) media), then harvested and stored at -80°C. Isolates were sequenced to find a PMS candidate free of spurious mutations. Two isolates were identified to be free of expressed (non-silent) mutations: one isolate was confirmed to be small plaque (M66 Proline) (Table 1), and the other contained a wt sequence (M66 Leucine) (Table 2). These two isolates were then grown in large flasks, aliquoted, and submitted to QC inventory as LP and SP PMS (P10) viruses.

Genetic stability of SP viruses produced at large scale

In order to determine if the S plaque phenotype is stable during a large scale manufacturing process, the small plaque PMS virus was passaged twice in a bioreactor by infecting Vero cells and growing under serum free conditions to produce the P12 virus. The P12 virus was harvested and plaqued in 6-well plates. The majority of the plaques were of small size. Twenty of the largest plaques available were picked, amplified on O-Vero (one passage), and the prME region was transcribed/amplified via Titan One-Tube RT-PCR kit (Roche). The cDNA fragments containing the M region were sequenced, and the morphology of the isolates was confirmed via immuno-staining using WN specific monoclonal antibodies. Thirteen of 20 plaques contained only M66 (the genetic marker responsible for SP morphology), and 5 isolates contained other mutations in addition to M66. Isolate #4 contained M63 (LP phenotype), and isolate #16 contained a mixed population of wt and M66. These data demonstrated that, despite the fact that some plaques appeared to be of large size, they contained the M66 mutation and upon amplification proved to be of S size. Only one plaque (#4) out of 20 appeared to be of L size, apparently due to a mutation at M63 from L to P. Plaque #16 appeared to produce a mixed population of large and small plaque size viruses containing both wt L and mutant P amino acids at position M66 (Table 3).

Growth of ChimeriVax™-WN virus variants in hepatic cells

Human hepatoma cell lines HepG2 and THLE-3 cells were infected with ChimeriVax™-WN01 (wild type prME), ChimeriVax™-WN02 P5 (containing

mutations at E107, E313, E316, E440, M66 mixed L/P amino acids, mixed S and L plaques), ChimeriVax™-WN LP (E107, E313, E316, and E440, WNL), and ChimeriVax™-WN SP (E107, E313, E316, E440, and M66P, WNS) at an MOI of 0.005. Supernatants were collected daily and titrated on O-Vero cells using the standard neutral red double agarose overlay procedure.

In HepG2 cells (Fig. 3) the highest virus growth ($7x10^6$ PFU/ml) was observed on Day 5 with WN01 (wild type prME), followed by that of LP ($2.7x10^6$ PFU/ml) on Day 5. The virus peak with YF-VAX® was reached on Day 3 ($1.17x10^6$ PFU/ml), followed by WN02 mixed vaccine virus ($6.4x10^5$ PFU/ml) on Day 4. The lowest growth was found with the SP virus (peak titer on Day 4 was $6.1x10^5$ PFU/ml), which contained a single amino acid substitution (L to P) at M66. In THLE-3 cells (Fig. 4), the same pattern as in HepG2 cells was observed with the exception that the titer of YF-VAX® was slightly higher than that of the LP virus. The highest titer was seen again with the WN01 ($1.3x10^5$ PFU/ml, Day 4), followed by those of LP ($5.7x10^4$ PFU/ml, Day 7), YF-VAX® ($8.8 x10^4$ PFU/ml, Day 4), and the mixed P5 virus ($1.8x10^4$ PFU/ml, Day 4). The lowest titer was observed again with the SP virus ($9.2x10^3$ PFU/ml, Day 4).

The induction of cytopathic effects (CPE) was recorded daily for each virus (Table 4). The CPE for WN 01 and the LP virus was first observed on Day 5 and was completed (100%) 2 days later, whereas SP or mixed plaque population induced CPE at an earlier time point (Day 3) and completely destroyed the cell monolayer one day earlier (Day 6) than WN01 or the LP. The induction of CPE with YF-VAX® was first observed on Day 3 and the monolayer was fully destroyed by Day 6 post inoculation. The induction of CPE in HepG2 cells may be due to apoptotic activity of the M protein, as has been shown with wild type dengue viruses (Catteau et al., J. Gen. Virol. 84:2781-2793, 2003). These data showed that the SP virus variant grows to a lower titer than those of mixed or LP viruses, indicating that the M66 mutation may have rendered the virus less hepatotropic for humans.

Lack of detection of ChimeriVax™-WN, SP viruses after inoculation of monkeys with mixed (SP and LP viruses) P5 vaccine virus

A total of 8 naïve cynomolgus monkeys that lacked detectable antibodies to Flaviviruses, such as WN, JE, and YF viruses (as determined by plaque reduction

neutralization test (PRNT)), were inoculated by the subcutaneous route with either ChimeriVax™-WN02 (P5) (n=4) or YF-VAX® (n=4). The purpose of this study was to evaluate viremia, biodistribution, and possible toxicity of the ChimeriVax™-WN02 vaccine during a 3 day observation period. The inoculated dose was ~$1.25x10^5$ PFU/0.5 mL and $5.5x10^4$ PFU/mL for ChimeriVax™-WN02 and YF-VAX®, respectively. Animals were bled daily and sacrificed on Day 4 post inoculation. Blood was used to determine the viremia level using a standard plaque assay on Vero cells, whereas collected tissues were either flash frozen for viral analysis or preserved for histopathological evaluations.

Viremia was assessed on monkey sera collected from Day 1 (before inoculation) through Day 4 (prior to euthanization). The assay was performed either by using agarose double overlay and neutral red staining (to isolate and sequence individual plaques) or by methyl cellulose overlay and crystal violet staining (to measure the level of viremia) as described (Monath et al., J. Virol. 74(4):1742-1751, 2000). The magnitude and duration of viremia in ChimeriVax™-WN02 inoculated monkeys were higher than those of YF-VAX® (Table 5). The highest titer of viremia for YF-VAX® was 200 PFU/mL (animal MF21157, Day 4). The highest titer of viremia for ChimeriVax™-WN P5 virus was 1000 PFU/mL (animal MF21191F, Day 4). All animals (4/4) inoculated with ChimeriVax™-WN02 virus were viremic for 3 days post inoculation, whereas only 2/4 animals inoculated with YF-VAX® became viremic (for only 2 days) (Table 5).

Because animals inoculated with ChimeriVax™-WN02 virus had received a mixture of SP and LP viruses, it was necessary to isolate various SP and LP viruses from sera to identify the virus variant (S or L) responsible for the high level of viremia. Sera of all 4 monkeys obtained from Day 2 to Day 4 post inoculation were diluted 1:2 and 1:10 and used to inoculate duplicate wells of 6-well plates seeded with Vero cells. After addition of the second agarose overlay with neutral red, individual plaques (4 S and 3 L plaques) were picked and directly sequenced to identify the presence of the M66 mutant virus (Table 6). None of the isolated plaques contained the M66 mutation (L to P substitution), indicating that the M66 mutant virus is not responsible for the high level of viremia that was detected in these animals. Interestingly, 3 other mutations were observed in the M region (M60, M61, and M63).

It is possible that either these virus variants had existed in low quantity in the ChimeriVax™-WN02 vaccine virus (which could not be detected by consensus sequencing), or that they have been generated *in vivo* (monkeys) by mutations in the genome of the LP virus variants.

Viremia and neutralizing antibody responses in hamsters inoculated with ChimeriVax™-WN SP (PMS, P10), LP (PMS, P10), or mixed (P5, SP, and LP) viruses

The animals used in this study were maintained in microseparators under BL2 and handled according to an animal protocol approved by the IACUC throughout the study. Three ChimeriVax™-WN02 viruses (SP, PMS, P10; LP, PMS, P10, and the mix SP and LP vaccine virus, P5) were used to infect 7 week-old female Golden Syrian hamsters (*Mesocricetus auratus*) from Harlan Sprague-Dawley. Each virus was injected into a group of 15 hamsters via the subcutaneous route in the inguinal area. The infection dose was 10^5 pfu, and the inoculum volume was 100 µl. An additional group of 5 animals was similarly injected with 100 µl of virus diluent as sham control. On the day of virus infection (Day 0) and each following day until 5 days post infection, blood samples were collected by retro orbital bleeding from all animals except the sham control group. The animals were anaesthetized by inhalation of isofluorane to effect prior to bleeding and inoculation. Virus concentration in the test samples were determined by direct plaquing of a 0.1 mL of 1:10 diluted serum sample in duplicate wells of Vero cell culture grown in 12-well-plates (Fig. 5).

As is shown in Fig. 5, a higher level (3 logs of pfu on average) of peak viremia was observed in serum samples collected from LP virus infected hamsters, while a very low level (< 10 pfu) of viremia was seen in blood samples of SP virus inoculated hamsters. When the proportion of SP virus was increased (to 50% as for the mixed plaque virus) in the inoculum, the peak viremia titer was lowered to approximately half of the LP virus induced viremia level. Additionally, the viremia peak time was delayed for at least 1 day to 4 days post infection.

These data demonstrated that the LP and SP variants isolated from the same parent virus, ChimeriVax™-WN02, have different biological properties. The LP virus replicated to a higher level at a faster rate, in comparison with the SP virus in

hamsters. In addition, mixing SP virus with LP (P5 virus) apparently counteracts some properties of the LP virus. This is shown in the hamster infection experiments, in which the presence of virus in blood was reduced to lower levels and the virus replication kinetics were slowed in mixed virus infected hamsters. In sum, the mutation at M66 (L to P) present in SP variant virus significantly reduced its viremia in hamsters.

Example 2: ChimeriVax™-JE and ChimeriVax™-DEN1-4

Background and Summary

In the study described below, we prepared and characterized a new ChimeriVax™-JE seed virus using Vero cells grown in serum-free (SF) media in order to eliminate any concerns about possible contamination of the vaccine with the prion agent of bovine transmissible encephalopathy. During propagation in SF culture, uncloned virus accumulated mutations not seen previously in serum-containing culture, which appeared to be adaptations to SF growth conditions increasing the rate of virus replication. These mutations occurred in the E or M proteins (E-107 F to L or M-60 R to C mutations) and suggested a functional significance of the M protein in the process of virus replication, which became noticeable during virus growth in SF conditions (see amino acid R at position 60 of the M-protein shown in Example 1 (ChimeriVax™-WN). The effects of mutations within the M (M60, M5 in ChimeriVax™-JE) or the E proteins (E-107 in ChimeriVax™-JE, E202/204 in ChimeriVax™-DEN1 and –DEN3 and E251 in ChimeriVax™-DEN2) on biological properties of the vaccine were defined. All of these chimeric viruses have already been tested in clinical trials.

Materials and methods

Cells and media

Vero cells were originally received from the American Type Culture Collection (ATCC; Manassas, VA; CCL 81; African green monkey kidney cells). These cells were adapted to grow in SF media and were obtained from Baxter (Orth, Austria) at passage 133, and then were used directly by seeding into flasks or seeded

starting from a cell bank at passage 136. In all experiments, the passage level of the Vero cells did not exceed passage 149. Cells and viruses were grown at 36°C under 7.5% CO_2. Cells were propagated under SF conditions.

ChimeriVax™-JE variants

The virus was initiated (passage P1) by electroporation of SF Vero cells with the same *in vitro* RNA transcripts (stored at -80°C) that were used previously for production of a non-SF ChimeriVax™-JE vaccine candidate tested in preclinical and clinical trials (Monath et al., Vaccine 20:1004-1018, 2002) and prepared as described previously (Chambers et al., J. Virol. 73:3095-3101, 1999). Amplification passages were generally done at an MOI of 0.001 pfu/cell and viral harvests were collected on days 3-4 postinfection (when CPE was ~ 10%), clarified by slow speed centrifugation, supplemented with 10% sorbitol, and stored at -80°C. Cloned variants were produced in Baxter Vero cells by three consecutive plaque purifications using a standard agar-neutral red overlay method in the presence of gamma-irradiated FBS (HyClone; FBS was used because the cells failed to form plaques under agar prepared with SF media) followed by amplification in SF conditions. Plaque assays to determine virus titers in indicated samples were performed using a single methyl cellulose overlay method with visualization of plaques by crystal violet on day 5 post-infection.

ChimeriVax™-DEN viruses

ChimeriVax™-DEN1-4 vaccine viruses were prepared by electroporation of Vero cells with RNA transcripts prepared from viral cDNA. Progeny viruses were subjected to three rounds of plaque purification to produce the Pre-Master Seed (PMS) viruses at passage 7 (P7). Three further passages were carried out using U.S. current Good Manufacturing Practices (cGMP) to produce the Vaccine lot (P10 viruses). Some mutations appeared in the E genes of the chimeras after multiple passages in Vero cells (Guirakhoo et al., J. Virol. 78:4761-4775, 2004). One of these mutations (E 204 in ChimeriVax™-DEN1) significantly reduced viscerotropism of the virus in non-human primates (Guirakhoo et al., J. Virol. 78:9998-10008, 2004).

Consensus sequencing

Consensus sequencing of indicated virus samples was performed as previously described (Pugachev et al., Vaccine 20:996-999, 2003). Briefly, virion RNA extracted with the TRIZOL LS reagent (Life Technologies-Gibco BRL) was amplified in five overlapping cDNA amplicons of ~ 2-3 kb in length with Titan One-Tube RT-PCR kit (Roche). Amplicons were sequenced using a collection of JE- and YF-specific oligonucleotide primers of both positive and negative orientation and CEQ Dye Terminator Cycle Sequencing kit (Beckman). Sequencing reaction products were resolved with a CEQ2000XL automated sequencer (Beckman Coulter). The data were aligned and analyzed with Sequencher 4.1.4 (GeneCodes) software. Nucleotide heterogeneities were registered when a heterogeneous signal was observed in all chromatograms representing both plus- and minus-strand sequencing reactions. For some viruses, only the first of the five cDNA amplicons (Fragment I) was sequenced that includes the structural genes.

Neurovirulence in suckling mice

The maintenance and care of mice was in compliance with the National Institutes of Health guidelines for the humane use of laboratory animals. Pregnant outbred ICR female mice were purchased from Taconic Farms (Germantown, NY). Newborn mice were fostered and mixed into new groups 6 days prior to inoculation. Groups of 8 day-old suckling mice were inoculated with 0.02 ml of the indicated virus samples by the intracerebral (IC) route. Serial 1:10 dilutions of viruses used for inoculations were done in MEM-10% FBS. Undiluted inocula were back-titrated and the exact doses of each dilution were calculated. Mortalities were recorded over a period of 21 days. The YF 17D control virus was YF-VAX® (Aventis Pasteur, Swiftwater, PA) reconstituted from a commercial vaccine vial.

Monkey safety and efficacy tests

Experiment 1. The neurovirulence/toxicity profile of new clone C (M-60) ChimeriVax™-JE Vaccine Master Viral Bank (MVB; P11) and Production Viral Bank (PVB; P12) stocks, as compared to YF-VAX® control (YF 17D vaccine virus), was studied according to GLP standards in cynomolgus monkeys. Thirty-three (33) experimentally-naïve, Flavivirus-seronegative cynomolgus monkeys (as determined

by HAI test) were assigned to treatment groups as shown in Table 9. All monkeys were dosed via a single IC injection on Day 1, observed for 30 days, and then euthanized and necropsied. The monkeys were evaluated for clinical signs (twice daily), and changes in food consumption (daily), body weight (weekly), and clinical pathology indices. Clinical scores were assigned according to a clinical scoring system, based on the World Health Organization (WHO) requirements for yellow fever vaccine (WHO, Technical Report Series, No. 872, 1998). Blood samples were collected pre-inoculation on Day 1 and on Days 3, 5, 7, 15, and 31 for clinical pathology analysis (serum chemistry and hematology parameters). Additional blood samples were collected on Day 1 (pre-dose) and Days 2-11 for quantitative viremia determinations, and on Day 1 (pre-dose) and Day 31 for neutralizing antibody titer analyses. A complete necropsy was performed on Day 31 and tissues collected for preservation. Tissue was prepared for histopathology of the liver, spleen, heart, kidney, and adrenal glands. Histopathology of the brain and spinal cord was performed according to the methods described by Levenbook et al. (J. Biol. Stand. 15:305, 1987) and incorporated into the WHO requirements for the yellow fever vaccine (WHO, 1998).

Experiment 2. This experiment was conducted to compare the viremia, immune response, and safety of ChimeriVax™-JE Vaccine [original uncloned vaccine P5 produced previously in LS5 Vero cells in the presence of FBS (BB-IND #9167, Serial #000) containing no mutations except for an E491 L to F change in the hydrophobic tail of E protein] and new Clone C (M-60 mutant) ChimeriVax™-JE purified vaccine bulk preparation (P13) over a 30-day period following a single subcutaneous (SC) administration in cynomolgus monkeys according to GLP standards. Eighteen (18) experimentally-naïve, Flavivirus-seronegative (by HAI test) cynomolgus monkeys were assigned to treatment groups as shown in Table 10. All monkeys were dosed once on Day 1 via SC injection at a single site in one arm. The monkeys were evaluated for clinical signs of toxicity (twice daily), changes in body weight (weekly), and serum chemistry, hematology, and coagulation parameters. Blood samples were collected on Day 1 (pre-inoculation) and Days 4, 7, 15, and 31 for serum chemistry, hematology, and coagulation parameter analysis. Additional

blood samples were collected on Day 1 (pre-inoculation) and Days 2-11 for quantitative viremia analysis, and on Day 1 (pre-inoculation) and Day 31 for Japanese encephalitis virus-specific serum antibody titer analysis.

pH threshold of virus inactivation (Indirect Fusion Assay)

One of the consequences of exposure of Flaviviruses to low pH (in the absence of cell membranes) is induction of irreversible conformational changes in the E protein and virus inactivation (loss of potency). In the presence of cell membranes, these conformational changes are necessary for fusion of viral membrane with those of cellular membranes, resulting in release of viral genome into the host cells. The pH threshold for fusion of mosquito-borne viruses such as WN, DEN, YF, and JE can be measured by fusion from within (FFWI) using the mosquito cell line C6/36 (Guirakhoo et al., Virology 169(1):90-99, 1989). We were not, however, able to demonstrate any FFWI with all of our ChimeriVax™ viruses, probably due to lack of sufficient growth of these viruses in mosquitoes and mosquito cell lines (Johnson et al., Am. J. Trop. Med. Hyg. 70(1):89-97, 2004). We therefore attempted to measure the loss of virus potency after exposure to different pH levels, in an assay designated here as an "Indirect Fusion Assay." This assay determines indirectly the pH threshold at which the fusion of viral membranes with those of cellular membranes occurs.

Fusion was performed at pH 7.0, 6.8, 6.6, 6.4, 6.2, 6.0, 5.8, 5.6, 5.4, and 5.0, using 1X MEM supplemented with 2 mM L-Glutamine, 2.7% sodium bicarbonate, 10% HI FBS, and 1% antibiotic/antimycotic solution [(100 U/ml of penicillin, 0.1 mg/ml of streptomycin, 0.25 μg/ml Amphotericin (Sigma)] adjusted to the proper pH with MES (Sigma). An aliquot of each virus at $1x10^4$ plaque forming unit (PFU)/ml was diluted (10^{-1} dilution) in each pH medium. After 10 minutes of exposure at each pH value, 50% heat inactivated (HI) FBS was added to each vial and the pH of each solution was neutralized with sodium bicarbonate. A volume of 100 μl of each virus at each pH value was used to infect Vero-cell monolayers (seeded at a density of $9x10^5$ cells/well, in 6-well plates) to determine its titer. Infection was performed in duplicate, so as to cause 50 PFU/well; two non-infected wells of cells were kept per plate and served as negative controls. The pH 7.0 and 6.8 samples were taken as references. Titers were analyzed using the standard plaque assay. In this assay, Vero cells were infected with serial dilutions of viruses (10^{-1} to 10^{-6}) into duplicate wells.

After infection, the Vero monolayers were overlaid with 1X MEM (Sigma) supplemented with 2 mM L-Glutamine, 2.7% sodium bicarbonate, 5% HI FBS, 1% antibiotic/antimycotic solution [100 U/ml of penicillin, 0.1 mg/ml of streptomycin, 0.25 μg/ml Amphotericin (Sigma)], and 44% of 0.6% agarose (Sigma). Cells were incubated for 4 days at 37°C, 5% CO_2, and were then overlaid with a second overlay containing 1X MEM supplemented with 2 mM L-Glutamine, 2.6% sodium bicarbonate, 2% HI FBS, 1% antibiotic/antimycotic solution, 44% of 0.6% agarose, and 3% of Neutral red solution (Sigma). The plaques were counted 24 hours after the addition of the second overlay to determine the titer of the virus defined in plaque forming unit (PFU) per milliliter.

Virus penetration assay according to Vlaycheva et al. (J. Virol. 76:6172-6184, 2002)

To demonstrate that the M-60 mutation (and E-107 mutation) facilitates penetration in SF Vero cells, SF Vero cells were infected with Clone A, C, and I viruses, appropriately diluted in SF medium, for 5, 10, 20, or 60 minutes, and then treated for 3 minutes with 0.1 M glycine, 0.1 M NaCl, pH 3.0, to inactivate extracellular virus. Wells were washed twice with PBS, and then monolayers were overlaid with methyl-cellulose, followed by staining plaques on day 5 with crystal violet. Efficiency of penetration was calculated as the percentage of observed plaque numbers after glycine treatment, as compared to control infected wells that were treated with PBS instead of glycine.

Clinical trials of ChimeriVax™-JE

A clinical study (protocol H-040-003) was performed. The vaccine administered to healthy adult male and female subjects had the native sequence at M60 (arginine). Healthy adult subjects/group received a subcutaneous dose of graded doses of ChimeriVax™-JE vaccine, and various control groups were included. Eleven to 33 subjects were tested per dose group. Viremia was measured daily by plaque assay in Vero cell monolayers. The same assay and laboratory determined viremia levels in both trials.

Safety assessments included the recording of adverse events, body temperature, physical examination, and laboratory tests (including measurement of viremia levels). Viremia was seen in the majority of subjects receiving ChimeriVax™-JE.

A second study (protocol H-040-007) was performed in healthy adult male and female subjects in which 31 or 32 subjects per group received graded subcutaneous doses (3, 4, or 5 $\log_{10}$ PFU) of ChimeriVax™-JE containing the M60 cysteine mutation. The dose range was similar to that in the previous study in subjects who had received 2.8, 3.8, and 4.8 $\log_{10}$ PFU.

Results

Adaptive mutations in uncloned SF ChimeriVax™-JE virus, and preparation of cloned variants

A diagram of virus samples produced in this study is shown in Fig. 6. The initial uncloned passage 2 (P2) sample (Pre-Master Seed candidate; PMS) was obtained in SF culture by transfecting cells with *in vitro* RNA transcripts that had been used to produce the vaccine in FBS-containing media for previous studies (Monath et al., Vaccine 20:1004-1018, 2002) followed by an additional amplification passage. The full genome of this virus was sequenced and shown not to contain any detectable mutations (Table 7) (note that the consensus sequencing approach does not detect minor subpopulations; detection limit of mutations is ~ 10%). Small-scale passages starting from this P2 virus to P10 level were performed in T25 flasks to analyze its genetic stability (g.s.) during prolonged propagation in SF culture (Fig. 6; g.s. passages). The full genome sequences of the g.s. P5 and g.s. P10 passages had one nucleotide change from C to T at nucleotide 935 resulting in an R to C amino acid substitution at residue M-60 (Table 7). This mutation was first detectable as heterogeneity at the g.s. P4 passage, but not g.s. P3.

Despite the results of small-scale genetic stability analysis, when three large scale manufacturing SF passages were performed from the uncloned P2 PMS in roller bottles to produce candidate uncloned Master Seed (P3) and the Production Seed (P4), and then in 100 L bioreactors to produce vaccine bulk (P5), a different mutation accumulated, an F to L amino acid change at residue E-107 due to a T to C change at nucleotide 1301 observed as a 50:50% heterogeneity (Table 7). This was an

unacceptable mutation because it is a reversion from the SA14-14-2 sequence to wild type JE sequence at a critical attenuating residue (Arroyo et al., J. Virol. 75:934-942, 2001) and thus could potentially compromise safety of the vaccine.

Based on considerations mentioned below, cloned PMS candidates were then generated by plaque purification, to stabilize the SF vaccine and prevent accumulation of undesirable mutations, such as E-107. Plaque purification removes random mutations in uncloned virus introduced by *in vitro* transcription characterized by low fidelity of RNA synthesis compared to viral RNA synthesis by YF 17D-specific RNA polymerase (Pugachev et al., J. Virol. 78:1032-1038, 2004). Starting from the uncloned P2 PMS virus, a biological clone at P7, Clone A virus, which did not have any amino acid substitutions was obtained by three sequential plaque purifications followed by two amplification passages in SF medium, and was designated non-mutant P7 Clone A PMS. Its genome contained two silent nucleotide changes, at nucleotides 6952 and 7147 (Table 7). These changes were acceptable because they did not change the amino acid sequence of viral proteins and were located outside *cis*-acting RNA elements essential for efficient virus replication. A Clone C P10 virus containing the M-60 mutation (designated M-60 P10 Clone C PMS variant) was produced similarly starting from the P5 g.s. virus (Fig. 6). In addition to the desired M-60 mutation, it only contained a silent nucleotide change at nucleotide 3616 (Table 7). Additionally, research-grade Clone I and Clone E viruses were later also isolated from the uncloned P5 vaccine bulk virus by a single plaque purification (selecting large plaque) and one amplification passage in Vero cells. The Clone I contained a single amino acid change at the E-107 residue, which was a reversion to wild type from amino acid F to amino acid L. Thus, Clone I represents a pure population of the E-107 revertant. Clone E contained a single amino acid mutation at the N-terminus of the M protein, a Q to P amino acid change at residue M-5.

To ascertain genetic stability of the cloned PMS variants, relatively large scale g.s. passages mimicking manufacturing events were performed in SF culture (Fig. 6) (sequential passages designated S were done in T-225 flasks, and passages designates F were done in a 5 or 15 L bioreactor in which Vero cells were grown on Cytodex I microcarrier beads). Sequencing of the prM-E region only (cDNA Fragment I) was performed for the SSS and SSF samples (obtained by three Static passages, or two Static plus one Fermenter passages, respectively) of both candidates, and the FFF

sample of the M-60 variant. None of these g.s. samples had any detectable mutations in the prM or E proteins of the viruses other than the M-60 mutation in Clone C. There was no trace of the E-107 mutation (Table 7). This indicated that an acceptable level of genetic stability was achieved due to plaque-purification. The high genetic stability of the M-60 variant was subsequently confirmed during manufacturing of new Master (P11) and Production Virus (P12) Seeds produced in cell factories and final vaccine bulk (P13) produced in a 50 L bioreactor, all of which retained the M-60 mutation, but had no other detectable changes in their full genomes by consensus sequencing.

Effects of the M-60 and E-107 mutations on virus growth in SF Vero cells

To compare growth kinetics of the non-mutant, M-60 mutant, and E-107 mutant viruses in SF culture, cells were infected at an MOI of 0.001 pfu/ml (confirmed by back-titration) with the uncloned P2 PMS, the uncloned P5 g.s. sample (M-60 mutant), or the uncloned P5 vaccine bulk variant (containing the E-107 mutation), as well as the uncloned P3 Master Seed and P4 Production Seed viruses also containing a proportion of the E-107 mutation. Daily aliquots of virus-containing media were harvested and titrated by plaque assay. As shown in Fig. 7, the M-60 virus grew faster than the non-mutant P2 virus and produced significantly (more than 10 times) higher titers on days 3 and 4 post-infection. The E-107 mutation also enhanced virus replication similarly to the M-60 mutation. Thus, both the M-60 and E-107 mutations clearly conferred a growth advantage in SF culture. In support of this conclusion, daily samples from the S, SSS, and SSF g.s. passages of both the non-mutant lone A and M-60 mutant clone C viruses (see Fig. 6) were collected and titered to analyze growth kinetics with the result that the M-60 mutant invariably produced up to 10 times higher peak titers (close to 8 $\log_{10}$ pfu/ml) compared to non-mutant. Additionally, this conclusion was confirmed by comparing growth curves of Clones A, C, and I viruses in small scale SF culture, as Clones C (M-60) and I (E-107) invariably grew to higher titers than Clone A (non-mutant).

Effects of the M-60 and E-107 mutations on neurovirulence of ChimeriVax™-JE in suckling mice

Mouse neurovirulence tests have been used to ensure that neurovirulence of ChimeriVax™ vaccine candidates does not exceed that of the YF 17D vector. The YF 17D vaccine is lethal for mice of all ages after IC inoculation. In contrast, ChimeriVax™ vaccines are significantly more attenuated. Since adult mice generally are not sensitive to detect subtle differences in neurovirulence, e.g., those due to a single amino acid change, a more sensitive suckling mouse model using survival analysis can be used for that purpose (Guirakhoo et al., Virology 257:363-372, 1999; Guirakhoo et al., Virology 298:146-159, 2002; Monath et al., J. Virol. 76:1932-1943, 2002).

Eight day-old suckling mice were inoculated IC with serial dilutions of the clone A P7 virus, clone C P10 virus (M-60 mutation), uncloned P5 vaccine bulk (E-107 mutation), as well as a previously produced FBS-containing control ChimeriVax™-JE virus (P5 Quality Control Reference Standard virus; no mutations), YF 17D positive control (YF-VAX®), or mock inoculated with diluent. Mortalities over a period of 21 days, median IC 50% lethal dose values (LD_{50}), and average survival times (AST) of mice that died are shown in Table 8. As expected, YF-VAX® was highly neurovirulent. Inoculation of 2.4 $\log_{10}$ PFU of this virus caused 100% mortality with a short AST of 8.8 days. Both the P7 non-mutant and P10 M-60 mutant clones were as highly attenuated as the original FBS-containing version of the chimera, with LD_{50} values > 5 $\log_{10}$ PFU and longer AST. Thus, the M-60 mutation does not change the highly attenuated phenotype of the vaccine in this animal model. The uncloned P5 vaccine bulk virus was significantly more virulent compared to the clones, with an IC LD_{50} of 3.1 $\log_{10}$ PFU, but was less virulent compared to YF-VAX®. Subsequently, manufacturing passages (Master Seed, Production Seed, and Vaccine bulk) of the cloned M-60 vaccine were examined in this test under GLP conditions, with similar results. This confirmed the high genetic/phenotypic stability that was achieved by plaque purification and the use of M-60 mutation.

Analysis of safety and efficacy in nonhuman primates

Experiment 1

In this experiment, neurovirulence of Clone C (M-60 mutant) ChimeriVax™-JE Vaccine Master Viral Bank (MVB) and Production Viral Bank (PVB) were compared after IC administration to cynomolgus monkeys, using YF-VAX® virus as a control (Table 9).

No vaccine-related clinical signs or changes in food consumption, body weight, or serum chemistry, and hematology parameters were observed. Lymphoid hyperplasia, consisting of increased size and number of lymphoid nodules in the spleen, was noted for 9 of 11, 4 of 11, and 8 of 11 monkeys from Groups 1-3, respectively. Although this finding is a common background finding in cynomolgus monkeys, the group incidences were greater than normal in these monkeys and were considered secondary to the expected immune response induced by the vaccines. It is noteworthy that similar changes occurred in both the ChimeriVax™-JE treatment groups and the YF-VAX® reference control group. [Some of the monkeys in all three groups developed low level postinoculation viremia of short duration, which was within acceptable limits, and all animals seroconverted to viruses used for inoculation. On Day 31, yellow fever virus-specific neutralizing antibody titers for the YF-VAX®-treated monkeys ranged from 2.07 to >6.13 in the LNI assay, and no YF-VAX®-treated monkeys had cross-reactive antibodies to JE virus in the $PRNT_{50}$ assay. All ChimeriVax™-JE MVB vaccine-treated monkeys had JE neutralizing antibody titers $\geq$ 320 (range 320 to >20480) and had no cross-reacting antibody to YF virus in the LNI assay. All ChimeriVax™-JE PVB vaccine-treated monkeys had JE neutralizing antibody titers $\geq$ 160 (range 160 to >20480) and had no cross-reacting antibody to YF virus. There was no discernible relation between magnitude or duration of detectable viremia and the magnitude of JE-neutralizing antibody titer induction].

The ChimeriVax™-JE MVB and PVB preparations exhibited minimal neurovirulence in this test. The most comprehensive measure of neurovirulence in the monkey neurovirulence test for Flavivirus vaccines is the combined group mean lesion score, representing the average of the mean target area and mean discriminator area scores. The target areas in cynomolgus monkeys are the substantia nigra and the cervical and lumbar enlargements of the spinal cord and represent regions of the central nervous system (CNS) that are injured by all Flaviviruses. The discriminator

areas are the globus pallidus, putamen, anterior and medial thalamic nuclei, and lateral thalamic nucleus, and represent regions of the CNS that are injured selectively by strains of YF 17D (and presumably other Flaviviruses) having different virulence properties, and that discriminate between a reference strain and a strain having increased neurovirulence. The combined mean lesion scores for monkeys treated with the ChimeriVax™-JE MVB and PVB preparations were significantly lower than for the YF-VAX® reference control group ($p<0.05$). The mean discriminator center scores for the two groups of monkeys treated with the ChimeriVax™-JE MVB and PVB were also significantly lower than for the YF-VAX® reference control group ($p<0.05$) (Table 9). There was no statistically significant difference between mean scores for the 2 groups of monkeys that received the ChimeriVax™-JE vaccine preparations, and both preparations demonstrated similarly low neurovirulence in the monkey neurovirulence test.

Thus, the results of the monkey neurovirulence test show that the new (M60, Clone C) plaque-purified MVB and PVB have a satisfactory safety profile. The test articles displayed no clinical toxicity, and had significantly lower discriminator and combined lesion scores on neuropathological examination than the reference control (YF-VAX®). The test articles did not differ from the reference control (YF-VAX®) in viscerotropism, as measured by quantitative viremia.

Experiment 2

This experiment was done to compare viremia, immune response, and safety of the original uncloned P5 ChimeriVax™-JE Vaccine [produced previously in Vero cells in the presence of FBS, had no mutation except for E491 L to F change located in the hydrophobic tail of the E protein, which appears to be a benign mutation in terms of biological phenotype, and it has already been tested in clinical trials (Monath et al., J. Infect. Dis. 188:1213-1230, 2003; Monath et al., Vaccine 20:1004-1018, 2002)] and the new Clone C (M-60 mutant) ChimeriVax™-JE purified vaccine bulk (P13) following a single subcutaneous (SC) administration in cynomolgus monkeys. ChimeriVax™ –JE virus was detected in the sera of 5 (100%) of 5 seronegative monkeys inoculated with original uncloned P5 ChimeriVax™ –JE vaccine. The

duration of viremia was 2-5 days with titers ranging from 20 to 790 PFU/mL. The mean peak viremia (±SD) was 244 (±310) PFU/mL, and the mean number of viremic days was 3.4 (± 1.34) (Table 10).

ChimeriVax™-JE virus was detected in the sera of 4 (100%) of 4 seronegative monkeys inoculated with the new P13 JE vaccine purified bulk. The duration of viremia was 2-5 days with titers ranging from 50 to 290 PFU/mL. The mean peak viremia (±SD) was 160 (±123) PFU/mL, and the mean number of viremic days was 3.75 (±1.26) (Table 10). Neither mean peak viremia nor number of viremic days differed significantly between the two treatment groups (p-values 0.6290 and 0.7016, respectively; ANOVA).

All seronegative monkeys seroconverted following treatment with the original uncloned P5 ChimeriVax™-JE Vaccine or P13 JE Vaccine Purified Bulk (Table 10). On Day 31, sera from 5 (100%) of 5 monkeys inoculated with uncloned P5 Vaccine had JE virus neutralizing antibody titers ranging from 640 to 5120 (geometric mean titer = 1689). Sera from 4 (100%) of 4 monkeys inoculated with P13 ChimeriVax™-JE Vaccine Purified Bulk had JE virus neutralizing antibody titers ranging from 320 to 2560 (geometric mean titer = 761). Antibody titers did not differ significantly between treatment groups ($p = 0.2986$, ANOVA).

Thus, the new M-60 vaccine was compared to the original uncloned ChimeriVax™-JE vaccine (no mutations except for E491) with respect to safety (viremia) and immunogenicity. The new vaccine was slightly less viscerotropic (a desirable feature) but still highly immunogenic. The differences in the magnitude of viremia and immunogenicity were not statistically significant.

Effects of M-5, M-60, and E-107 mutations on the pH threshold of virus infectivity

ChimeriVax™-JE vaccine was produced by insertion of prM and E genes from SA14-14-2 strain of JE virus into backbone of YF 17D virus. The envelope of SA14-14-2 virus (present in ChimeriVax™-JE) differed from its parent SA14 virus by 10 amino acids: E107 L to F, E138 E to K, E176 I to V, E177 T to A, E227 P to S, E244 E to G, E264 Q to H, E279 K to M, E315 A to V, and E439 K to R (Guirakhoo et al., Virology 257:363-372, 1999). By site-directed mutagenesis it was shown that some of these residues were involved in attenuation of ChimeriVax™-JE virus.

Mutants or revertants of ChimeriVax™-JE were selected to identify whether mutations have altered the pH threshold of these viruses. To determine whether the M-60, E-107, or M-5 mutations affect virus infectivity in a pH-dependent fashion, a standard assay for pH threshold of infectivity was performed as described in Materials and Methods. The following viruses were tested: (1) ChimeriVax™-JE non-mutant (clone A, P7 containing all 10 SA14-14-2 mutations in the E protein); (2) ChimeriVax™-JE E107 F to L revertant (clone I P6, containing 9 E protein mutations); (3) ChimeriVax™-JE M60 R to C mutant (clone C, P10 containing all 10 E protein mutations), and (4) M-5 Q to P mutant (clone E, P6 containing all 10 E protein mutations) (Table 12).

Non-mutant clone A P7 virus, M-60 mutant clone C P10 virus, M-5 mutant clone E, and uncloned P5 virus containing the E-107 mutation were treated with a range of decreasing pHs followed by titration of residual viral infectivity. Infectivity of three viruses (clone A control virus, Clone C M60 mutant, and Clone I E-107 mutant) started to drop uniformly after pH 6.0 and was lost at pH 5.8 (pH threshold 5.9), except for M5 mutant Clone E virus. The M-5 mutant had a significantly higher pH threshold (pH 6.3) compared to all other viruses (pH 5.9) (Fig. 8A). This is the first direct evidence that the ectodomain of M protein plays an essential role in the process of infection of cells by a Flavivirus. Thus, the N-terminus of M protein may function in the process of fusion triggered by a low pH in endosomes following virus adsorption and internalization, which is a function attributed previously solely to the envelope E protein.

The pH threshold of 5.9 for fusion of ChimeriVax™-JE viruses is lower than those described for other wild-type (wt) Flaviviruses (Guirakhoo et al., J. Gen. Virol. 72:1323-1329, 1991) and may be involved in attenuation of the virus.

These data demonstrated that the E-107 mutation in the E region of ChimeriVax™-JE did not change the pH threshold for fusion. Generally, a low pH threshold means that more protonization of specific amino acids is required for conformational changes in the E-protein to occur that are necessary for transition from dimer to trimer. It is likely that one or more SA14-14-2 specific mutations (other than the E107 mutation, which is located within the conserved fusion peptide) are responsible for retaining the low pH threshold (pH 5.9) for fusion and consequently attenuated phenotype of the virus for the host. Apparently, the M-5 mutation is

capable of increasing this threshold from 5.9 to 6.3, which is closer to those of wt Flaviviruses (Guirakhoo et al., Virology:169(1):90-99, 1989; Guirakhoo et al., J. Gen. Virol. 72:1323-1329, 1991). An increase in pH threshold for fusion should theoretically decrease the attenuated phenotype of the virus, because the viruses can fuse at higher pHs with less protonization required for transition to a fusion active state. This appeared to be true, since M5 virus inoculated at 1.4 $\log_{10}$ PFU into 3-4 day old suckling mice by the intracerebral route was significantly more virulent than the control virus (ChimeriVax™-JE vaccine virus without the M5 mutation) inoculated at 1.7 $\log_{10}$ PFU (p=0056) (Fig. 8B). Nevertheless, the M5 mutant virus (at a dose of 1.4 $\log_{10}$ PFU) remained significantly less neurovirulent than YF-VAX® (at a dose of 0.9 $\log_{10}$ PFU) in 3-4 day old suckling mice (Fig. 8C), indicating than the SA14-14-2 mutations within the envelope protein of the vaccine virus are still providing a sufficient level of attenuation for this virus.

Mutations in other chimeras that affect pH threshold for fusion

The Indirect Fusion Assay was performed using two groups of each ChimeriVax™-DEN vaccines viruses: ChimeriVax™-DEN1-4 P7 containing no E protein mutations and ChimeriVax™-DEN1-4 P10 which contained single mutations in the E protein, except for ChimeriVax™-DEN4 P10. Viruses were incubated with media of different pH for 10 minutes at room temperature. The titers were determined, after returning the pH to the neutral pH, using a standard plaque assay. As shown in Table 13, the threshold for virus inactivation (fusion) was similar between P7 and P10 of ChimeriVax™-DEN2 and DEN4 viruses (pH 6.4). In contrast, the pH threshold for ChimeriVax™-DEN1 P10 was 0.4 units lower than that of ChimeriVax™-DEN1 P7 virus (pH 6.0 vs. pH 6.4). The difference in pH threshold was less dramatic for ChimeriVax™-DEN3 P10 virus (pH 6.4 vs. pH 6.2).

The maximum virus inactivation occurred at pH 6.2 for all P7 of ChimeriVax™-DEN viruses except for that of ChimeriVax™-DEN4, which was slightly lower (pH 6.0). It appeared that ChimeriVax™-DEN1 P10 required a significantly lower pH for complete inactivation (pH 5.6). Both ChimeriVax™-DEN1 and –DEN3 viruses contain an amino acid substitution at E-204 from K to R (the E-protein of DEN3 is 2 amino acids shorter than other 3 serotypes, therefore, the E-202 residue in this virus is homologous to E-204 in DEN1). The less dramatic

difference in fusion threshold for the DEN3 chimera might be due to presence of WT (K) and mutant R amino acids (E204K/R) in P10 virus stock as was shown by consensus sequencing (K:R=50:50) (Pugachev et al., J. Virol. 78:1032-1038, 2004). Since no change in threshold for virus inactivation was observed with DEN2 P10 chimera, despite the E251 mutation, it can be concluded that the mutation at this residue is not involved in viral fusion process (Fig. 8D).

In order to determine if the presence of K/R heterogeneity in P10 of ChimeriVax™-DEN3 was responsible for its non-dramatic change in pH threshold for fusion, the indirect fusion assay was performed using P7 (no mutation, E202K), P10 (50% mutation, E202K/R), and P15 (complete mutation, E202R) viruses. As shown in Fig. 8E, the pH threshold for inactivation (fusion) of ChimeriVax™-DEN3 P10 was at pH 6.2, which was between those for ChimeriVax™-DEN3 P7 (pH 6.4) and ChimeriVax™-DEN3 P15 (pH 6.0) viruses. Since the E202 K to R mutation was the only amino acid substitution detected in E-protein of these chimeras, it is most likely that this mutation is responsible for a 0.4 pH shift in pH threshold for fusion of the P15 virus.

As mentioned above, the E204 K to R mutation, which occurred during cell culture manufacture of the vaccine, lowered the pH threshold for fusion by 0.4 units of pH. The E204 K to R mutation appears to generate new intramolecular H bonds and a new salt bridge, which might have a significant impact on the dissociation of the E dimers. The structure of the ChimeriVax™-DEN1 (PMS, P7) E protein was modelled based on the atomic coordinates of 394 residues of the DEN2 E-protein ectodomain (S1 strain) determined in the presence of the detergent *n*-octyl-β-D-glucoside (Modis et al., Proc. Natl. Acad. Sci. U.S.A. 100:6986-6991, 2003). The K residue at position 204 was changed to R to mimic the mutant virus, and the modelling was repeated to represent the E-protein structure of the ChimeriVax™-DEN1 (VL, P10) virus (Guirakhoo et al., J. Virol. 78:9998-10008, 2004). The K residue at position 204 (204K) is located within a short loop, in a hydrophobic pocket lined by residues, which have been shown to influence neurovirulence or the pH threshold for fusion (Lee et al., Virology 232:281-290, 1997; Lindenbach et al., 2001 Flaviviridae: the viruses and their replication. *Fields Virology*, eds. Knipe D.M., and Howley P.M. [Lippincott Williams and Wilkins, Philadelphia], 1, 991-1004; Monath et al., J. Virol. 76:1932-1943, 2002). In Fig. 8F, the homology model of the E-

homodimer structure of the vaccine virus (204R) is compared to that of the PMS (204K) virus. The side chains of 204K and 261H of one of E monomer appeared to make H bonds with the backbone atoms of 252V and 253L residues, respectively, on the opposite monomer. At position 204, the R in the E protein of the vaccine virus (VL P10) is predicted to reorient itself so that these hydrogen (H) bonds are lost. Instead the side chain of the mutant R is in proximity with 261H and 257E, resulting in the generation of new intramolecular H bonds between 204R and 261H, and probably of a new salt bridge between 204R and 257E. Since the *pk* of Histidine could be approximately 6.0, which is slightly below the fusion threshold (pH ~6.4), the initial hypothesis by Guirakhoo et al., (J. Virol. 78:9998-10008, 2004) was that the predicted new H bonds between 204R and 261H and the salt bridge between 204R and 257E, might affect the pH threshold of fusion. This theory turned out to be true, since the experiments described here revealed that the threshold for fusion of ChimeriVax™-DEN1 is around 6.0, which is 0.4 pH units lower than its P7 virus (pH 6.4). Apparently, the new intermolecular bonds introduced by R at residue 204 strengthen the association of the E-dimer so that the transition to low pH requires more protonization of appropriate residues (e.g., H 261). The lower threshold for fusion affects viscerotropism of the virus in monkeys and reduces neurovirulence for suckling mice inoculated by the i.c. route (Guirakhoo et al., J. Virol. 78:9998-10008, 2004).

The E202 K to R substitution in the E-protein of the ChimeriVax™-DEN3 P10 vaccine is homologous to the E204 mutation in the ChimeriVax™-DEN1 P10 vaccine. As with ChimeriVax™-DEN1 P10, ChimeriVax™-DEN3 P10 (heterogenous at residue 202 containing both K and R residue) showed a lower pH threshold (~0.2 pH unit) for fusion when compared to P7. The pH threshold for fusion was further lowered (0.4 pH unit, similar to ChimeriVax™-DEN1 P10) when the mutation was fixed at P15 of ChimeriVax™-DEN3. This data showed that the residue 202/204 may be a universal determinant of attenuation in all dengue viruses. Currently, ChimeriVax™-DEN3 and –DEN4 P10 vaccine viruses do not contain this mutation and both viruses induce a higher viremia levels in monkeys (Guirakhoo et al., J. Virol. 78:4761-4775, 2004) inoculated with a tetravalent vaccine formulation. It remains to be seen if K to R mutation in ChimeriVax™-DEN3 or ChimeriVax™-DEN4 would lower their viscerotropism in their hosts.

It was previously reported that WT-JE had a pH threshold for fusion of 6.4 (Guirakhoo et al., J. Gen. Virol. 72:1323-1329, 1991). In this study, all variants of ChimeriVax™-JE had a pH threshold of 5.9. The low pH threshold observed in these experiments is likely due to the presence of one or more of the 10 attenuating mutations in the envelope protein of ChimeriVax™-JE. This mutation might strengthen the association of the E-protein dimer so that a lower pH is required for dissociation and transition to trimer structure and subsequent fusion. The data presented here showed that neither the E107 F to L mutation (located in the cd-loop of the domain II of the E-protein) nor the E279 M to K mutation (located within the hydrophobic pocket of the domain II) was responsible for lowering the pH threshold. It is possible that other mutations in the JE E protein may affect the pH threshold for fusion. Analysis of the crystal structure of TBE virus E protein, which closely resembles the JE E protein, can help to predict the residues that, if altered, could change the pH threshold for fusion. Based on this model, it is likely that the mutations in residues E244 G and/or E264 H are responsible for a lower pH threshold, than the WT JE, for fusion of ChimeriVax™-JE virus.

Effect of the M-60 and E-107 mutations on efficiency of virus penetration

The effects of the M-60 (Clone C virus) and E-107 (Clone I virus) mutations on virus penetration into SF Vero cells were examined using the method of Chambers (Vlaycheva et al., J. Virol. 76:6172-6184, 2002). In this experiment, SF Vero cells were infected with appropriately diluted viruses (to yield ~ 50 plaques/well at each time point) for 5, 10, 20, or 60 minutes. Un-internalized virus is inactivated by addition of acidic glycine silution, while control parallel wells are treated with PBS (neutral pH). Cells are washed with PBS and overlaid with methyl-cellulose overlay, followed by visualization and counting of plaques on day 5. The efficiency of penetration is presented as a percentage of the average number of plaques in glycine-treated wells relative to the number of plaques in control, PBS treated wells. A preliminary penetration test result is shown in Fig. 9A. It is important that the percentages of penetrated Clone C and Clone I viruses were higher than the non-mutant Clone A virus at 5 and 10 minute time points, at which effects of mutations on penetration are more likely to be detected. The result is not statistically significant as evidenced by standard deviation bars and needs to be confirmed in additional repeat

tests. Nevertheless, this experiment suggested that both the M-60 and E-107 mutations could improve the efficiency of membrane fusion of ChimeriVax™-JE virus to cells grown in SF conditions. A possible mechanism of the effect of the M-60 and E-107 residues on process of membrane fusion is illustrated in Fig. 9B. The M-60 residue is located in the viral membrane, while the E-107 residue inserts into the cell membrane, and the two membranes are forced to fuse following low pH-dependent rearrangement of the E protein (which based on our data could be facilitated by the M protein ectodomain). A more appropriate amino acid at either of these two residues may facilitate fusion of the membranes.

Because our data establish for the first time that both the ectodomain of the M protein and its transmembrane domain are of functional significance, the entire M protein can now be considered an attractive target for mutagenesis to attenuate Flaviviruses for the purpose of developing new live attenuated vaccines. For example, random or specific (following further analysis of protein structure) amino acid changes, or deletions of increasing length, e.g., of 1, 2, 3, 4, 5, etc., amino acids, can be incorporated throughout the protein with the expectation that biological phenotype of the virus will be altered, resulting in significant attenuation.

Results from clinical trial

The viremia profiles of ChimeriVax™-JE with the arginine and cysteine M60 residues as obtained from the clinical trials noted above are compared in Tables 11 A and B. In subjects receiving ChimeriVax™-JE M60 arginine, 67-100% of the subjects were viremic on one or more days, compared to 29-50% for subjects receiving ChimeriVax™-JE M60 cysteine. The mean maximum viremia levels in subjects receiving ChimeriVax™-JE M60 arginine ranged from 13 to 40 PFU/ml, compared to mean maximum viremia levels of 3.5-6.3 PFU/ml in the case of ChimeriVax™-JE M60 cysteine. The duration of viremia was also notably longer in the case of ChimeriVax™-JE M60 arginine.

These data demonstrated that the level of viremia is notably lower in the case of the vaccine containing the M60 mutation. Viremia is a measure of viscerotropism (virulence) of the vaccine virus. A vaccine with reduced viremia is considered safer, since cell damage and dysfunction of organs sustaining virus replication and contributing to viremia is reduced, as is the likelihood that the virus will cross the

blood brain barrier and invade the central nervous system. In other experiments, it was shown that the M60 mutant was as highly immunogenic in humans as the non-mutant.

Table 1. Consensus sequence of small plaque (P10 PMS) (P/N IT-0116; L/N I020504A) (plaque purified from p5 Run 1 Vaccine Lot).

Position	**Amino Acid change**	NT position	**NT change**
M(66)	**L**eucine → **P**roline	954	CTA → CCA
E(313)	**G**lycine → a**R**ginine	1919	GGG → AGG
	Asparagine (silent)	2926	AAC → AAT
	Glycine (silent)	7126	GGA → GGG

Table 2. Consensus sequence of large plaque PMS (P10, PMS) (P/N IT-0117; L/N I030804A) (derived from p5 Run 1 Vaccine Lot).

Position	**Amino Acid change**	NT position	**NT change**
E(313)	**G**lycine → a**R**ginine	1919	GGG → AGG
	Glycine (Silent)	7126	GGA → GGG

Table 3. Sequence of large plaques isolated after 2 additional passages of the S plaque PMS (p10) in Vero cells under serum free conditions.

LP Isolate	Position	Amino Acid Change	NT #	NT change	Immuno-Stain
#3, #7, #8, #9, #10, #11, #12, #13, #14, #18, #19, #20	M66	Leucine → Proline	954	CTA → CCA	SP
#1	M62	Valine → Methionine	941	TGT → TAT	SP
	M66	Leucine → Proline	954	CTA → CCA	
#2	M62	Valine → Glycine	942	GTG → GGG	SP
		Valine → Glutamic Acid	942	GTG → GAG	
	M66	Leucine → Proline	954	CTA → CCA	
#4	M63	Phenylalanine → Serine	945	TTT → TCT	LP
#5	M62 M66	Valine → Alanine Leucine → Proline	942	GTG → GCG	SP
#6	M66	Leucine → Proline	954	CTA → CCA	SP
	M64	Valine (Silent)	949	GTC → GTT	
#15	M62	Valine → Alanine	942	GTG → GCG	SP
	M66	Leucine → Proline	954	CTA → CCA	
#16	wt	Leucine	N/A	CTA	LP/SP
	M66	Leucine → Proline	954	CTA → CCA	
#17	M64	Valine → Isoleucine	947	GTC → ATC	SP
	M66	Leucine → Proline	954	CTA → CCA	

Table 4. Observed CPE for HepG2.

Days Post Infection	0	1	2	3	4	5	6	7	8
WN01	0%	0%	0%	0%	0%	30%	90%	~100%	100%
WN02 P5	0%	0%	0%	5%	30%	50%	~100%	100%	
WNL	0%	0%	0%	0%	0%	30%	90%	~100%	100%
WNS	0%	0%	0%	5%	30%	50%	~100%	100%	
YF/17D	0%	0%	0%	20%	50%	70%	~100%	100%	

Table 5. Viremia in monkeys inoculated with ChimeriVax™-WN02 vaccine or YF-VAX®.

Treatment Group	Monkey Number	Day 1**	Day 2	Day 3	Day 4
YF-Vax®	**MF21157M**	0	0	20	200
YF-Vax®	**MF21214F**	0	0	0	0
YF-Vax®	**MF21151M**	0	0	10	60
YF-Vax®	**MF21252F**	0	0	0	0
ChimeriVax™-WN Vaccine (P5)	**MF2808M**	0	30	790	820
ChimeriVax™-WN Vaccine (P5)	**MF21205F**	0	50	160	100
ChimeriVax™-WN Vaccine (P5)	**MF21139M**	0	10	180	70
ChimeriVax™-WN Vaccine (P5)	**MF21191F**	0	80	970	1000

*Viremia expressed as pfu/mL

**Day 1: Study Day 1, monkeys inoculated on Study Day 1

Zero PFU/mL means below the limit of detection, theoretical assay cutoff = 10 PFU/mL

Table 6. Sequence of the M region of YF-WN chimera obtained directly from a plaque isolate from viremic monkeys inoculated with WN02 vaccine virus.

Monkey #	*Day of viremia*	*Plaque Isolate #*	*Visible Plaque Morphology (at time of picking)*	*M66 Present?*	Additional M Mutations
21205	4	#4	SP	NO	NO
2808	3	#8	SP	NO	NO
2808	3	#9	LP	NO	M60 (R to G)
21191	2	#10	LP	NO	NO
21191	1	#14	SP	NO	M61 (V to A)
21191	1	#15	SP	NO	NO
21191	1	#16	LP	NO	M63 (F to S)

Table 7. Nucleotide and amino acid sequences of the uncloned and cloned SF ChimeriVax™-JE samples (see Fig. 6).

Candidate	Passage	Part of genome sequenced	Protein – a.a. No.[b]	Nt No.[a]	Nucleotide change/ heterogeneity	Amino acid change/ heterogen.	Comments
Uncloned	**P2 (PMS)**	Full genome	-	-	-	-	No mutations
	P3 g. s. from PMS	a.a. M-60 only	-	-	-	-	No M-60 mutation
	P4 g. s. from PMS	a.a. M-60 only	M-60	935	c/ T	R/C	M-60 mutation first detectable and dominant
	P5 g. s. from PMS	Full genome	M-60	935	C to T	R to C	M-60 mutation located in the cytoplasmic hydrophilic stretch of the M protein
	P10 g. s. from PMS	95% full genome	M-60	935	C to T	R to C	M-60 is the only detected mutation
	cGMP P3 (MS) Baxter	prM-E	E-107	1301	T/c	F/L	Reversion to WT first detectable
	cGMP P4 (PS) Baxter	prM-E	E-107	1301	T/c	F/L	Reversion to WT
	cGMP P5 (VB) Baxter	Full genome	E-107	1301	T/C	F/L	Reversion to WT (~50%).
M-60 mutant **clone C**	**P10 PMS**	Full genome	M-60 NS2A-26	935 3616	C to T A to G	R to C -	Desired/expected Silent
	SSS P13 g.s.	prM-E	M-60	935	C to T	R to C	No subpopulations detected
	SSF P13 g.s.	prM-E	M-60	935	C to T	R to C	No subpopulations detected
	FFF P13 g.s.	prM-E	M-60	935	C to T	R to C	No subpopulations detected
Non-mutant **clone A**	**P7 PMS**	Full genome	NS4B-12 NS4B-77	6952 7147	C to T T to C	- -	Silent Silent
	SSS P10 g.s.	prM-E	-	-	-	-	No subpopulations detected
	SSF P10 g.s.	prM-E	-	-	-	-	No subpopulations detected

[a]: From the beginning of the genome [b]: From the N-terminus of indicated protein

Table 8. Neurovirulence of clone A P7, clone C P10, uncloned P5, FBS-containing standard, and YF-VAX® viruses in 8 day-old suckling mice.

Virus	a.a. change	Dilution	Inoculation Dose Log_{10} PFU	Mortality No. dead/No. inoculated (% mortality)	LD_{50} Log_{10} PFU	AST days
Clone A P7 PMS	None	Neat	5.1	1/11 (9%)	>5.1	11
		10^{-1}	4.1	3/11 (27%)		14
		10^{-2}	3.1	1/10 (10%)		14
		10^{-3}	2.1	1/12 (8.3%)		11
		10^{-4}	1.1	0/12 (0%)		N/A
Clone C P10 PMS	M-60	Neat	5.5	2/11 (18%)	>5.5	11
		10^{-1}	4.5	0/10 (0%)		N/A
		10^{-2}	3.5	1/12 (8.3%)		13
		10^{-3}	2.5	0/12 (0%)		N/A
		10^{-4}	1.5	0/12 (0%)		N/A
Uncloned P5 VB	E-107	Neat	5.3	9/10 (90%)	3.1	9.4
		10^{-1}	4.3	10/11 (91%)		10.7
		10^{-2}	3.3	9/11 (82%)		11.8
		10^{-3}	2.3	1/11 (9%)		14
		10^{-4}	1.3	1/10 (10%)		9
FBS-containing standard virus	none	Neat	5.3	0/10 (0%)	>5.3	N/A
		10^{-1}	4.3	0/10 (0%)		N/A
		10^{-2}	3.3	2/9 (22%)		16.5
		10^{-3}	2.3	0/11 (0%)		N/A
YF-VAX®	N/A	10^{-1}	2.4	10/10 (100%)	<2.4	8.8
Sham (MEM-10%FBS)	N/A	N/A	N/A	0/10 (0%)	N/A	N/A

Table 9. Neurovirulence for Cynomolgus Monkeys of M-60 (Clone C) Master and Production seeds vs. YF-VAX® control.

Group No.	Number Male/Female	Treatment	Dose (PFU[1]/0.25 mL inoculum)	Lesion scores (group mean; SD (ran		
				Target areas	Discriminator Areas	Combined
1	6/5	YF-VAX® (Commercial Yellow Fever Vaccine)	5.5×10^4	0.436 SD 0.190 (0.25-0.81)	0.610 SD 0.417 (0.25-1.38)	0.526 SD 0.194 (0.29-0.87)
2	5/6	ChimeriVax™-JE Vaccine Master Viral Bank P11 (M-60)	1.0×10^5	0.196 SD 0.210 (0-0.56)	0.183 SD 0.177 (0-0.44)	0.191 SD 0.163 (0-0.47)
3	6/5	ChimeriVax™-JE Vaccine Production Viral Bank P12 (M-60)	1.0×10^5	0.223 SD 0.349 (0-0.56)	0.106 SD 0.138 (0-0.31)	0.167 SD 0.231 (0-0.63)

[1] PFU = plaque-forming units

[2] 4 of 11, 2 of 11, and 1 of 11 animals in groups 1, 2, and 3, respectively, were excluded from score calculations because they were found to be JE-seropositive on day 1 (pre-inoculation) in a retrospective PRNT50 test, which is more sensitive than HAI test used for prescreening.

Table 10. Comparison of magnitudes of viremia and immunogenicity in cynomolgus monkeys inoculated SC with the original uncloned P5 ChimeriVax™-JE vaccine produced in FBS-containing medium (containing no mutations except for E491) and the new Clone C P13 purified vaccine bulk (M-60 mutant).

Group No.	Number of Male/Female	Sample	Dose (PFU)	Viremia[1]		Neutralizing antibody titer on day 31 (geometric mean $PRNT_{50}$ titer (min., max))[1]
				Mean peak titer ± SD (PFU/ml)	Mean duration ± SD (days)	
1	3/3	Diluent	0	0	0	N/D
2	3/3	ChimeriVax™-JE original uncloned P5 Vaccine	1.0×10^4	244 ± 310	3.4 ± 1.34	1689 (640, 5120
3	3/3	Clone C (M-60) ChimeriVax™-JE vaccine, purified bulk, P13	1.0×10^4	160 ± 123	3.75 ± 1.26	761 (320, 2560)

[1]2 of 6, 1 of 6, and 2 of 6 animals in groups 1, 2, and 3, respectively, were excluded from calculations of the values because they were found to be JE-seropositive on day 1 (pre-inoculation) in a retrospective PRNT50 test, which is more sensitive than HAI test used for prescreening.

Table 11A. Viremia profiles in subjects enrolled in Study H-040-003 in which ChimeriVax™-JE with the M60 arginine amino acid was administered. The dose range in bold is similar to that given in another study (H-040-007) in which the mutant M-60 cysteine vaccine was administered.

Viremia	Dose Log_{10} PFU ChimeriVax™-JE M60 arginine				
	5.8	4.8	3.8	2.8	1.8
	(n=10)	(n=33)	(n=11)	(n=11)	(n=11)
Viremic on 1 or more days [No. viremic/total (%)]	5/10 (50%)	**22/33 (67%)**	**9/11 (82%)**	**11/11 (100%)**	9/11 (82%)
Mean peak viremia (PFU/mL)	7.0	**13.0**	**16.4**	**40.9**	18.2
Range in peak viremia (PFU/mL)	0 - 20	**0 - 40**	**0 - 50**	**0 - 220**	0 - 50
Mean duration (days)	0.9	**1.6**	**1.4**	**2.7**	2.2
Range in duration (days)	0 - 4	**0 - 5**	**0 - 3**	**1 - 6**	0 - 5

Table 11B. Viremia profiles in subjects enrolled in Study H-040-007 in which ChimeriVax™-JE with the M60 cysteine amino acid was administered.

Viremia	Dose Log_{10} PFU ChimeriVax™-JE M60 cysteine		
	5.0	4.0	3.0
	N=31	N=32	N=32
Viremic on 1 or more days [No. viremic/total (%)]	9/31 (29%)	16/32 (50%)	13/32 41%)
Mean peak viremia (PFU/mL)	3.5	6.3	4.4
Range in peak viremia (PFU/mL)	0-20	0-30	0-10
Mean duration (days)	0.3	0.8	0.6
Range in duration (days)	0-2	0-4	0-3

Table 12. Values of pH threshold for fusion found with the fusion assay for each ChimeriVax™-JE vaccine.

Virus	pH threshold for fusion
ChimeriVax™-JE parent, clone A P7 (contains all 10 E mutations)	5.9
ChimeriVax™-JE clone C P10 (M60 R to C mutant, contains all 10 E mutations)	5.9
ChimeriVax™-JE clone I P6 (E107 F to L revertant, contains 9 E mutations)	5.9
ChimeriVax™-JE clone E P6 (M5 Q to P mutant, contains all 10 E mutations)	6.3

Table 13. Values of pH threshold for fusion found with the indirect fusion assay for each couple of ChimeriVax™-DEN P7 and P10.

Virus	pH Threshold for fusion
ChimeriVax™-DEN1 PMS P7	6.4
ChimeriVax™-DEN1 VL P10	6.0
ChimeriVax™-DEN2 PMS P7	6.4
ChimeriVax™-DEN2 VL P10	6.4
ChimeriVax™-DEN3 PMS P7	6.4
ChimeriVax™-DEN3 VL P10	6.2
ChimeriVax™-DEN4 PMS P7	6.4
ChimeriVax™-DEN4 VL P10	6.4

Table 14

Engineering of YF/Flavivirus chimeras

Virus	Chimeric C/prM junction[1]	Chimeric E/NS1 junction[2]	5' ligation[3]	3' ligation[4]	Sites[5] eliminated or (created)
YF/WN	X-cactgggagagcttgaaggtc (SEQ ID NO:1)	aaagccagttgcagccgcggtttaa (SEQ ID NO:2)	*Aat*II	*Nsi*I	
YF/DEN-1	X-aaggtagactggtgggctccc (SEQ ID NO:3)	gatcctcagtaccaaccgcggtttaa (SEQ ID NO:4)	*Aat*II	*Sph*I	*Sph*I in DEN
YF/DEN-2	X-aaggtagattggtgtgcattg (SEQ ID NO:5)	aaccctcagtaccaccccgcggtttaa (SEQ ID NO:6)	*Aat*II	*Sph*I	
YF/DEN-3	X-aaggtgaattgaagtgctcta (SEQ ID NO:7)	acccccagcaccaccccgcggtttaa (SEQ ID NO:8)	*Aat*II	*Sph*I	*Xho*I in DEN (*Sph*I in DEN)
YF/DEN-4	X-aaaaggaacagttgttctcta (SEQ ID NO:9)	acccgaagtgtcaaccgcggtttaa (SEQ ID NO:10)	*Aat*II	*Nsi*I	
YF/SLE	X-aacgtgaatagttggatagtc (SEQ ID NO:11)	accgttggtcgcacccgcggtttaa (SEQ ID NO:12)	*Aat*II	*Sph*I	*Aat*II in SLE
YF/MVE	X-aatttcgaaaggtggaaggtc (SEQ ID NO:13)	gaccggtgtttacagccgcggtttaa (SEQ ID NO:14)	*Aat*II	*Age*I	(*Age*I in YF)
YF/TBE	X-tactgcgaacgacgttgccac (SEQ ID NO:15)	actgggaacctcacccgcggtttaa (SEQ ID NO:16)	*Aat*II	AgeI	(*Age*I in YF)

1,2: The column illustrates the oligonucleotide used to generate chimeric YF/Flavivirus primers corresponding to the C/prM or E/NS1 junction. (See text). X = carboxyl terminal coding sequence of the YF capsid. The underlined region corresponds to the targeted heterologous sequence immediately upstream of the *Nar*I site (antisense - ccgcgg). This site allows insertion of PCR products into the Yfm5.2 (*Nar*I) plasmid required for generating full-length cDNA templates. Other nucleotides are specific to the heterologous virus. Oligonucleotide primers are listed 5' to 3'.

3,4: The unique restriction sites used for creating restriction fragments that can be isolated and ligated *in vitro* to produce full-length chimeric cDNA templates are listed. Because some sequences do not contain convenient sites, engineering of appropriate sites is required in some cases (footnote 5).

5: In parentheses are the restriction enzyme sites that must be created either in the YF backbone or the heterologous virus to allow efficient *in vitro* ligation. Sites not in parentheses must be eliminated. All such modifications are done by silent mutagenesis of the cDNA for the respective clone. Blank spaces indicate that no modification of the cDNA clones is required.

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```
1      NGTAAATCCT GTGTGCTAAT TGAGGTGCAT TGGTCTGCAA

41     ATCGAGTTGC TAGGCAATAA ACACATTTGG ATTAATTTTA

81     ATCGTTCGTT GAGCGATTAG CAGAGAACTG ACCAGAACAT
                                                 M
121    GTCTGGTCGT AAAGCTCAGG GAAAAACCCT GGGCGTCAAT
         S  G  R  K  A  Q  G  K  T  L  G  V  N
161    ATGGTACGAC GAGGAGTTCG CTCCTTGTCA AACAAAATAA
         M  V  R  R  G  V  R  S  L  S  N  K  I
201    AACAAAAAAC AAAACAAATT GGAAACAGAC CTGGACCTTC
       K  Q  K  T  K  Q  I  G  N  R  P  G  P  S
241    AAGAGGTGTT CAAGGATTTA TCTTTTTCTT TTTGTTCAAC
         R  G  V  Q  G  F  I  F  F  F  L  F  N
281    ATTTTGACTG GAAAAAAGAT CACAGCCCAC CTAAAGAGGT
         I  L  T  G  K  K  I  T  A  H  L  K  R
321    TGTGGAAAAT GCTGGACCCA AGACAAGGCT TGGCTGTTCT
       L  W  K  M  L  D  P  R  Q  G  L  A  V  L
361    AAGGAAAGTC AAGAGAGTGG TGGCCAGTTT GATGAGAGGA
         R  K  V  K  R  V  V  A  S  L  M  R  G
401    TTGTCCTCAA GGAAACGCCG TTCCCATGAT GTTCTGACTG
         L  S  S  R  K  R  R  S  H  D  V  L  T
441    TGCAATTCCT AATTTTGGGA ATGCTGTTGA TGACGGGTGG
       V  Q  F  L  I  L  G  M  L  L  M  T  G  G
481    AGTTACCCTC TCTAACTTCC AAGGGAAGGT GATGATGACG
         V  T  L  S  N  F  Q  G  K  V  M  M  T
521    GTAAATGCTA CTGACGTCAC AGATGTCATC ACGATTCCAA
         V  N  A  T  D  V  T  D  V  I  T  I  P
561    CAGCTGCTGG AAAGAACCTA TGCATTGTCA GAGCAATGGA
       T  A  A  G  K  N  L  C  I  V  R  A  M  D
601    TGTGGGATAC ATGTGCGATG ATACTATCAC TTATGAATGC
         V  G  Y  M  C  D  D  T  I  T  Y  E  C
641    CCAGTGCTGT CGGCTGGTAA TGATCCAGAA GACATCGACT
         P  V  L  S  A  G  N  D  P  E  D  I  D
```

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```
681   GTTGGTGCAC AAAGTCAGCA GTCTACGTCA GGTATGGAAG
        C  W  C  T  K  S  A  V  Y  V  R  Y  G  R
721   ATGCACCAAG ACACGCCACT CAAGACGCAG TCGGAGGTCA
        C  T  K  T  R  H  S  R  R  S  R  R  S
761   CTGACAGTGC AGACACACGG AGAAAGCACT CTAGCGAACA
        L  T  V  Q  T  H  G  E  S  T  L  A  N
801   AGAAGGGGGC TTGGATGGAC AGCACCAAGG CCACAAGGTA
        K  K  G  A  W  M  D  S  T  K  A  T  R  Y
841   TTTGGTAAAA ACAGAATCAT GGATCTTGAG GAACCCTGGA
        L  V  K  T  E  S  W  I  L  R  N  P  G
881   TATGCCCTGG TGGCAGCCGT CATTGGTTGG ATGCTTGGGA
        Y  A  L  V  A  A  V  I  G  W  M  L  G
921   GCAACACCAT GCAGAGAGTT GTGTTTGTCG TGCTATTGCT
        S  N  T  M  Q  R  V  V  F  V  V  L  L  L
961   TTTGGTGGCC CCAGCTTACA GCTTCAACTG CCTTGGAATG
        L  V  A  P  A  Y  S  F  N  C  L  G  M
1001  AGCAACAGAG ACTTCTTGGA AGGAGTGTCT GGAGCAACAT
        S  N  R  D  F  L  E  G  V  S  G  A  T
1041  GGGTGGATTT GGTTCTCGAA GGCGACAGCT GCGTGACTAT
        W  V  D  L  V  L  E  G  D  S  C  V  T  I
1081  CATGTCTAAG GACAAGCCTA CCATCGACGT CAAGATGATG
        M  S  K  D  K  P  T  I  D  V  K  M  M
1121  AATATGGAGG CGGCCAACCT GGCAGAGGTC CGCAGTTATT
        N  M  E  A  A  N  L  A  E  V  R  S  Y
1161  GCTATTTGGC TACCGTCAGC GATCTCTCCA CCAAAGCTGC
        C  Y  L  A  T  V  S  D  L  S  T  K  A  A
1201  ATGCCCGACC ATGGGAGAAG CTCACAATGA CAAACGTGCT
        C  P  T  M  G  E  A  H  N  D  K  R  A
1241  GACCCAGCTT TTGTGTGCAG ACAAGGAGTG GTGGACAGGG
        D  P  A  F  V  C  R  Q  G  V  V  D  R
1281  GCTGGGGCAA CGGCTGCGGA TTTTTTGGCA AAGGATCCAT
        G  W  G  N  G  C  G  F  F  G  K  G  S  I
1321  TGACACATGC GCCAAATTTG CCTGCTCTAC CAAGGCAATA
        D  T  C  A  K  F  A  C  S  T  K  A  I
```

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```
1361  GGAAGAACCA TCTTGAAAGA GAATATCAAG TACGAAGTGG
        G  R  T  I  L  K  E  N  I  K  Y  E  V
1401  CCATTTTTGT CCATGGACCA ACTACTGTGG AGTCGCACGG
      A  I  F  V  H  G  P  T  T  V  E  S  H  G
1441  AAATTACTCC ACACAGGTTG GAGCCACTCA GGCCGGCCGA
        N  Y  S  T  Q  V  G  A  T  Q  A  G  R
1481  TTCAGCATCA CTCCTGCTGC GCCTTCATAC ACACTAAAGC
        F  S  I  T  P  A  A  P  S  Y  T  L  K
1521  TTGGAGAATA TGGAGAGGTG ACAGTGGACT GTGAACCACG
      L  G  E  Y  G  E  V  T  V  D  C  E  P  R
1561  GTCAGGGATT GACACCAATG CATACTACGT GATGACTGTT
        S  G  I  D  T  N  A  Y  Y  V  M  T  V
1601  GGAACAAAGA CGTTCTTGGT CCATCGTGAG TGGTTCATGG
        G  T  K  T  F  L  V  H  R  E  W  F  M
1641  ACCTCAACCT CCCTTGGAGC AGTGCTGGAA GTACTGTGTG
      D  L  N  L  P  W  S  S  A  G  S  T  V  W
1681  GAGGAACAGA GAGACGTTAA TGGAGTTTGA GGAACCACAC
        R  N  R  E  T  L  M  E  F  E  E  P  H
1721  GCCACGAAGC AGTCTGTGAT AGCATTGGGC TCACAAGAGG
        A  T  K  Q  S  V  I  A  L  G  S  Q  E
1761  GAGCTCTGCA TCAAGCTTTG GCTGGAGCCA TTCCTGTGGA
      G  A  L  H  Q  A  L  A  G  A  I  P  V  E
1801  ATTTTCAAGC AACACTGTCA AGTTGACGTC GGGTCATTTG
        F  S  S  N  T  V  K  L  T  S  G  H  L
1841  AAGTGTAGAG TGAAGATGGA AAAATTGCAG TTGAAGGGAA
        K  C  R  V  K  M  E  K  L  Q  L  K  G
1881  CAACCTATGG CGTCTGTTCA AAGGCTTTCA AGTTTCTTAG
      T  T  Y  G  V  C  S  K  A  F  K  F  L  R
1921  GACTCCCGTG GACACCGGTC ACGGCACTGT GGTGTTGGAA
        T  P  V  D  T  G  H  G  T  V  V  L  E
1961  TTGCAGTACA CTGGCACGGA TGGACCTTGC AAAGTTCCTA
        L  Q  Y  T  G  T  D  G  P  C  K  V  P
2001  TCTCGTCAGT GGCTTCATTG AACGACCTAA CGCCAGTGGG
      I  S  S  V  A  S  L  N  D  L  T  P  V  G
```

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```
2041  CAGATTGGTC ACTGTCAACC CTTTTGTTTC AGTGGCCACG
        R  L  V  T  V  N  P  F  V  S  V  A  T
2081  GCCAACGCTA AGGTCCTGAT TGAATTGGAA CCACCCTTTG
        A  N  A  K  V  L  I  E  L  E  P  P  F
2121  GAGACTCATA CATAGTGGTG GGCAGAGGAG AACAACAGAT
        G  D  S  Y  I  V  V  G  R  G  E  Q  Q  I
2161  CAATCACCAT TGGCACAAGT CTGGAAGCAG CATTGGCAAA
        N  H  H  W  H  K  S  G  S  S  I  G  K
2201  GCCTTTACAA CCACCCTCAA AGGAGCGCAG AGACTAGCCG
        A  F  T  T  T  L  K  G  A  Q  R  L  A
2241  CTCTAGGAGA CACAGCTTGG GACTTTGGAT CAGTTGGAGG
        A  L  G  D  T  A  W  D  F  G  S  V  G  G
2281  GGTGTTCACT AGTGTTGGGC GGGCTGTCCA TCAAGTGTTC
        V  F  T  S  V  G  R  A  V  H  Q  V  F
2321  GGAGGAGCAT TCCGCTCACT GTTCGGAGGC ATGTCCTGGA
        G  G  A  F  R  S  L  F  G  G  M  S  W
2361  TAACGCAAGG ATTGCTGGGG GCTCTCCTGT TGTGGATGGG
        I  T  Q  G  L  L  G  A  L  L  L  W  M  G
2401  CATCAATGCT CGTGATAGGT CCATAGCTCT CACGTTTCTC
        I  N  A  R  D  R  S  I  A  L  T  F  L
2441  GCAGTTGGAG GAGTTCTGCT CTTCCTCTCC GTGAACGTGG
        A  V  G  G  V  L  L  F  L  S  V  N  V
2481  GCGCCGATCA AGGATGCGCC ATCAACTTTG GCAAGAGAGA
        G  A  D  Q  G  C  A  I  N  F  G  K  R  E
2521  GCTCAAGTGC GGAGATGGTA TCTTCATATT TAGAGACTCT
        L  K  C  G  D  G  I  F  I  F  R  D  S
2561  GATGACTGGC TGAACAAGTA CTCATACTAT CCAGAAGATC
        D  D  W  L  N  K  Y  S  Y  Y  P  E  D
2601  CTGTGAAGCT TGCATCAATA GTGAAAGCCT CTTTTGAAGA
        P  V  K  L  A  S  I  V  K  A  S  F  E  E
2641  AGGGAAGTGT GGCCTAAATT CAGTTGACTC CCTTGAGCAT
        G  K  C  G  L  N  S  V  D  S  L  E  H
2681  GAGATGTGGA GAAGCAGGGC AGATGAGATC AATGCCATTT
        E  M  W  R  S  R  A  D  E  I  N  A  I
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```
2721  TTGAGGAAAA CGAGGTGGAC ATTTCTGTTG TCGTGCAGGA
        F  E  E  N   E  V  D   I  S  V  V   V  Q  D
2761  TCCAAAGAAT GTTTACCAGA GAGGAACTCA TCCATTTTCC
        P  K  N   V  Y  Q  R   G  T  H   P  F  S
2801  AGAATTCGGG ATGGTCTGCA GTATGGTTGG AAGACTTGGG
        R  I  R  D   G  L  Q   Y  G  W   K  T  W
2841  GTAAGAACCT TGTGTTCTCC CCAGGGAGGA AGAATGGAAG
        G  K  N  L   V  F  S   P  G  R  K   N  G  S
2881  CTTCATCATA GATGGAAAGT CCAGGAAAGA ATGCCCGTTT
        F  I  I   D  G  K  S   R  K  E   C  P  F
2921  TCAAACCGGG TCTGGAATTC TTTCCAGATA GAGGAGTTTG
        S  N  R  V   W  N  S   F  Q  I   E  E  F
2961  GGACGGGAGT GTTCACCACA CGCGTGTACA TGGACGCAGT
        G  T  G  V   F  T  T   R  V  Y  M   D  A  V
3001  CTTTGAATAC ACCATAGACT GCGATGGATC TATCTTGGGT
        F  E  Y   T  I  D  C   D  G  S   I  L  G
3041  GCAGCGGTGA ACGGAAAAAA GAGTGCCCAT GGCTCTCCAA
        A  A  V  N   G  K  K   S  A  H   G  S  P
3081  CATTTTGGAT GGGAAGTCAT GAAGTAAATG GGACATGGAT
        T  F  W  M   G  S  H   E  V  N  G   T  W  M
3121  GATCCACACC TTGGAGGCAT TAGATTACAA GGAGTGTGAG
        I  H  T   L  E  A  L   D  Y  K   E  C  E
3161  TGGCCACTGA CACATACGAT TGGAACATCA GTTGAAGAGA
        W  P  L  T   H  T  I   G  T  S   V  E  E
3201  GTGAAATGTT CATGCCGAGA TCAATCGGAG GCCCAGTTAG
        S  E  M  F   M  P  R   S  I  G  G   P  V  S
3241  CTCTCACAAT CATATCCCTG GATACAAGGT TCAGACGAAC
        S  H  N   H  I  P  G   Y  K  V   Q  T  N
3281  GGACCTTGGA TGCAGGTACC ACTAGAAGTG AAGAGAGAAG
        G  P  W  M   Q  V  P   L  E  V   K  R  E
3321  CTTGCCCAGG GACTAGCGTG ATCATTGATG GCAACTGTGA
        A  C  P  G   T  S  V   I  I  D  G   N  C  D
3361  TGGACGGGGA AAATCAACCA GATCCACCAC GGATAGCGGG
        G  R  G   K  S  T  R   S  T  T   D  S  G
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
3401  AAAGTTATTC CTGAATGGTG TTGCCGCTCC TGCACAATGC
        K  V  I  P  E  W  C  C  R  S  C  T  M
3441  CGCCTGTGAG CTTCCATGGT AGTGATGGGT GTTGGTATCC
      P  P  V  S  F  H  G  S  D  G  C  W  Y  P
3481  CATGGAAATT AGGCCAAGGA AAACGCATGA AGCCATCTG
        M  E  I  R  P  R  K  T  H  E  S  H  L
3521  GTGCGCTCCT GGGTTACAGC TGGAGAAATA CATGCTGTCC
        V  R  S  W  V  T  A  G  E  I  H  A  V
3561  CTTTTGGTTT GGTGAGCATG ATGATAGCAA TGGAAGTGGT
      P  F  G  L  V  S  M  M  I  A  M  E  V  V
3601  CCTAAGGAAA AGACAGGGAC CAAAGCAAAT GTTGGTTGGA
        L  R  K  R  Q  G  P  K  Q  M  L  V  G
3641  GGAGTAGTGC TCTTGGGAGC AATGCTGGTC GGGCAAGTAA
        G  V  V  L  L  G  A  M  L  V  G  Q  V
3681  CTCTCCTTGA TTTGCTGAAA CTCACAGTGG CTGTGGGATT
      T  L  L  D  L  L  K  L  T  V  A  V  G  L
3721  GCATTTCCAT GAGATGAACA ATGGAGGAGA CGCCATGTAT
        H  F  H  E  M  N  N  G  G  D  A  M  Y
3761  ATGGCGTTGA TTGCTGCCTT TTCAATCAGA CCAGGGCTGC
        M  A  L  I  A  A  F  S  I  R  P  G  L
3801  TCATCGGCTT TGGGCTCAGG ACCCTATGGA GCCCTCGGGA
      L  I  G  F  G  L  R  T  L  W  S  P  R  E
3841  ACGCCTTGTG CTGACCCTAG GAGCAGCCAT GGTGGAGATT
        R  L  V  L  T  L  G  A  A  M  V  E  I
3881  GCCTTGGGTG GCGTGATGGG CGGCCTGTGG AAGTATCTAA
        A  L  G  G  V  M  G  G  L  W  K  Y  L
3921  ATGCAGTTTC TCTCTGCATC CTGACAATAA ATGCTGTTGC
      N  A  V  S  L  C  I  L  T  I  N  A  V  A
3961  TTCTAGGAAA GCATCAAATA CCATCTTGCC CCTCATGGCT
        S  R  K  A  S  N  T  I  L  P  L  M  A
4001  CTGTTGACAC CTGTCACTAT GGCTGAGGTG AGACTTGCCG
        L  L  T  P  V  T  M  A  E  V  R  L  A
4041  CAATGTTCTT TTGTGCCATG GTTATCATAG GGGTCCTTCA
      A  M  F  F  C  A  M  V  I  I  G  V  L  H
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
4081  CCAGAATTTC AAGGACACCT CCATGCAGAA GACTATACCT
        Q  N  F   K  D  T  S   M  Q  K   T  I  P
4121  CTGGTGGCCC TCACACTCAC ATCTTACCTG GGCTTGACAC
        L  V  A   L  T  L  T   S  Y  L   G  L  T
4161  AACCTTTTTT GGGCCTGTGT GCATTTCTGG CAACCCGCAT
        Q  P  F   L  G  L  C   A  F  L   A  T  R  I
4201  ATTTGGGCGA AGGAGTATCC CAGTGAATGA GGCACTCGCA
        F  G  R   R  S  I  P   V  N  E   A  L  A
4241  GCAGCTGGTC TAGTGGGAGT GCTGGCAGGA CTGGCTTTTC
        A  A  G   L  V  G  V   L  A  G   L  A  F
4281  AGGAGATGGA GAACTTCCTT GGTCCGATTG CAGTTGGAGG
        Q  E  M   E  N  F  L   G  P  I   A  V  G  G
4321  ACTCCTGATG ATGCTGGTTA GCGTGGCTGG GAGGGTGGAT
        L  L  M   M  L  V  S   V  A  G   R  V  D
4361  GGGCTAGAGC TCAAGAAGCT TGGTGAAGTT TCATGGGAAG
        G  L  E   L  K  K  L   G  E  V   S  W  E
4401  AGGAGGCGGA GATCAGCGGG AGTTCCGCCC GCTATGATGT
        E  E  A   E  I  S  G   S  S  A   R  Y  D  V
4441  GGCACTCAGT GAACAAGGGG AGTTCAAGCT GCTTTCTGAA
        A  L  S   E  Q  G  E   F  K  L   L  S  E
4481  GAGAAAGTGC CATGGGACCA GGTTGTGATG ACCTCGCTGG
        E  K  V   P  W  D  Q   V  V  M   T  S  L
4521  CCTTGGTTGG GGCTGCCCTC CATCCATTTG CTCTTCTGCT
        A  L  V   G  A  A  L   H  P  F   A  L  L  L
4561  GGTCCTTGCT GGGTGGCTGT TTCATGTCAG GGGAGCTAGG
        V  L  A   G  W  L  F   H  V  R   G  A  R
4601  AGAAGTGGGG ATGTCTTGTG GGATATTCCC ACTCCTAAGA
        R  S  G   D  V  L  W   D  I  P   T  P  K
4641  TCATCGAGGA ATGTGAACAT CTGGAGGATG GGATTTATGG
        I  I  E   E  C  E  H   L  E  D   G  I  Y  G
4681  CATATTCCAG TCAACCTTCT TGGGGGCCTC CCAGCGAGGA
        I  F  Q   S  T  F  L   G  A  S   Q  R  G
4721  GTGGGAGTGG CACAGGGAGG GGTGTTCCAC ACAATGTGGC
        V  G  V   A  Q  G  G   V  F  H   T  M  W
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
4761  ATGTCACAAG AGGAGCTTTC CTTGTCAGGA ATGGCAAGAA
        H  V  T  R  G  A  F  L  V  R  N  G  K  K
4801  GTTGATTCCA TCTTGGGCTT CAGTAAAGGA AGACCTTGTC
        L  I  P  S  W  A  S  V  K  E  D  L  V
4841  GCCTATGGTG GCTCATGGAA GTTGGAAGGC AGATGGGATG
        A  Y  G  G  S  W  K  L  E  G  R  W  D
4881  GAGAGGAAGA GGTCCAGTTG ATCGCGGCTG TTCCAGGAAA
        G  E  E  E  V  Q  L  I  A  A  V  P  G  K
4921  GAACGTGGTC AACGTCCAGA CAAAACCGAG CTTGTTCAAA
        N  V  V  N  V  Q  T  K  P  S  L  F  K
4961  GTGAGGAATG GGGGAGAAAT CGGGGCTGTC GCTCTTGACT
        V  R  N  G  G  E  I  G  A  V  A  L  D
5001  ATCCGAGTGG CACTTCAGGA TCTCCTATTG TTAACAGGAA
        Y  P  S  G  T  S  G  S  P  I  V  N  R  N
5041  CGGAGAGGTG ATTGGGCTGT ACGGCAATGG CATCCTTGTC
        G  E  V  I  G  L  Y  G  N  G  I  L  V
5081  GGTGACAACT CCTTCGTGTC CGCCATATCC CAGACTGAGG
        G  D  N  S  F  V  S  A  I  S  Q  T  E
5121  TGAAGGAAGA AGGAAAGGAG GAGCTCCAAG AGATCCCGAC
        V  K  E  E  G  K  E  E  L  Q  E  I  P  T
5161  AATGCTAAAG AAAGGAATGA CAACTGTCCT TGATTTTCAT
        M  L  K  K  G  M  T  T  V  L  D  F  H
5201  CCTGGAGCTG GGAAGACAAG ACGTTTCCTC CCACAGATCT
        P  G  A  G  K  T  R  R  F  L  P  Q  I
5241  TGGCCGAGTG CGCACGGAGA CGCTTGCGCA CTCTTGTGTT
        L  A  E  C  A  R  R  R  L  R  T  L  V  L
5281  GGCCCCCACC AGGGTTGTTC TTTCTGAAAT GAAGGAGGCT
        A  P  T  R  V  V  L  S  E  M  K  E  A
5321  TTTCACGGCC TGGACGTGAA ATTCCACACA CAGGCTTTTT
        F  H  G  L  D  V  K  F  H  T  Q  A  F
5361  CCGCTCACGG CAGCGGGAGA GAAGTCATTG ATGCCATGTG
        S  A  H  G  S  G  R  E  V  I  D  A  M  C
5401  CCATGCCACC CTAACTTACA GGATGTTGGA ACCAACTAGG
        H  A  T  L  T  Y  R  M  L  E  P  T  R
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
5441  GTTGTTAACT GGGAAGTGAT CATTATGGAT GAAGCCCATT
        V  V  N  W   E  V  I   I  M  D   E  A  H
5481  TTTTGGATCC AGCCAGCATA GCCGCTAGAG GTTGGGCAGC
      F  L  D  P   A  S  I   A  A  R  G   W  A  A
5521  GCACAGAGCT AGGGCAAATG AAAGTGCAAC AATCTTGATG
        H  R  A   R  A  N  E   S  A  T   I  L  M
5561  ACAGCCACAC CGCCTGGGAC TAGTGATGAA TTTCCACATT
        T  A  T   P  P  G  T   S  D  E   F  P  H
5601  CAAATGGTGA AATAGAAGAT GTTCAAACGG ACATACCCAG
      S  N  G   E  I  E  D   V  Q  T   D  I  P  S
5641  TGAGCCCTGG AACACAGGGC ATGACTGGAT CCTGGCTGAC
        E  P  W   N  T  G  H   D  W  I   L  A  D
5681  AAAAGGCCCA CGGCATGGTT CCTTCCATCC ATCAGAGCTG
        K  R  P   T  A  W  F   L  P  S   I  R  A
5721  CAAATGTCAT GGCTGCCTCT TTGCGTAAGG CTGGAAAGAG
      A  N  V   M  A  A  S   L  R  K  A   G  K  S
5761  TGTGGTGGTC CTGAACAGGA AAACCTTTGA GAGAGAATAC
        V  V  V   L  N  R  K   T  F  E   R  E  Y
5801  CCCACGATAA AGCAGAAGAA ACCTGACTTT ATATTGGCCA
        P  T  I   K  Q  K  K   P  D  F   I  L  A
5841  CTGACATAGC TGAAATGGGA GCCAACCTTT GCGTGGAGCG
      T  D  I   A  E  M  G   A  N  L  C   V  E  R
5881  AGTGCTGGAT TGCAGGACGG CTTTTAAGCC TGTGCTTGTG
        V  L  D   C  R  T  A   F  K  P   V  L  V
5921  GATGAAGGGA GGAAGGTGGC AATAAAAGGG CCACTTCGTA
        D  E  G   R  K  V  A   I  K  G   P  L  R
5961  TCTCCGCATC CTCTGCTGCT CAAAGGAGGG GGCGCATTGG
      I  S  A   S  S  A  A   Q  R  R  G   R  I  G
6001  GAGAAATCCC AACAGAGATG GAGACTCATA CTACTATTCT
        R  N  P   N  R  D  G   D  S  Y   Y  Y  S
6041  GAGCCTACAA GTGAAAATAA TGCCCACCAC GTCTGCTGGT
        E  P  T   S  E  N  N   A  H  H   V  C  W
6081  TGGAGGCCTC AATGCTCTTG GACAACATGG AGGTGAGGGG
      L  E  A   S  M  L  L   D  N  M  E   V  R  G
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
6121  TGGAATGGTC GCCCCACTCT ATGGCGTTGA AGGAACTAAA
        G  M  V   A  P  L  Y   G  V  E   G  T  K
6161  ACACCAGTTT CCCCTGGTGA AATGAGACTG AGGGATGACC
        T  P  V   S  P  G  E   M  R  L   R  D  D
6201  AGAGGAAAGT CTTCAGAGAA CTAGTGAGGA ATTGTGACCT
        Q  R  K   V  F  R  E   L  V  R   N  C  D  L
6241  GCCCGTTTGG CTTTCGTGGC AAGTGGCCAA GGCTGGTTTG
        P  V  W   L  S  W  Q   V  A  K   A  G  L
6281  AAGACGAATG ATCGTAAGTG GTGTTTTGAA GGCCCTGAGG
        K  T  N   D  R  K  W   C  F  E   G  P  E
6321  AACATGAGAT CTTGAATGAC AGCGGTGAAA CAGTGAAGTG
        E  H  E   I  L  N  D   S  G  E   T  V  K  C
6361  CAGGGCTCCT GGAGGAGCAA AGAAGCCTCT GCGCCCAAGG
        R  A  P   G  G  A  K   K  P  L   R  P  R
6401  TGGTGTGATG AAAGGGTGTC ATCTGACCAG AGTGCGCTGT
        W  C  D   E  R  V  S   S  D  Q   S  A  L
6441  CTGAATTTAT TAAGTTTGCT GAAGGTAGGA GGGGAGCTGC
        S  E  F   I  K  F  A   E  G  R   R  G  A  A
6481  TGAAGTGCTA GTTGTGCTGA GTGAACTCCC TGATTTCCTG
        E  V  L   V  V  L  S   E  L  P   D  F  L
6521  GCTAAAAAAG GTGGAGAGGC AATGGATACC ATCAGTGTGT
        A  K  K   G  G  E  A   M  D  T   I  S  V
6561  TCCTCCACTC TGAGGAAGGC TCTAGGGCTT ACCGCAATGC
        F  L  H   S  E  E  G   S  R  A   Y  R  N  A
6601  ACTATCAATG ATGCCTGAGG CAATGACAAT AGTCATGCTG
        L  S  M   M  P  E  A   M  T  I   V  M  L
6641  TTTATACTGG CTGGACTACT GACATCGGGA ATGGTCATCT
        F  I  L   A  G  L  L   T  S  G   M  V  I
6681  TTTTCATGTC TCCCAAAGGC ATCAGTAGAA TGTCTATGGC
        F  F  M   S  P  K  G   I  S  R   M  S  M  A
6721  GATGGGCACA ATGGCCGGCT GTGGATATCT CATGTTCCTT
        M  G  T   M  A  G  C   G  Y  L   M  F  L
6761  GGAGGCGTCA AACCCACTCA CATCTCCTAT GTCATGCTCA
        G  G  V   K  P  T  H   I  S  Y   V  M  L
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
6801   TATTCTTTGT CCTGATGGTG GTTGTGATCC CCGAGCCAGG
         I  F  F  V   L  M  V   V  V  I  P   E  P  G
6841   GCAACAAAGG TCCATCCAAG ACAACCAAGT GGCATACCTC
         Q  Q  R   S  I  Q  D   N  Q  V   A  Y  L
6881   ATTATTGGCA TCCTGACGCT GGTTTCAGCG GTGGCAGCCA
         I  I  G   I  L  T  L   V  S  A   V  A  A
6921   ACGAGCTAGG CATGCTGGAG AAAACCAAAG AGGACCTCTT
         N  E  L   G  M  L  E   K  T  K   E  D  L  F
6961   TGGGAAGAAG AACTTAATTC CATCTAGTGC TTCACCCTGG
         G  K  K   N  L  I  P   S  S  A   S  P  W
7001   AGTTGGCCGG ATCTTGACCT GAAGCCAGGA GCTGCCTGGA
         S  W  P   D  L  D  L   K  P  G   A  A  W
7041   CAGTGTACGT TGGCATTGTT ACAATGCTCT CTCCAATGTT
         T  V  Y   V  G  I  V   T  M  L   S  P  M  L
7081   GCACCACTGG ATCAAAGTCG AATATGGCAA CCTGTCTCTG
         H  H  W   I  K  V  E   Y  G  N   L  S  L
7121   TCTGGAATAG CCCAGTCAGC CTCAGTCCTT TCTTTCATGG
         S  G  I   A  Q  S  A   S  V  L   S  F  M
7161   ACAAGGGGAT ACCATTCATG AAGATGAATA TCTCGGTCAT
         D  K  G   I  P  F  M   K  M  N   I  S  V  I
7201   AATGCTGCTG GTCAGTGGCT GGAATTCAAT AACAGTGATG
         M  L  L   V  S  G  W   N  S  I   T  V  M
7241   CCTCTGCTCT GTGGCATAGG GTGCGCCATG CTCCACTGGT
         P  L  L   C  G  I  G   C  A  M   L  H  W
7281   CTCTCATTTT ACCTGGAATC AAAGCGCAGC AGTCAAAGCT
         S  L  I   L  P  G  I   K  A  Q   Q  S  K  L
7321   TGCACAGAGA AGGGTGTTCC ATGGCGTTGC CAAGAACCCT
         A  Q  R   R  V  F  H   G  V  A   K  N  P
7361   GTGGTTGATG GGAATCCAAC AGTTGACATT GAGGAAGCTC
         V  V  D   G  N  P  T   V  D  I   E  E  A
7401   CTGAAATGCC TGCCCTTTAT GAGAAGAAAC TGGCTCTATA
         P  E  M   P  A  L  Y   E  K  K   L  A  L  Y
7441   TCTCCTTCTT GCTCTCAGCC TAGCTTCTGT TGCCATGTGC
         L  L  L   A  L  S  L   A  S  V   A  M  C
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
7481   AGAACGCCCT TTTCATTGGC TGAAGGCATT GTCCTAGCAT
         R  T  P  F  S  L  A  E  G  I  V  L  A
7521   CAGCTGCCTT AGGGCCGCTC ATAGAGGGAA ACACCAGCCT
       S  A  A  L  G  P  L  I  E  G  N  T  S  L
7561   TCTTTGGAAT GGACCCATGG CTGTCTCCAT GACAGGAGTC
         L  W  N  G  P  M  A  V  S  M  T  G  V
7601   ATGAGGGGGA ATCACTATGC TTTTGTGGGA GTCATGTACA
         M  R  G  N  H  Y  A  F  V  G  V  M  Y
7641   ATCTATGGAA GATGAAAACT GGACGCCGGG GGAGCGCGAA
       N  L  W  K  M  K  T  G  R  R  G  S  A  N
7681   TGGAAAAACT TTGGGTGAAG TCTGGAAGAG GGAACTGAAT
         G  K  T  L  G  E  V  W  K  R  E  L  N
7721   CTGTTGGACA AGCGACAGTT TGAGTTGTAT AAAAGGACCG
         L  L  D  K  R  Q  F  E  L  Y  K  R  T
7761   ACATTGTGGA GGTGGATCGT GATACGGCAC GCAGGCATTT
       D  I  V  E  V  D  R  D  T  A  R  R  H  L
7801   GGCCGAAGGG AAGGTGGACA CCGGGGTGGC GGTCTCCAGG
         A  E  G  K  V  D  T  G  V  A  V  S  R
7841   GGGACCGCAA AGTTAAGGTG GTTCCATGAG CGTGGCTATG
         G  T  A  K  L  R  W  F  H  E  R  G  Y
7881   TCAAGCTGGA AGGTAGGGTG ATTGACCTGG GGTGTGGCCG
       V  K  L  E  G  R  V  I  D  L  G  C  G  R
7921   CGGAGGCTGG TGTTACTACG CTGCTGCGCA AAAGGAAGTG
         G  G  W  C  Y  Y  A  A  A  Q  K  E  V
7961   AGTGGGGTCA AAGGATTTAC TCTTGGAAGA GACGGCCATG
         S  G  V  K  G  F  T  L  G  R  D  G  H
8001   AGAAACCCAT GAATGTGCAA AGTCTGGGAT GGAACATCAT
       E  K  P  M  N  V  Q  S  L  G  W  N  I  I
8041   CACCTTCAAG GACAAAACTG ATATCCACCG CCTAGAACCA
         T  F  K  D  K  T  D  I  H  R  L  E  P
8081   GTGAAATGTG ACACCCTTTT GTGTGACATT GGAGAGTCAT
         V  K  C  D  T  L  L  C  D  I  G  E  S
8121   CATCGTCATC GGTCACAGAG GGGGAAAGGA CCGTGAGAGT
       S  S  S  S  V  T  E  G  E  R  T  V  R  V
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
8161  TCTTGATACT GTAGAAAAAT GGCTGGCTTG TGGGGTTGAC
        L  D  T  V  E  K  W  L  A  C  G  V  D
8201  AACTTCTGTG TGAAGGTGTT AGCTCCATAC ATGCCAGATG
        N  F  C  V  K  V  L  A  P  Y  M  P  D
8241  TTCTTGAGAA ACTGGAATTG CTCCAAAGGA GGTTTGGCGG
        V  L  E  K  L  E  L  L  Q  R  R  F  G  G
8281  AACAGTGATC AGGAACCCTC TCTCCAGGAA TTCCACTCAT
        T  V  I  R  N  P  L  S  R  N  S  T  H
8321  GAAATGTACT ACGTGTCTGG AGCCCGCAGC AATGTCACAT
        E  M  Y  Y  V  S  G  A  R  S  N  V  T
8361  TTACTGTGAA CCAAACATCC CGCCTCCTGA TGAGGAGAAT
        F  T  V  N  Q  T  S  R  L  L  M  R  R  M
8401  GAGGCGTCCA ACTGGAAAAG TGACCCTGGA GGCTGACGTC
        R  R  P  T  G  K  V  T  L  E  A  D  V
8441  ATCCTCCCAA TTGGGACACG CAGTGTTGAG ACAGACAAGG
        I  L  P  I  G  T  R  S  V  E  T  D  K
8481  GACCCCTGGA CAAAGAGGCC ATAGAAGAAA GGGTTGAGAG
        G  P  L  D  K  E  A  I  E  E  R  V  E  R
8521  GATAAAATCT GAGTACATGA CCTCTTGGTT TTATGACAAT
        I  K  S  E  Y  M  T  S  W  F  Y  D  N
8561  GACAACCCCT ACAGGACCTG GCACTACTGT GGCTCCTATG
        D  N  P  Y  R  T  W  H  Y  C  G  S  Y
8601  TCACAAAAAC CTCCGGAAGT GCGGCGAGCA TGGTAAATGG
        V  T  K  T  S  G  S  A  A  S  M  V  N  G
8641  TGTTATTAAA ATTCTGACAT ATCCATGGGA CAGGATAGAG
        V  I  K  I  L  T  Y  P  W  D  R  I  E
8681  GAGGTCACAA GAATGGCAAT GACTGACACA ACCCCTTTTG
        E  V  T  R  M  A  M  T  D  T  T  P  F
8721  GACAGCAAAG AGTGTTTAAA GAAAAAGTTG ACACCAGAGC
        G  Q  Q  R  V  F  K  E  K  V  D  T  R  A
8761  AAAGGATCCA CCAGCGGGAA CTAGGAAGAT CATGAAAGTT
        K  D  P  P  A  G  T  R  K  I  M  K  V
8801  GTCAACAGGT GGCTGTTCCG CCACCTGGCC AGAGAAAAGA
        V  N  R  W  L  F  R  H  L  A  R  E  K
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
8841  ACCCCAGACT GTGCACAAAG GAAGAATTTA TTGCAAAAGT
        N  P  R  L  C  T  K  E  E  F  I  A  K  V
8881  CCGAAGTCAT GCAGCCATTG GAGCTTACCT GGAAGAACAA
        R  S  H  A  A  I  G  A  Y  L  E  E  Q
8921  GAACAGTGGA AGACTGCCAA TGAGGCTGTC CAAGACCCAA
        E  Q  W  K  T  A  N  E  A  V  Q  D  P
8961  AGTTCTGGGA ACTGGTGGAT GAAGAAAGGA AGCTGCACCA
        K  F  W  E  L  V  D  E  E  R  K  L  H  Q
9001  ACAAGGCAGG TGTCGGACTT GTGTGTACAA CATGATGGGG
        Q  G  R  C  R  T  C  V  Y  N  M  M  G
9041  AAAAGAGAGA AGAAGCTGTC AGAGTTTGGG AAAGCAAAGG
        K  R  E  K  K  L  S  E  F  G  K  A  K
9081  GAAGCCGTGC CATATGGTAT ATGTGGCTGG GAGCGCGGTA
        G  S  R  A  I  W  Y  M  W  L  G  A  R  Y
9121  TCTTGAGTTT GAGGCCCTGG GATTCCTGAA TGAGGACCAT
        L  E  F  E  A  L  G  F  L  N  E  D  H
9161  TGGGCTTCCA GGGAAAACTC AGGAGGAGGA GTGGAAGGCA
        W  A  S  R  E  N  S  G  G  G  V  E  G
9201  TTGGCTTACA ATACCTAGGA TATGTGATCA GAGACCTGGC
        I  G  L  Q  Y  L  G  Y  V  I  R  D  L  A
9241  TGCAATGGAT GGTGGTGGAT TCTACGCGGA TGACACCGCT
        A  M  D  G  G  G  F  Y  A  D  D  T  A
9281  GGATGGGACA CGCGCATCAC AGAGGCAGAC CTTGATGATG
        G  W  D  T  R  I  T  E  A  D  L  D  D
9321  AACAGGAGAT CTTGAACTAC ATGAGCCCAC ATCACAAAAA
        E  Q  E  I  L  N  Y  M  S  P  H  H  K  K
9361  ACTGGCACAA GCAGTGATGG AAATGACATA CAAGAACAAA
        L  A  Q  A  V  M  E  M  T  Y  K  N  K
9401  GTGGTGAAAG TGTTGAGACC AGCCCCAGGA GGGAAAGCCT
        V  V  K  V  L  R  P  A  P  G  G  K  A
9441  ACATGGATGT CATAAGTCGA CGAGACCAGA GAGGATCCGG
        Y  M  D  V  I  S  R  R  D  Q  R  G  S  G
9481  GCAGGTAGTG ACTTATGCTC TGAACACCAT CACCAACTTG
        Q  V  V  T  Y  A  L  N  T  I  T  N  L
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
9521   AAAGTCCAAT TGATCAGAAT GGCAGAAGCA GAGATGGTGA
         K  V  Q  L  I  R  M  A  E  A  E  M  V
9561   TACATCACCA ACATGTTCAA GATTGTGATG AATCAGTTCT
       I  H  H  Q  H  V  Q  D  C  D  E  S  V  L
9601   GACCAGGCTG GAGGCATGGC TCACTGAGCA CGGATGTGAC
         T  R  L  E  A  W  L  T  E  H  G  C  D
9641   AGACTGAAGA GGATGGCGGT GAGTGGAGAC GACTGTGTGG
         R  L  K  R  M  A  V  S  G  D  D  C  V
9681   TCCGGCCCAT CGATGACAGG TTCGGCCTGG CCCTGTCCCA
       V  R  P  I  D  D  R  F  G  L  A  L  S  H
9721   TCTCAACGCC ATGTCCAAGG TTAGAAAGGA CATATCTGAA
         L  N  A  M  S  K  V  R  K  D  I  S  E
9761   TGGCAGCCAT CAAAAGGGTG GAATGATTGG GAGAATGTGC
         W  Q  P  S  K  G  W  N  D  W  E  N  V
9801   CCTTCTGTTC CCACCACTTC CATGAACTAC AGCTGAAGGA
       P  F  C  S  H  H  F  H  E  L  Q  L  K  D
9841   TGGCAGGAGG ATTGTGGTGC CTTGCCGAGA ACAGGACGAG
         G  R  R  I  V  V  P  C  R  E  Q  D  E
9881   CTCATTGGGA GAGGAAGGGT GTCTCCAGGA AACGGCTGGA
         L  I  G  R  G  R  V  S  P  G  N  G  W
9921   TGATCAAGGA AACAGCTTGC CTCAGCAAAG CCTATGCCAA
       M  I  K  E  T  A  C  L  S  K  A  Y  A  N
9961   CATGTGGTCA CTGATGTATT TTCACAAAAG GGACATGAGG
         M  W  S  L  M  Y  F  H  K  R  D  M  R
10001  CTACTGTCAT TGGCTGTTTC CTCAGCTGTT CCCACCTCAT
         L  L  S  L  A  V  S  S  A  V  P  T  S
10041  GGGTTCCACA AGGACGCACA ACATGGTCGA TTCATGGGAA
       W  V  P  Q  G  R  T  T  W  S  I  H  G  K
10081  AGGGGAGTGG ATGACCACGG AAGACATGCT TGAGGTGTGG
         G  E  W  M  T  T  E  D  M  L  E  V  W
10121  AACAGAGTAT GGATAACCAA CAACCCACAC ATGCAGGACA
         N  R  V  W  I  T  N  N  P  H  M  Q  D
10161  AGACAATGGT GAAAAAATGG AGAGATGTCC CTTATCTAAC
       K  T  M  V  K  K  W  R  D  V  P  Y  L  T
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

```
10201  CAAGAGACAA GACAAGCTGT GCGGATCACT GATTGGAATG
         K  R  Q   D  K  L  C   G  S  L   I  G  M
10241  ACCAATAGGG CCACCTGGGC CTCCCACATC CATTTAGTCA
         T  N  R  A   T  W  A   S  H  I   H  L  V
10281  TCCATCGTAT CCGAACGCTG ATTGGACAGG AGAAATACAC
         I  H  R  I   R  T  L   I  G  Q  E   K  Y  T
10321  TGACTACCTA ACAGTCATGG ACAGGTATTC TGTGGATGCT
         D  Y  L   T  V  M  D   R  Y  S   V  D  A
10361  GACCTGCAAC TGGGTGAGCT TATCTGAAAC ACCATCTAAC
         D  L  Q  L   G  E  L   I
10401  AGGAATAACC GGGATACAAA CCACGGGTGG AGAACCGGAC

10441  TCCCCACAAC CTGAAACCGG GATATAAACC ACGGCTGGAG

10481  AACCGGACTC CGCACTTAAA ATGAAACAGA AACCGGGATA

10521  AAAACTACGG ATGGAGAACC GGACTCCACA CATTGAGACA

10561  GAAGAAGTTG TCAGCCCAGA ACCCCACACG AGTTTTGCCA

10601  CTGCTAAGCT GTGAGGCAGT GCAGGCTGGG ACAGCCGACC

10641  TCCAGGTTGC GAAAAACCTG GTTTCTGGGA CCTCCCACCC

10681  CAGAGTAAAA AGAACGGAGC CTCCGCTACC ACCCTCCCAC

10721  GTGGTGGTAG AAAGACGGGG TCTAGAGGTT AGAGGAGACC

10761  CTCCAGGGAA CAAATAGTGG GACCATATTG ACGCCAGGGA

10801  AAGACCGGAG TGGTTCTCTG CTTTTCCTCC AGAGGTCTGT

10841  GAGCACAGTT TGCTCAAGAA TAAGCAGACC TTTGGATGAC
```

ChimerivaxWN02 Final Product Bottled (Run 1) L/N# 02H01; P/N# FP-0008
[Strand]

10881 AAACACAAAA CCACAA

Chimerivax WN02 M66 variant
[Strand]

```
1      NGTAAATCCT GTGTGCTAAT TGAGGTGCAT TGGTCTGCAA

41     ATCGAGTTGC TAGGCAATAA ACACATTTGG ATTAATTTTA

81     ATCGTTCGTT GAGCGATTAG CAGAGAACTG ACCAGAACAT
                                                 M
121    GTCTGGTCGT AAAGCTCAGG GAAAAACCCT GGGCGTCAAT
         S  G  R  K  A  Q  G  K  T  L  G  V  N
161    ATGGTACGAC GAGGAGTTCG CTCCTTGTCA AACAAAATAA
        M  V  R  R  G  V  R  S  L  S  N  K  I
201    AACAAAAAAC AAAACAAATT GGAAACAGAC CTGGACCTTC
       K  Q  K  T  K  Q  I  G  N  R  P  G  P  S
241    AAGAGGTGTT CAAGGATTTA TCTTTTTCTT TTTGTTCAAC
         R  G  V  Q  G  F  I  F  F  F  L  F  N
281    ATTTTGACTG GAAAAAAGAT CACAGCCCAC CTAAAGAGGT
        I  L  T  G  K  K  I  T  A  H  L  K  R
321    TGTGGAAAAT GCTGGACCCA AGACAAGGCT TGGCTGTTCT
       L  W  K  M  L  D  P  R  Q  G  L  A  V  L
361    AAGGAAAGTC AAGAGAGTGG TGGCCAGTTT GATGAGAGGA
         R  K  V  K  R  V  V  A  S  L  M  R  G
401    TTGTCCTCAA GGAAACGCCG TTCCCATGAT GTTCTGACTG
        L  S  S  R  K  R  R  S  H  D  V  L  T
441    TGCAATTCCT AATTTTGGGA ATGCTGTTGA TGACGGGTGG
       V  Q  F  L  I  L  G  M  L  L  M  T  G  G
481    AGTTACCCTC TCTAACTTCC AAGGGAAGGT GATGATGACG
         V  T  L  S  N  F  Q  G  K  V  M  M  T
521    GTAAATGCTA CTGACGTCAC AGATGTCATC ACGATTCCAA
        V  N  A  T  D  V  T  D  V  I  T  I  P
561    CAGCTGCTGG AAAGAACCTA TGCATTGTCA GAGCAATGGA
       T  A  A  G  K  N  L  C  I  V  R  A  M  D
601    TGTGGGATAC ATGTGCGATG ATACTATCAC TTATGAATGC
         V  G  Y  M  C  D  D  T  I  T  Y  E  C
641    CCAGTGCTGT CGGCTGGTAA TGATCCAGAA GACATCGACT
        P  V  L  S  A  G  N  D  P  E  D  I  D
```

Chimerivax WN02 M66 variant
[Strand]

```
681   GTTGGTGCAC AAAGTCAGCA GTCTACGTCA GGTATGGAAG
        C  W  C  T  K  S  A  V  Y  V  R  Y  G  R
721   ATGCACCAAG ACACGCCACT CAAGACGCAG TCGGAGGTCA
        C  T  K  T  R  H  S  R  R  S  R  R  S
761   CTGACAGTGC AGACACACGG AGAAAGCACT CTAGCGAACA
        L  T  V  Q  T  H  G  E  S  T  L  A  N
801   AGAAGGGGGC TTGGATGGAC AGCACCAAGG CCACAAGGTA
        K  K  G  A  W  M  D  S  T  K  A  T  R  Y
841   TTTGGTAAAA ACAGAATCAT GGATCTTGAG GAACCCTGGA
        L  V  K  T  E  S  W  I  L  R  N  P  G
881   TATGCCCTGG TGGCAGCCGT CATTGGTTGG ATGCTTGGGA
        Y  A  L  V  A  A  V  I  G  W  M  L  G
921   GCAACACCAT GCAGAGAGTT GTGTTTGTCG TGCCATTGCT
        S  N  T  M  Q  R  V  V  F  V  V  P  L  L
961   TTTGGTGGCC CCAGCTTACA GCTTCAACTG CCTTGGAATG
        L  V  A  P  A  Y  S  F  N  C  L  G  M
1001  AGCAACAGAG ACTTCTTGGA AGGAGTGTCT GGAGCAACAT
        S  N  R  D  F  L  E  G  V  S  G  A  T
1041  GGGTGGATTT GGTTCTCGAA GGCGACAGCT GCGTGACTAT
        W  V  D  L  V  L  E  G  D  S  C  V  T  I
1081  CATGTCTAAG GACAAGCCTA CCATCGACGT CAAGATGATG
        M  S  K  D  K  P  T  I  D  V  K  M  M
1121  AATATGGAGG CGGCCAACCT GGCAGAGGTC CGCAGTTATT
        N  M  E  A  A  N  L  A  E  V  R  S  Y
1161  GCTATTTGGC TACCGTCAGC GATCTCTCCA CCAAAGCTGC
        C  Y  L  A  T  V  S  D  L  S  T  K  A  A
1201  ATGCCCGACC ATGGGAGAAG CTCACAATGA CAAACGTGCT
        C  P  T  M  G  E  A  H  N  D  K  R  A
1241  GACCCAGCTT TTGTGTGCAG ACAAGGAGTG GTGGACAGGG
        D  P  A  F  V  C  R  Q  G  V  V  D  R
1281  GCTGGGGCAA CGGCTGCGGA TTTTTTGGCA AAGGATCCAT
        G  W  G  N  G  C  G  F  F  G  K  G  S  I
1321  TGACACATGC GCCAAATTTG CCTGCTCTAC CAAGGCAATA
        D  T  C  A  K  F  A  C  S  T  K  A  I
```

Chimerivax WN02 M66 variant
[Strand]

```
1361  GGAAGAACCA TCTTGAAAGA GAATATCAAG TACGAAGTGG
        G  R  T  I  L  K  E  N  I  K  Y  E  V
1401  CCATTTTTGT CCATGGACCA ACTACTGTGG AGTCGCACGG
        A  I  F  V  H  G  P  T  T  V  E  S  H  G
1441  AAATTACTCC ACACAGGTTG GAGCCACTCA GGCCGGCCGA
        N  Y  S  T  Q  V  G  A  T  Q  A  G  R
1481  TTCAGCATCA CTCCTGCTGC GCCTTCATAC ACACTAAAGC
        F  S  I  T  P  A  A  P  S  Y  T  L  K
1521  TTGGAGAATA TGGAGAGGTG ACAGTGGACT GTGAACCACG
        L  G  E  Y  G  E  V  T  V  D  C  E  P  R
1561  GTCAGGGATT GACACCAATG CATACTACGT GATGACTGTT
        S  G  I  D  T  N  A  Y  Y  V  M  T  V
1601  GGAACAAAGA CGTTCTTGGT CCATCGTGAG TGGTTCATGG
        G  T  K  T  F  L  V  H  R  E  W  F  M
1641  ACCTCAACCT CCCTTGGAGC AGTGCTGGAA GTACTGTGTG
        D  L  N  L  P  W  S  S  A  G  S  T  V  W
1681  GAGGAACAGA GAGACGTTAA TGGAGTTTGA GGAACCACAC
        R  N  R  E  T  L  M  E  F  E  E  P  H
1721  GCCACGAAGC AGTCTGTGAT AGCATTGGGC TCACAAGAGG
        A  T  K  Q  S  V  I  A  L  G  S  Q  E
1761  GAGCTCTGCA TCAAGCTTTG GCTGGAGCCA TTCCTGTGGA
        G  A  L  H  Q  A  L  A  G  A  I  P  V  E
1801  ATTTTCAAGC AACACTGTCA AGTTGACGTC GGGTCATTTG
        F  S  S  N  T  V  K  L  T  S  G  H  L
1841  AAGTGTAGAG TGAAGATGGA AAAATTGCAG TTGAAGGGAA
        K  C  R  V  K  M  E  K  L  Q  L  K  G
1881  CAACCTATGG CGTCTGTTCA AAGGCTTTCA AGTTTCTTAG
        T  T  Y  G  V  C  S  K  A  F  K  F  L  R
1921  GACTCCCGTG GACACCGGTC ACGGCACTGT GGTGTTGGAA
        T  P  V  D  T  G  H  G  T  V  V  L  E
1961  TTGCAGTACA CTGGCACGGA TGGACCTTGC AAAGTTCCTA
        L  Q  Y  T  G  T  D  G  P  C  K  V  P
2001  TCTCGTCAGT GGCTTCATTG AACGACCTAA CGCCAGTGGG
        I  S  S  V  A  S  L  N  D  L  T  P  V  G
```

Chimerivax WN02 M66 variant
[Strand]

```
2041  CAGATTGGTC ACTGTCAACC CTTTTGTTTC AGTGGCCACG
        R  L  V   T  V  N  P   F  V  S   V  A  T
2081  GCCAACGCTA AGGTCCTGAT TGAATTGGAA CCACCCTTTG
        A  N  A   K  V  L  I   E  L  E   P  P  F
2121  GAGACTCATA CATAGTGGTG GGCAGAGGAG AACAACAGAT
        G  D  S   Y  I  V  V   G  R  G   E  Q  Q  I
2161  CAATCACCAT TGGCACAAGT CTGGAAGCAG CATTGGCAAA
        N  H  H   W  H  K  S   G  S  S   I  G  K
2201  GCCTTTACAA CCACCCTCAA AGGAGCGCAG AGACTAGCCG
        A  F  T   T  T  L  K   G  A  Q   R  L  A
2241  CTCTAGGAGA CACAGCTTGG GACTTTGGAT CAGTTGGAGG
        A  L  G   D  T  A  W   D  F  G   S  V  G  G
2281  GGTGTTCACT AGTGTTGGGC GGGCTGTCCA TCAAGTGTTC
        V  F  T   S  V  G  R   A  V  H   Q  V  F
2321  GGAGGAGCAT TCCGCTCACT GTTCGGAGGC ATGTCCTGGA
        G  G  A   F  R  S  L   F  G  G   M  S  W
2361  TAACGCAAGG ATTGCTGGGG GCTCTCCTGT TGTGGATGGG
        I  T  Q   G  L  L  G   A  L  L   L  W  M  G
2401  CATCAATGCT CGTGATAGGT CCATAGCTCT CACGTTTCTC
        I  N  A   R  D  R  S   I  A  L   T  F  L
2441  GCAGTTGGAG GAGTTCTGCT CTTCCTCTCC GTGAACGTGG
        A  V  G   G  V  L  L   F  L  S   V  N  V
2481  GCGCCGATCA AGGATGCGCC ATCAACTTTG GCAAGAGAGA
        G  A  D   Q  G  C  A   I  N  F   G  K  R  E
2521  GCTCAAGTGC GGAGATGGTA TCTTCATATT TAGAGACTCT
        L  K  C   G  D  G  I   F  I  F   R  D  S
2561  GATGACTGGC TGAACAAGTA CTCATACTAT CCAGAAGATC
        D  D  W   L  N  K  Y   S  Y  Y   P  E  D
2601  CTGTGAAGCT TGCATCAATA GTGAAAGCCT CTTTTGAAGA
        P  V  K   L  A  S  I   V  K  A   S  F  E  E
2641  AGGGAAGTGT GGCCTAAATT CAGTTGACTC CCTTGAGCAT
        G  K  C   G  L  N  S   V  D  S   L  E  H
2681  GAGATGTGGA GAAGCAGGGC AGATGAGATC AATGCCATTT
        E  M  W   R  S  R  A   D  E  I   N  A  I
```

Chimerivax WN02 M66 variant
[Strand]

```
2721  TTGAGGAAAA CGAGGTGGAC ATTTCTGTTG TCGTGCAGGA
        F  E  E  N  E  V  D  I  S  V  V  V  Q  D
2761  TCCAAAGAAT GTTTACCAGA GAGGAACTCA TCCATTTTCC
        P  K  N  V  Y  Q  R  G  T  H  P  F  S
2801  AGAATTCGGG ATGGTCTGCA GTATGGTTGG AAGACTTGGG
        R  I  R  D  G  L  Q  Y  G  W  K  T  W
2841  GTAAGAACCT TGTGTTCTCC CCAGGGAGGA AGAATGGAAG
        G  K  N  L  V  F  S  P  G  R  K  N  G  S
2881  CTTCATCATA GATGGAAAGT CCAGGAAAGA ATGCCCGTTT
        F  I  I  D  G  K  S  R  K  E  C  P  F
2921  TCAAACCGGG TCTGGAATTC TTTCCAGATA GAGGAGTTTG
        S  N  R  V  W  N  S  F  Q  I  E  E  F
2961  GGACGGGAGT GTTCACCACA CGCGTGTACA TGGACGCAGT
        G  T  G  V  F  T  T  R  V  Y  M  D  A  V
3001  CTTTGAATAC ACCATAGACT GCGATGGATC TATCTTGGGT
        F  E  Y  T  I  D  C  D  G  S  I  L  G
3041  GCAGCGGTGA ACGGAAAAAA GAGTGCCCAT GGCTCTCCAA
        A  A  V  N  G  K  K  S  A  H  G  S  P
3081  CATTTTGGAT GGGAAGTCAT GAAGTAAATG GGACATGGAT
        T  F  W  M  G  S  H  E  V  N  G  T  W  M
3121  GATCCACACC TTGGAGGCAT TAGATTACAA GGAGTGTGAG
        I  H  T  L  E  A  L  D  Y  K  E  C  E
3161  TGGCCACTGA CACATACGAT TGGAACATCA GTTGAAGAGA
        W  P  L  T  H  T  I  G  T  S  V  E  E
3201  GTGAAATGTT CATGCCGAGA TCAATCGGAG GCCCAGTTAG
        S  E  M  F  M  P  R  S  I  G  G  P  V  S
3241  CTCTCACAAT CATATCCCTG GATACAAGGT TCAGACGAAC
        S  H  N  H  I  P  G  Y  K  V  Q  T  N
3281  GGACCTTGGA TGCAGGTACC ACTAGAAGTG AAGAGAGAAG
        G  P  W  M  Q  V  P  L  E  V  K  R  E
3321  CTTGCCCAGG GACTAGCGTG ATCATTGATG GCAACTGTGA
        A  C  P  G  T  S  V  I  I  D  G  N  C  D
3361  TGGACGGGGA AAATCAACCA GATCCACCAC GGATAGCGGG
        G  R  G  K  S  T  R  S  T  T  D  S  G
```

Chimerivax WN02 M66 variant
[Strand]

```
3401  AAAGTTATTC CTGAATGGTG TTGCCGCTCC TGCACAATGC
        K  V  I  P  E  W  C  C  R  S  C  T  M
3441  CGCCTGTGAG CTTCCATGGT AGTGATGGGT GTTGGTATCC
      P  P  V  S  F  H  G  S  D  G  C  W  Y  P
3481  CATGGAAATT AGGCCAAGGA AAACGCATGA AAGCCATCTG
        M  E  I  R  P  R  K  T  H  E  S  H  L
3521  GTGCGCTCCT GGGTTACAGC TGGAGAAATA CATGCTGTCC
        V  R  S  W  V  T  A  G  E  I  H  A  V
3561  CTTTTGGTTT GGTGAGCATG ATGATAGCAA TGGAAGTGGT
      P  F  G  L  V  S  M  M  I  A  M  E  V  V
3601  CCTAAGGAAA AGACAGGGAC CAAAGCAAAT GTTGGTTGGA
        L  R  K  R  Q  G  P  K  Q  M  L  V  G
3641  GGAGTAGTGC TCTTGGGAGC AATGCTGGTC GGGCAAGTAA
        G  V  V  L  L  G  A  M  L  V  G  Q  V
3681  CTCTCCTTGA TTTGCTGAAA CTCACAGTGG CTGTGGGATT
      T  L  L  D  L  L  K  L  T  V  A  V  G  L
3721  GCATTTCCAT GAGATGAACA ATGGAGGAGA CGCCATGTAT
        H  F  H  E  M  N  N  G  G  D  A  M  Y
3761  ATGGCGTTGA TTGCTGCCTT TTCAATCAGA CCAGGGCTGC
        M  A  L  I  A  A  F  S  I  R  P  G  L
3801  TCATCGGCTT TGGGCTCAGG ACCCTATGGA GCCCTCGGGA
      L  I  G  F  G  L  R  T  L  W  S  P  R  E
3841  ACGCCTTGTG CTGACCCTAG GAGCAGCCAT GGTGGAGATT
        R  L  V  L  T  L  G  A  A  M  V  E  I
3881  GCCTTGGGTG GCGTGATGGG CGGCCTGTGG AAGTATCTAA
        A  L  G  G  V  M  G  G  L  W  K  Y  L
3921  ATGCAGTTTC TCTCTGCATC CTGACAATAA ATGCTGTTGC
      N  A  V  S  L  C  I  L  T  I  N  A  V  A
3961  TTCTAGGAAA GCATCAAATA CCATCTTGCC CCTCATGGCT
        S  R  K  A  S  N  T  I  L  P  L  M  A
4001  CTGTTGACAC CTGTCACTAT GGCTGAGGTG AGACTTGCCG
        L  L  T  P  V  T  M  A  E  V  R  L  A
4041  CAATGTTCTT TTGTGCCATG GTTATCATAG GGGTCCTTCA
      A  M  F  F  C  A  M  V  I  I  G  V  L  H
```

Chimerivax WN02 M66 variant
[Strand]

```
4081  CCAGAATTTC AAGGACACCT CCATGCAGAA GACTATACCT
        Q  N  F  K  D  T  S  M  Q  K  T  I  P
4121  CTGGTGGCCC TCACACTCAC ATCTTACCTG GGCTTGACAC
        L  V  A  L  T  L  T  S  Y  L  G  L  T
4161  AACCTTTTTT GGGCCTGTGT GCATTTCTGG CAACCCGCAT
        Q  P  F  L  G  L  C  A  F  L  A  T  R  I
4201  ATTTGGGCGA AGGAGTATCC CAGTGAATGA GGCACTCGCA
        F  G  R  R  S  I  P  V  N  E  A  L  A
4241  GCAGCTGGTC TAGTGGGAGT GCTGGCAGGA CTGGCTTTTC
        A  A  G  L  V  G  V  L  A  G  L  A  F
4281  AGGAGATGGA GAACTTCCTT GGTCCGATTG CAGTTGGAGG
        Q  E  M  E  N  F  L  G  P  I  A  V  G  G
4321  ACTCCTGATG ATGCTGGTTA GCGTGGCTGG GAGGGTGGAT
        L  L  M  M  L  V  S  V  A  G  R  V  D
4361  GGGCTAGAGC TCAAGAAGCT TGGTGAAGTT TCATGGGAAG
        G  L  E  L  K  K  L  G  E  V  S  W  E
4401  AGGAGGCGGA GATCAGCGGG AGTTCCGCCC GCTATGATGT
        E  E  A  E  I  S  G  S  S  A  R  Y  D  V
4441  GGCACTCAGT GAACAAGGGG AGTTCAAGCT GCTTTCTGAA
        A  L  S  E  Q  G  E  F  K  L  L  S  E
4481  GAGAAAGTGC CATGGGACCA GGTTGTGATG ACCTCGCTGG
        E  K  V  P  W  D  Q  V  V  M  T  S  L
4521  CCTTGGTTGG GGCTGCCCTC CATCCATTTG CTCTTCTGCT
        A  L  V  G  A  A  L  H  P  F  A  L  L  L
4561  GGTCCTTGCT GGGTGGCTGT TTCATGTCAG GGGAGCTAGG
        V  L  A  G  W  L  F  H  V  R  G  A  R
4601  AGAAGTGGGG ATGTCTTGTG GGATATTCCC ACTCCTAAGA
        R  S  G  D  V  L  W  D  I  P  T  P  K
4641  TCATCGAGGA ATGTGAACAT CTGGAGGATG GGATTTATGG
        I  I  E  E  C  E  H  L  E  D  G  I  Y  G
4681  CATATTCCAG TCAACCTTCT TGGGGGCCTC CCAGCGAGGA
        I  P  Q  S  T  F  L  G  A  S  Q  R  G
4721  GTGGGAGTGG CACAGGGAGG GGTGTTCCAC ACAATGTGGC
        V  G  V  A  Q  G  G  V  F  H  T  M  W
```

Chimerivax WN02 M66 variant
[Strand]

```
4761  ATGTCACAAG AGGAGCTTTC CTTGTCAGGA ATGGCAAGAA
        H  V  T  R  G  A  F  L  V  R  N  G  K  K
4801  GTTGATTCCA TCTTGGGCTT CAGTAAAGGA AGACCTTGTC
        L  I  P  S  W  A  S  V  K  E  D  L  V
4841  GCCTATGGTG GCTCATGGAA GTTGGAAGGC AGATGGGATG
        A  Y  G  G  S  W  K  L  E  G  R  W  D
4881  GAGAGGAAGA GGTCCAGTTG ATCGCGGCTG TTCCAGGAAA
        G  E  E  E  V  Q  L  I  A  A  V  P  G  K
4921  GAACGTGGTC AACGTCCAGA CAAAACCGAG CTTGTTCAAA
        N  V  V  N  V  Q  T  K  P  S  L  F  K
4961  GTGAGGAATG GGGGAGAAAT CGGGGCTGTC GCTCTTGACT
        V  R  N  G  G  E  I  G  A  V  A  L  D
5001  ATCCGAGTGG CACTTCAGGA TCTCCTATTG TTAACAGGAA
        Y  P  S  G  T  S  G  S  P  I  V  N  R  N
5041  CGGAGAGGTG ATTGGGCTGT ACGGCAATGG CATCCTTGTC
        G  E  V  I  G  L  Y  G  N  G  I  L  V
5081  GGTGACAACT CCTTCGTGTC CGCCATATCC CAGACTGAGG
        G  D  N  S  F  V  S  A  I  S  Q  T  E
5121  TGAAGGAAGA AGGAAAGGAG GAGCTCCAAG AGATCCCGAC
        V  K  E  E  G  K  E  E  L  Q  E  I  P  T
5161  AATGCTAAAG AAAGGAATGA CAACTGTCCT TGATTTTCAT
        M  L  K  K  G  M  T  T  V  L  D  F  H
5201  CCTGGAGCTG GGAAGACAAG ACGTTTCCTC CCACAGATCT
        P  G  A  G  K  T  R  R  F  L  P  Q  I
5241  TGGCCGAGTG CGCACGGAGA CGCTTGCGCA CTCTTGTGTT
        L  A  E  C  A  R  R  R  L  R  T  L  V  L
5281  GGCCCCCACC AGGGTTGTTC TTTCTGAAAT GAAGGAGGCT
        A  P  T  R  V  V  L  S  E  M  K  E  A
5321  TTTCACGGCC TGGACGTGAA ATTCCACACA CAGGCTTTTT
        F  H  G  L  D  V  K  F  H  T  Q  A  F
5361  CCGCTCACGG CAGCGGGAGA GAAGTCATTG ATGCCATGTG
        S  A  H  G  S  G  R  E  V  I  D  A  M  C
5401  CCATGCCACC CTAACTTACA GGATGTTGGA ACCAACTAGG
        H  A  T  L  T  Y  R  M  L  E  P  T  R
```

Chimerivax WN02 M66 variant
[Strand]

```
5441  GTTGTTAACT GGGAAGTGAT CATTATGGAT GAAGCCCATT
        V  V  N  W  E  V  I  I  M  D  E  A  H
5481  TTTTGGATCC AGCCAGCATA GCCGCTAGAG GTTGGGCAGC
        F  L  D  P  A  S  I  A  A  R  G  W  A  A
5521  GCACAGAGCT AGGGCAAATG AAAGTGCAAC AATCTTGATG
        H  R  A  R  A  N  E  S  A  T  I  L  M
5561  ACAGCCACAC CGCCTGGGAC TAGTGATGAA TTTCCACATT
        T  A  T  P  P  G  T  S  D  E  F  P  H
5601  CAAATGGTGA AATAGAAGAT GTTCAAACGG ACATACCCAG
        S  N  G  E  I  E  D  V  Q  T  D  I  P  S
5641  TGAGCCCTGG AACACAGGGC ATGACTGGAT CCTGGCTGAC
        E  P  W  N  T  G  H  D  W  I  L  A  D
5681  AAAAGGCCCA CGGCATGGTT CCTTCCATCC ATCAGAGCTG
        K  R  P  T  A  W  F  L  P  S  I  R  A
5721  CAAATGTCAT GGCTGCCTCT TTGCGTAAGG CTGGAAAGAG
        A  N  V  M  A  A  S  L  R  K  A  G  K  S
5761  TGTGGTGGTC CTGAACAGGA AAACCTTTGA GAGAGAATAC
        V  V  V  L  N  R  K  T  F  E  R  E  Y
5801  CCCACGATAA AGCAGAAGAA ACCTGACTTT ATATTGGCCA
        P  T  I  K  Q  K  K  P  D  F  I  L  A
5841  CTGACATAGC TGAAATGGGA GCCAACCTTT GCGTGGAGCG
        T  D  I  A  E  M  G  A  N  L  C  V  E  R
5881  AGTGCTGGAT TGCAGGACGG CTTTTAAGCC TGTGCTTGTG
        V  L  D  C  R  T  A  F  K  P  V  L  V
5921  GATGAAGGGA GGAAGGTGGC AATAAAAGGG CCACTTCGTA
        D  E  G  R  K  V  A  I  K  G  P  L  R
5961  TCTCCGCATC CTCTGCTGCT CAAAGGAGGG GGCGCATTGG
        I  S  A  S  S  A  A  Q  R  R  G  R  I  G
6001  GAGAAATCCC AACAGAGATG GAGACTCATA CTACTATTCT
        R  N  P  N  R  D  G  D  S  Y  Y  Y  S
6041  GAGCCTACAA GTGAAAATAA TGCCCACCAC GTCTGCTGGT
        E  P  T  S  E  N  N  A  H  H  V  C  W
6081  TGGAGGCCTC AATGCTCTTG GACAACATGG AGGTGAGGGG
        L  E  A  S  M  L  L  D  N  M  E  V  R  G
```

Chimerivax WN02 M66 variant
[Strand]

```
6121   TGGAATGGTC GCCCCACTCT ATGGCGTTGA AGGAACTAAA
         G  M  V   A  P  L  Y   G  V  E   G  T  K
6161   ACACCAGTTT CCCCTGGTGA AATGAGACTG AGGGATGACC
         T  P  V   S  P  G  E   M  R  L   R  D  D
6201   AGAGGAAAGT CTTCAGAGAA CTAGTGAGGA ATTGTGACCT
         Q  R  K   V  F  R  E   L  V  R   N  C  D  L
6241   GCCCGTTTGG CTTTCGTGGC AAGTGGCCAA GGCTGGTTTG
         P  V  W   L  S  W  Q   V  A  K   A  G  L
6281   AAGACGAATG ATCGTAAGTG GTGTTTTGAA GGCCCTGAGG
         K  T  N   D  R  K  W   C  F  E   G  P  E
6321   AACATGAGAT CTTGAATGAC AGCGGTGAAA CAGTGAAGTG
         E  H  E   I  L  N  D   S  G  E   T  V  K  C
6361   CAGGGCTCCT GGAGGAGCAA AGAAGCCTCT GCGCCCAAGG
         R  A  P   G  G  A  K   K  P  L   R  P  R
6401   TGGTGTGATG AAAGGGTGTC ATCTGACCAG AGTGCGCTGT
         W  C  D   E  R  V  S   S  D  Q   S  A  L
6441   CTGAATTTAT TAAGTTTGCT GAAGGTAGGA GGGGAGCTGC
         S  E  F   I  K  F  A   E  G  R   R  G  A  A
6481   TGAAGTGCTA GTTGTGCTGA GTGAACTCCC TGATTTCCTG
         E  V  L   V  V  L  S   E  L  P   D  F  L
6521   GCTAAAAAAG GTGGAGAGGC AATGGATACC ATCAGTGTGT
         A  K  K   G  G  E  A   M  D  T   I  S  V
6561   TCCTCCACTC TGAGGAAGGC TCTAGGGCTT ACCGCAATGC
         F  L  H   S  E  E  G   S  R  A   Y  R  N  A
6601   ACTATCAATG ATGCCTGAGG CAATGACAAT AGTCATGCTG
         L  S  M   M  P  E  A   M  T  I   V  M  L
6641   TTTATACTGG CTGGACTACT GACATCGGGA ATGGTCATCT
         F  I  L   A  G  L  L   T  S  G   M  V  I
6681   TTTTCATGTC TCCCAAAGGC ATCAGTAGAA TGTCTATGGC
         F  F  M   S  P  K  G   I  S  R   M  S  M  A
6721   GATGGGCACA ATGGCCGGCT GTGGATATCT CATGTTCCTT
         M  G  T   M  A  G  C   G  Y  L   M  F  L
6761   GGAGGCGTCA AACCCACTCA CATCTCCTAT GTCATGCTCA
         G  G  V   K  P  T  H   I  S  Y   V  M  L
```

Chimerivax WN02 M66 variant
[Strand]

```
6801  TATTCTTTGT CCTGATGGTG GTTGTGATCC CCGAGCCAGG
        I  F  F  V  L  M  V  V  V  I  P  E  P  G
6841  GCAACAAAGG TCCATCCAAG ACAACCAAGT GGCATACCTC
        Q  Q  R  S  I  Q  D  N  Q  V  A  Y  L
6881  ATTATTGGCA TCCTGACGCT GGTTTCAGCG GTGGCAGCCA
        I  I  G  I  L  T  L  V  S  A  V  A  A
6921  ACGAGCTAGG CATGCTGGAG AAAACCAAAG AGGACCTCTT
        N  E  L  G  M  L  E  K  T  K  E  D  L  F
6961  TGGGAAGAAG AACTTAATTC CATCTAGTGC TTCACCCTGG
        G  K  K  N  L  I  P  S  S  A  S  P  W
7001  AGTTGGCCGG ATCTTGACCT GAAGCCAGGA GCTGCCTGGA
        S  W  P  D  L  D  L  K  P  G  A  A  W
7041  CAGTGTACGT TGGCATTGTT ACAATGCTCT CTCCAATGTT
        T  V  Y  V  G  I  V  T  M  L  S  P  M  L
7081  GCACCACTGG ATCAAAGTCG AATATGGCAA CCTGTCTCTG
        H  H  W  I  K  V  E  Y  G  N  L  S  L
7121  TCTGGAATAG CCCAGTCAGC CTCAGTCCTT TCTTTCATGG
        S  G  I  A  Q  S  A  S  V  L  S  F  M
7161  ACAAGGGGAT ACCATTCATG AAGATGAATA TCTCGGTCAT
        D  K  G  I  P  F  M  K  M  N  I  S  V  I
7201  AATGCTGCTG GTCAGTGGCT GGAATTCAAT AACAGTGATG
        M  L  L  V  S  G  W  N  S  I  T  V  M
7241  CCTCTGCTCT GTGGCATAGG GTGCGCCATG CTCCACTGGT
        P  L  L  C  G  I  G  C  A  M  L  H  W
7281  CTCTCATTTT ACCTGGAATC AAAGCGCAGC AGTCAAAGCT
        S  L  I  L  P  G  I  K  A  Q  Q  S  K  L
7321  TGCACAGAGA AGGGTGTTCC ATGGCGTTGC CAAGAACCCT
        A  Q  R  R  V  F  H  G  V  A  K  N  P
7361  GTGGTTGATG GGAATCCAAC AGTTGACATT GAGGAAGCTC
        V  V  D  G  N  P  T  V  D  I  E  E  A
7401  CTGAAATGCC TGCCCTTTAT GAGAAGAAAC TGGCTCTATA
        P  E  M  P  A  L  Y  E  K  K  L  A  L  Y
7441  TCTCCTTCTT GCTCTCAGCC TAGCTTCTGT TGCCATGTGC
        L  L  L  A  L  S  L  A  S  V  A  M  C
```

Chimerivax WN02 M66 variant
[Strand]

```
7481  AGAACGCCCT TTTCATTGGC TGAAGGCATT GTCCTAGCAT
        R  T  P  F  S  L  A  E  G  I  V  L  A
7521  CAGCTGCCTT AGGGCCGCTC ATAGAGGGAA ACACCAGCCT
        S  A  A  L  G  P  L  I  E  G  N  T  S  L
7561  TCTTTGGAAT GGACCCATGG CTGTCTCCAT GACAGGAGTC
        L  W  N  G  P  M  A  V  S  M  T  G  V
7601  ATGAGGGGGA ATCACTATGC TTTTGTGGGA GTCATGTACA
        M  R  G  N  H  Y  A  F  V  G  V  M  Y
7641  ATCTATGGAA GATGAAAACT GGACGCCGGG GGAGCGCGAA
        N  L  W  K  M  K  T  G  R  R  G  S  A  N
7681  TGGAAAAACT TTGGGTGAAG TCTGGAAGAG GGAACTGAAT
        G  K  T  L  G  E  V  W  K  R  E  L  N
7721  CTGTTGGACA AGCGACAGTT TGAGTTGTAT AAAAGGACCG
        L  L  D  K  R  Q  F  E  L  Y  K  R  T
7761  ACATTGTGGA GGTGGATCGT GATACGGCAC GCAGGCATTT
        D  I  V  E  V  D  R  D  T  A  R  R  H  L
7801  GGCCGAAGGG AAGGTGGACA CCGGGGTGGC GGTCTCCAGG
        A  E  G  K  V  D  T  G  V  A  V  S  R
7841  GGGACCGCAA AGTTAAGGTG GTTCCATGAG CGTGGCTATG
        G  T  A  K  L  R  W  F  H  E  R  G  Y
7881  TCAAGCTGGA AGGTAGGGTG ATTGACCTGG GGTGTGGCCG
        V  K  L  E  G  R  V  I  D  L  G  C  G  R
7921  CGGAGGCTGG TGTTACTACG CTGCTGCGCA AAAGGAAGTG
        G  G  W  C  Y  Y  A  A  A  Q  K  E  V
7961  AGTGGGGTCA AAGGATTTAC TCTTGGAAGA GACGGCCATG
        S  G  V  K  G  F  T  L  G  R  D  G  H
8001  AGAAACCCAT GAATGTGCAA AGTCTGGGAT GGAACATCAT
        E  K  P  M  N  V  Q  S  L  G  W  N  I  I
8041  CACCTTCAAG GACAAAACTG ATATCCACCG CCTAGAACCA
        T  F  K  D  K  T  D  I  H  R  L  E  P
8081  GTGAAATGTG ACACCCTTTT GTGTGACATT GGAGAGTCAT
        V  K  C  D  T  L  L  C  D  I  G  E  S
8121  CATCGTCATC GGTCACAGAG GGGGAAAGGA CCGTGAGAGT
        S  S  S  S  V  T  E  G  E  R  T  V  R  V
```

Chimerivax WN02 M66 variant
[Strand]

```
8161  TCTTGATACT GTAGAAAAAT GGCTGGCTTG TGGGGTTGAC
        L  D  T  V  E  K  W  L  A  C  G  V  D
8201  AACTTCTGTG TGAAGGTGTT AGCTCCATAC ATGCCAGATG
        N  F  C  V  K  V  L  A  P  Y  M  P  D
8241  TTCTTGAGAA ACTGGAATTG CTCCAAAGGA GGTTTGGCGG
        V  L  E  K  L  E  L  L  Q  R  R  F  G  G
8281  AACAGTGATC AGGAACCCTC TCTCCAGGAA TTCCACTCAT
        T  V  I  R  N  P  L  S  R  N  S  T  H
8321  GAAATGTACT ACGTGTCTGG AGCCCGCAGC AATGTCACAT
        E  M  Y  Y  V  S  G  A  R  S  N  V  T
8361  TTACTGTGAA CCAAACATCC CGCCTCCTGA TGAGGAGAAT
        F  T  V  N  Q  T  S  R  L  L  M  R  R  M
8401  GAGGCGTCCA ACTGGAAAAG TGACCCTGGA GGCTGACGTC
        R  R  P  T  G  K  V  T  L  E  A  D  V
8441  ATCCTCCCAA TTGGGACACG CAGTGTTGAG ACAGACAAGG
        I  L  P  I  G  T  R  S  V  E  T  D  K
8481  GACCCCTGGA CAAAGAGGCC ATAGAAGAAA GGGTTGAGAG
        G  P  L  D  K  E  A  I  E  E  R  V  E  R
8521  GATAAAATCT GAGTACATGA CCTCTTGGTT TTATGACAAT
        I  K  S  E  Y  M  T  S  W  F  Y  D  N
8561  GACAACCCCT ACAGGACCTG GCACTACTGT GGCTCCTATG
        D  N  P  Y  R  T  W  H  Y  C  G  S  Y
8601  TCACAAAAAC CTCCGGAAGT GCGGCGAGCA TGGTAAATGG
        V  T  K  T  S  G  S  A  A  S  M  V  N  G
8641  TGTTATTAAA ATTCTGACAT ATCCATGGGA CAGGATAGAG
        V  I  K  I  L  T  Y  P  W  D  R  I  E
8681  GAGGTCACAA GAATGGCAAT GACTGACACA ACCCCTTTTG
        E  V  T  R  M  A  M  T  D  T  T  P  F
8721  GACAGCAAAG AGTGTTTAAA GAAAAAGTTG ACACCAGAGC
        G  Q  Q  R  V  F  K  E  K  V  D  T  R  A
8761  AAAGGATCCA CCAGCGGGAA CTAGGAAGAT CATGAAAGTT
        K  D  P  P  A  G  T  R  K  I  M  K  V
8801  GTCAACAGGT GGCTGTTCCG CCACCTGGCC AGAGAAAAGA
        V  N  R  W  L  F  R  H  L  A  R  E  K
```

Chimerivax WN02 M66 variant
[Strand]

```
8841  ACCCCAGACT GTGCACAAAG GAAGAATTTA TTGCAAAAGT
        N  P  R  L  C  T  K  E  E  F  I  A  K  V
8881  CCGAAGTCAT GCAGCCATTG GAGCTTACCT GGAAGAACAA
        R  S  H  A  A  I  G  A  Y  L  E  E  Q
8921  GAACAGTGGA AGACTGCCAA TGAGGCTGTC CAAGACCCAA
        E  Q  W  K  T  A  N  E  A  V  Q  D  P
8961  AGTTCTGGGA ACTGGTGGAT GAAGAAAGGA AGCTGCACCA
        K  F  W  E  L  V  D  E  E  R  K  L  H  Q
9001  ACAAGGCAGG TGTCGGACTT GTGTGTACAA CATGATGGGG
        Q  G  R  C  R  T  C  V  Y  N  M  M  G
9041  AAAAGAGAGA AGAAGCTGTC AGAGTTTGGG AAAGCAAAGG
        K  R  E  K  K  L  S  E  F  G  K  A  K
9081  GAAGCCGTGC CATATGGTAT ATGTGGCTGG GAGCGCGGTA
        G  S  R  A  I  W  Y  M  W  L  G  A  R  Y
9121  TCTTGAGTTT GAGGCCCTGG GATTCCTGAA TGAGGACCAT
        L  E  F  E  A  L  G  F  L  N  E  D  H
9161  TGGGCTTCCA GGGAAAACTC AGGAGGAGGA GTGGAAGGCA
        W  A  S  R  E  N  S  G  G  G  V  E  G
9201  TTGGCTTACA ATACCTAGGA TATGTGATCA GAGACCTGGC
        I  G  L  Q  Y  L  G  Y  V  I  R  D  L  A
9241  TGCAATGGAT GGTGGTGGAT TCTACGCGGA TGACACCGCT
        A  M  D  G  G  G  F  Y  A  D  D  T  A
9281  GGATGGGACA CGCGCATCAC AGAGGCAGAC CTTGATGATG
        G  W  D  T  R  I  T  E  A  D  L  D  D
9321  AACAGGAGAT CTTGAACTAC ATGAGCCCAC ATCACAAAAA
        E  Q  E  I  L  N  Y  M  S  P  H  H  K  K
9361  ACTGGCACAA GCAGTGATGG AAATGACATA CAAGAACAAA
        L  A  Q  A  V  M  E  M  T  Y  K  N  K
9401  GTGGTGAAAG TGTTGAGACC AGCCCCAGGA GGGAAAGCCT
        V  V  K  V  L  R  P  A  P  G  G  K  A
9441  ACATGGATGT CATAAGTCGA CGAGACCAGA GAGGATCCGG
        Y  M  D  V  I  S  R  R  D  Q  R  G  S  G
9481  GCAGGTAGTG ACTTATGCTC TGAACACCAT CACCAACTTG
        Q  V  V  T  Y  A  L  N  T  I  T  N  L
```

Chimerivax WN02 M66 variant
[Strand]

```
9521   AAAGTCCAAT TGATCAGAAT GGCAGAAGCA GAGATGGTGA
         K  V  Q  L  I  R  M  A  E  A  E  M  V
9561   TACATCACCA ACATGTTCAA GATTGTGATG AATCAGTTCT
       I  H  H  Q  H  V  Q  D  C  D  E  S  V  L
9601   GACCAGGCTG GAGGCATGGC TCACTGAGCA CGGATGTGAC
         T  R  L  E  A  W  L  T  E  H  G  C  D
9641   AGACTGAAGA GGATGGCGGT GAGTGGAGAC GACTGTGTGG
         R  L  K  R  M  A  V  S  G  D  D  C  V
9681   TCCGGCCCAT CGATGACAGG TTCGGCCTGG CCCTGTCCCA
       V  R  P  I  D  D  R  F  G  L  A  L  S  H
9721   TCTCAACGCC ATGTCCAAGG TTAGAAAGGA CATATCTGAA
         L  N  A  M  S  K  V  R  K  D  I  S  E
9761   TGGCAGCCAT CAAAAGGGTG GAATGATTGG GAGAATGTGC
         W  Q  P  S  K  G  W  N  D  W  E  N  V
9801   CCTTCTGTTC CCACCACTTC CATGAACTAC AGCTGAAGGA
       P  F  C  S  H  H  F  H  E  L  Q  L  K  D
9841   TGGCAGGAGG ATTGTGGTGC CTTGCCGAGA ACAGGACGAG
         G  R  R  I  V  V  P  C  R  E  Q  D  E
9881   CTCATTGGGA GAGGAAGGGT GTCTCCAGGA AACGGCTGGA
         L  I  G  R  G  R  V  S  P  G  N  G  W
9921   TGATCAAGGA AACAGCTTGC CTCAGCAAAG CCTATGCCAA
       M  I  K  E  T  A  C  L  S  K  A  Y  A  N
9961   CATGTGGTCA CTGATGTATT TTCACAAAAG GGACATGAGG
         M  W  S  L  M  Y  F  H  K  R  D  M  R
10001  CTACTGTCAT TGGCTGTTTC CTCAGCTGTT CCCACCTCAT
         L  L  S  L  A  V  S  S  A  V  P  T  S
10041  GGGTTCCACA AGGACGCACA ACATGGTCGA TTCATGGGAA
       W  V  P  Q  G  R  T  T  W  S  I  H  G  K
10081  AGGGGAGTGG ATGACCACGG AAGACATGCT TGAGGTGTGG
         G  E  W  M  T  T  E  D  M  L  E  V  W
10121  AACAGAGTAT GGATAACCAA CAACCCACAC ATGCAGGACA
         N  R  V  W  I  T  N  N  P  H  M  Q  D
10161  AGACAATGGT GAAAAAATGG AGAGATGTCC CTTATCTAAC
       K  T  M  V  K  K  W  R  D  V  P  Y  L  T
```

Chimerivax WN02 M66 variant
[Strand]

```
10201  CAAGAGACAA GACAAGCTGT GCGGATCACT GATTGGAATG
         K  R  Q  D  K  L  C  G  S  L  I  G  M
10241  ACCAATAGGG CCACCTGGGC CTCCCACATC CATTTAGTCA
         T  N  R  A  T  W  A  S  H  I  H  L  V
10281  TCCATCGTAT CCGAACGCTG ATTGGACAGG AGAAATACAC
         I  H  R  I  R  T  L  I  G  Q  E  K  Y  T
10321  TGACTACCTA ACAGTCATGG ACAGGTATTC TGTGGATGCT
         D  Y  L  T  V  M  D  R  Y  S  V  D  A
10361  GACCTGCAAC TGGGTGAGCT TATCTGAAAC ACCATCTAAC
         D  L  Q  L  G  E  L  I
10401  AGGAATAACC GGGATACAAA CCACGGGTGG AGAACCGGAC

10441  TCCCCACAAC CTGAAACCGG GATATAAACC ACGGCTGGAG

10481  AACCGGACTC CGCACTTAAA ATGAAACAGA AACCGGGATA

10521  AAAACTACGG ATGGAGAACC GGACTCCACA CATTGAGACA

10561  GAAGAAGTTG TCAGCCCAGA ACCCCACACG AGTTTTGCCA

10601  CTGCTAAGCT GTGAGGCAGT GCAGGCTGGG ACAGCCGACC

10641  TCCAGGTTGC GAAAAACCTG GTTTCTGGGA CCTCCCACCC

10681  CAGAGTAAAA AGAACGGAGC CTCCGCTACC ACCCTCCCAC

10721  GTGGTGGTAG AAAGACGGGG TCTAGAGGTT AGAGGAGACC

10761  CTCCAGGGAA CAAATAGTGG GACCATATTG ACGCCAGGGA

10801  AAGACCGGAG TGGTTCTCTG CTTTTCCTCC AGAGGTCTGT

10841  GAGCACAGTT TGCTCAAGAA TAAGCAGACC TTTGGATGAC
```

Chimerivax WN02 M66 variant
[Strand]

10881 AAACACAAAA CCACAA

DNA Strider™ 1.3f7

WN 02 x M66 Variant => DNA Alignment

```
DNA  sequence     10896  bp   *GTAAATCCTGT ... ACAAAACCACAA   linear

DNA  sequence     10896  bp   *GTAAATCCTGT ... ACAAAACCACAA   linear

   Layout:           Compacted
   Method:           Blocks (Martinez)
   Mismatch penalty: Smaller (1)
   Gap penalty:      Medium  (2)
   Translation:      Off

     1 *GTAAATCCTGTGTGCTAATTGAGGTGCATTGGTCTGCAAATCGAGTTGCTAGGCAATAAACACATTTGGATTAATTTTA 80
     1 ..................................................................................  80

    81 ATCGTTCGTTGAGCGATTAGCAGAGAACTGACCAGAACATGTCTGGTCGTAAAGCTCAGGGAAAAACCCTGGGCGTCAAT 160
    81 ..................................................................................  160

   161 ATGGTACGACGAGGAGTTCGCTCCTTGTCAAACAAAATAAAACAAAAAACAAAACAAATTGGAAACAGACCTGGACCTTC 240
   161 ..................................................................................  240

   241 AAGAGGTGTTCAAGGATTTATCTTTTTCTTTTTGTTCAACATTTTGACTGGAAAAAAGATCACAGCCCACCTAAAGAGGT 320
   241 ..................................................................................  320

   321 TGTGGAAAATGCTGGACCCAAGACAAGGCTTGGCTGTTCTAAGGAAAGTCAAGAGAGTGGTGGCCAGTTTGATGAGAGGA 400
   321 ..................................................................................  400

   401 TTGTCCTCAAGGAAACGCCGTTCCCATGATGTTCTGACTGTGCAATTCCTAATTTTGGGAATGCTGTTGATGACGGGTGG 480
   401 ..................................................................................  480

   481 AGTTACCCTCTCTAACTTCCAAGGGAAGGTGATGATGACGGTAAATGCTACTGACGTCACAGATGTCATCACGATTCCAA 560
   481 ..................................................................................  560

   561 CAGCTGCTGGAAAGAACCTATGCATTGTCAGAGCAATGGATGTGGGATACATGTGCGATGATACTATCACTTATGAATGC 640
   561 ..................................................................................  640

   641 CCAGTGCTGTCGGCTGGTAATGATCCAGAAGACATCGACTGTTGGTGCACAAAGTCAGCAGTCTACGTCAGGTATGGAAG 720
   641 ..................................................................................  720

   721 ATGCACCAAGACACGCCACTCAAGACGCAGTCGGAGGTCACTGACAGTGCAGACACACGGAGAAAGCACTCTAGCGAACA 800
   721 ..................................................................................  800

   801 AGAAGGGGGCTTGGATGGACAGCACCAAGGCCACAAGGTATTTGGTAAAAACAGAATCATGGATCTTGAGGAACCCTGGA 880
   801 ..................................................................................  880

   881 TATGCCCTGGTGGCAGCCGTCATTGGTTGGATGCTTGGGAGCAACACCATGCAGAGAGTTGTGTTTGTCGTGCTATTGCT 960
   881 .....................................................................C........  960

   961 TTTGGTGGCCCCAGCTTACAGCTTCAACTGCCTTGGAATGAGCAACAGAGACTTCTTGGAAGGAGTGTCTGGAGCAACAT 1040
   961 ..................................................................................  1040

  1041 GGGTGGATTTGGTTCTCGAAGGCGACAGCTGCGTGACTATCATGTCTAAGGACAAGCCTACCATCGACGTCAAGATGATG 1120
  1041 ..................................................................................  1120

  1121 AATATGGAGGCGGCCAACCTGGCAGAGGTCCGCAGTTATTGCTATTTGGCTACCGTCAGCGATCTCTCCACCAAAGCTGC 1200
  1121 ..................................................................................  1200

  1201 ATGCCCGACCATGGGAGAAGCTCACAATGACAAACGTGCTGACCCAGCTTTTGTGTGCAGACAAGGAGTGGTGGACAGGG 1280
  1201 ..................................................................................  1280

  1281 GCTGGGGCAACGGCTGCGGATTTTTTGGCAAAGGATCCATTGACACATGCGCCAAATTTGCCTGCTCTACCAAGGCAATA 1360
  1281 ..................................................................................  1360

  1361 GGAAGAACCATCTTGAAAGAGAATATCAAGTACGAAGTGGCCATTTTTGTCCATGGACCAACTACTGTGGAGTCGCACGG 1440
  1361 ..................................................................................  1440
```

WN 02 x M66 Variant => DNA Alignment

```
1441 AAATTACTCCACACAGGTTGGAGCCACTCAGGCCGGCCGATTCAGCATCACTCCTGCTGCGCCTTCATACACACTAAAGC 1520
1441 ....................................................................................... 1520

1521 TTGGAGAATATGGAGAGGTGACAGTGGACTGTGAACCACGGTCAGGGATTGACACCAATGCATACTACGTGATGACTGTT 1600
1521 ....................................................................................... 1600

1601 GGAACAAAGACGTTCTTGGTCCATCGTGAGTGGTTCATGGACCTCAACCTCCCTTGGAGCAGTGCTGGAAGTACTGTGTG 1680
1601 ....................................................................................... 1680

1681 GAGGAACAGAGAGACGTTAATGGAGTTTGAGGAACCACACGCCACGAAGCAGTCTGTGATAGCATTGGGCTCACAAGAGG 1760
1681 ....................................................................................... 1760

1761 GAGCTCTGCATCAAGCTTTGGCTGGAGCCATTCCTGTGGAATTTTCAAGCAACACTGTCAAGTTGACGTCGGGTCATTTG 1840
1761 ....................................................................................... 1840

1841 AAGTGTAGAGTGAAGATGGAAAAATTGCAGTTGAAGGGAACAACCTATGGCGTCTGTTCAAAGGCTTTCAAGTTTCTTAG 1920
1841 ....................................................................................... 1920

1921 GACTCCCGTGGACACCGGTCACGGCACTGTGGTGTTGGAATTGCAGTACACTGGCACGGATGGACCTTGCAAAGTTCCTA 2000
1921 ....................................................................................... 2000

2001 TCTCGTCAGTGGCTTCATTGAACGACCTAACGCCAGTGGGCAGATTGGTCACTGTCAACCCTTTTGTTTCAGTGGCCACG 2080
2001 ....................................................................................... 2080

2081 GCCAACGCTAAGGTCCTGATTGAATTGGAACCACCCTTTGGAGACTCATACATAGTGGTGGGCAGAGGAGAACAACAGAT 2160
2081 ....................................................................................... 2160

2161 CAATCACCATTGGCACAAGTCTGGAAGCAGCATTGGCAAAGCCTTTACAACCACCCTCAAAGGAGCGCAGAGACTAGCCG 2240
2161 ....................................................................................... 2240

2241 CTCTAGGAGACACAGCTTGGGACTTTGGATCAGTTGGAGGGGTGTTCACTAGTGTTGGGCGGGCTGTCCATCAAGTGTTC 2320
2241 ....................................................................................... 2320

2321 GGAGGAGCATTCCGCTCACTGTTCGGAGGCATGTCCTGGATAACGCAAGGATTGCTGGGGGCTCTCCTGTTGTGGATGGG 2400
2321 ....................................................................................... 2400

2401 CATCAATGCTCGTGATAGGTCCATAGCTCTCACGTTTCTCGCAGTTGGAGGAGTTCTGCTCTTCCTCTCCGTGAACGTGG 2480
2401 ....................................................................................... 2480

2481 GCGCCGATCAAGGATGCGCCATCAACTTTGGCAAGAGAGAGCTCAAGTGCGGAGATGGTATCTTCATATTTAGAGACTCT 2560
2481 ....................................................................................... 2560

2561 GATGACTGGCTGAACAAGTACTCATACTATCCAGAAGATCCTGTGAAGCTTGCATCAATAGTGAAAGCCTCTTTTGAAGA 2640
2561 ....................................................................................... 2640

2641 AGGGAAGTGTGGCCTAAATTCAGTTGACTCCCTTGAGCATGAGATGTGGAGAAGCAGGGCAGATGAGATCAATGCCATTT 2720
2641 ....................................................................................... 2720

2721 TTGAGGAAAACGAGGTGGACATTTCTGTTGTCGTGCAGGATCCAAAGAATGTTTACCAGAGAGGAACTCATCCATTTTCC 2800
2721 ....................................................................................... 2800

2801 AGAATTCGGGATGGTCTGCAGTATGGTTGGAAGACTTGGGGTAAGAACCTTGTGTTCTCCCCAGGGAGGAAGAATGGAAG 2880
2801 ....................................................................................... 2880

2881 CTTCATCATAGATGGAAAGTCCAGGAAAGAATGCCCGTTTTCAAACCGGGTCTGGAATTCTTTCCAGATAGAGGAGTTTG 2960
2881 ....................................................................................... 2960

2961 GGACGGGAGTGTTCACCACACGCGTGTACATGGACGCAGTCTTTGAATACACCATAGACTGCGATGGATCTATCTTGGGT 3040
2961 ....................................................................................... 3040

3041 GCAGCGGTGAACGGAAAAAAGAGTGCCCATGGCTCTCCAACATTTTGGATGGGAAGTCATGAAGTAAATGGGACATGGAT 3120
3041 ....................................................................................... 3120

3121 GATCCACACCTTGGAGGCATTAGATTACAAGGAGTGTGAGTGGCCACTGACACATACGATTGGAACATCAGTTGAAGAGA 3200
3121 ....................................................................................... 3200
```

WN 02 x M66 Variant => DNA Alignment

```
3201 GTGAAATGTTCATGCCGAGATCAATCGGAGGCCCAGTTAGCTCTCACAATCATATCCCTGGATACAAGGTTCAGACGAAC 3280
3201 ..................................................................................... 3280

3281 GGACCTTGGATGCAGGTACCACTAGAAGTGAAGAGAGAAGCTTGCCCAGGGACTAGCGTGATCATTGATGGCAACTGTGA 3360
3281 ..................................................................................... 3360

3361 TGGACGGGGAAAATCAACCAGATCCACCACGGATAGCGGGAAAGTTATTCCTGAATGGTGTTGCCGCTCCTGCACAATGC 3440
3361 ..................................................................................... 3440

3441 CGCCTGTGAGCTTCCATGGTAGTGATGGGTGTTGGTATCCCATGGAAATTAGGCCAAGGAAAACGCATGAAAGCCATCTG 3520
3441 ..................................................................................... 3520

3521 GTGCGCTCCTGGGTTACAGCTGGAGAAATACATGCTGTCCCTTTTGGTTTGGTGAGCATGATGATAGCAATGGAAGTGGT 3600
3521 ..................................................................................... 3600

3601 CCTAAGGAAAAGACAGGGACCAAAGCAAATGTTGGTTGGAGGAGTAGTGCTCTTGGGAGCAATGCTGGTCGGGCAAGTAA 3680
3601 ..................................................................................... 3680

3681 CTCTCCTTGATTTGCTGAAACTCACAGTGGCTGTGGGATTGCATTTCCATGAGATGAACAATGGAGGAGACGCCATGTAT 3760
3681 ..................................................................................... 3760

3761 ATGGCGTTGATTGCTGCCTTTTCAATCAGACCAGGGCTGCTCATCGGCTTTGGGCTCAGGACCCTATGGAGCCCTCGGGA 3840
3761 ..................................................................................... 3840

3841 ACGCCTTGTGCTGACCCTAGGAGCAGCCATGGTGGAGATTGCCTTGGGTGGCGTGATGGGCGGCCTGTGGAAGTATCTAA 3920
3841 ..................................................................................... 3920

3921 ATGCAGTTTCTCTCTGCATCCTGACAATAAATGCTGTTGCTTCTAGGAAAGCATCAAATACCATCTTGCCCCTCATGGCT 4000
3921 ..................................................................................... 4000

4001 CTGTTGACACCTGTCACTATGGCTGAGGTGAGACTTGCCGCAATGTTCTTTTGTGCCATGGTTATCATAGGGGTCCTTCA 4080
4001 ..................................................................................... 4080

4081 CCAGAATTTCAAGGACACCTCCATGCAGAAGACTATACCTCTGGTGGCCCTCACACTCACATCTTACCTGGGCTTGACAC 4160
4081 ..................................................................................... 4160

4161 AACCTTTTTTGGGCCTGTGTGCATTTCTGGCAACCCGCATATTTGGGCGAAGGAGTATCCCAGTGAATGAGGCACTCGCA 4240
4161 ..................................................................................... 4240

4241 GCAGCTGGTCTAGTGGGAGTGCTGGCAGGACTGGCTTTTCAGGAGATGGAGAACTTCCTTGGTCCGATTGCAGTTGGAGG 4320
4241 ..................................................................................... 4320

4321 ACTCCTGATGATGCTGGTTAGCGTGGCTGGGAGGGTGGATGGGCTAGAGCTCAAGAAGCTTGGTGAAGTTTCATGGGAAG 4400
4321 ..................................................................................... 4400

4401 AGGAGGCGGAGATCAGCGGGAGTTCCGCCCGCTATGATGTGGCACTCAGTGAACAAGGGGAGTTCAAGCTGCTTTCTGAA 4480
4401 ..................................................................................... 4480

4481 GAGAAAGTGCCATGGGACCAGGTTGTGATGACCTCGCTGGCCTTGGTTGGGGCTGCCCTCCATCCATTTGCTCTTCTGCT 4560
4481 ..................................................................................... 4560

4561 GGTCCTTGCTGGGTGGCTGTTTCATGTCAGGGGAGCTAGGAGAAGTGGGGATGTCTTGTGGGATATTCCCACTCCTAAGA 4640
4561 ..................................................................................... 4640

4641 TCATCGAGGAATGTGAACATCTGGAGGATGGGATTTATGGCATATTCCAGTCAACCTTCTTGGGGGCCTCCCAGCGAGGA 4720
4641 ..................................................................................... 4720

4721 GTGGGAGTGGCACAGGGAGGGGTGTTCCACACAATGTGGCATGTCACAAGAGGAGCTTTCCTTGTCAGGAATGGCAAGAA 4800
4721 ..................................................................................... 4800

4801 GTTGATTCCATCTTGGGCTTCAGTAAAGGAAGACCTTGTCGCCTATGGTGGCTCATGGAAGTTGGAAGGCAGATGGGATG 4880
4801 ..................................................................................... 4880

4881 GAGAGGAAGAGGTCCAGTTGATCGCGGCTGTTCCAGGAAAGAACGTGGTCAACGTCCAGACAAAACCGAGCTTGTTCAAA 4960
4881 ..................................................................................... 4960
```

WN 02 x M66 Variant => DNA Alignment

```
4961 GTGAGGAATGGGGGAGAAATCGGGGCTGTCGCTCTTGACTATCCGAGTGGCACTTCAGGATCTCCTATTGTTAACAGGAA 5040
4961 ..................................................................................... 5040

5041 CGGAGAGGTGATTGGGCTGTACGGCAATGGCATCCTTGTCGGTGACAACTCCTTCGTGTCCGCCATATCCCAGACTGAGG 5120
5041 ..................................................................................... 5120

5121 TGAAGGAAGAAGGAAAGGAGGAGCTCCAAGAGATCCCGACAATGCTAAAGAAAGGAATGACAACTGTCCTTGATTTTCAT 5200
5121 ..................................................................................... 5200

5201 CCTGGAGCTGGGAAGACAAGACGTTTCCTCCCACAGATCTTGGCCGAGTGCGCACGGAGACGCTTGCGCACTCTTGTGTT 5280
5201 ..................................................................................... 5280

5281 GGCCCCCACCAGGGTTGTTCTTTCTGAAATGAAGGAGGCTTTTCACGGCCTGGACGTGAAATTCCACACACAGGCTTTTT 5360
5281 ..................................................................................... 5360

5361 CCGCTCACGGCAGCGGGAGAGAAGTCATTGATGCCATGTGCCATGCCACCCTAACTTACAGGATGTTGGAACCAACTAGG 5440
5361 ..................................................................................... 5440

5441 GTTGTTAACTGGGAAGTGATCATTATGGATGAAGCCCATTTTTTGGATCCAGCCAGCATAGCCGCTAGAGGTTGGGCAGC 5520
5441 ..................................................................................... 5520

5521 GCACAGAGCTAGGGCAAATGAAAGTGCAACAATCTTGATGACAGCCACACCGCCTGGGACTAGTGATGAATTTCCACATT 5600
5521 ..................................................................................... 5600

5601 CAAATGGTGAAATAGAAGATGTTCAAACGGACATACCCAGTGAGCCCTGGAACACAGGGCATGACTGGATCCTGGCTGAC 5680
5601 ..................................................................................... 5680

5681 AAAAGGCCCACGGCATGGTTCCTTCCATCCATCAGAGCTGCAAATGTCATGGCTGCCTCTTTGCGTAAGGCTGGAAAGAG 5760
5681 ..................................................................................... 5760

5761 TGTGGTGGTCCTGAACAGGAAAACCTTTGAGAGAGAATACCCCACGATAAAGCAGAAGAAACCTGACTTTATATTGGCCA 5840
5761 ..................................................................................... 5840

5841 CTGACATAGCTGAAATGGGAGCCAACCTTTGCGTGGAGCGAGTGCTGGATTGCAGGACGGCTTTTAAGCCTGTGCTTGTG 5920
5841 ..................................................................................... 5920

5921 GATGAAGGGAGGAAGGTGGCAATAAAAGGGCCACTTCGTATCTCCGCATCCTCTGCTGCTCAAAGGAGGGGGCGCATTGG 6000
5921 ..................................................................................... 6000

6001 GAGAAATCCCAACAGAGATGGAGACTCATACTACTATTCTGAGCCTACAAGTGAAAATAATGCCCACCACGTCTGCTGGT 6080
6001 ..................................................................................... 6080

6081 TGGAGGCCTCAATGCTCTTGGACAACATGGAGGTGAGGGGTGGAATGGTCGCCCCACTCTATGGCGTTGAAGGAACTAAA 6160
6081 ..................................................................................... 6160

6161 ACACCAGTTTCCCCTGGTGAAATGAGACTGAGGGATGACCAGAGGAAAGTCTTCAGAGAACTAGTGAGGAATTGTGACCT 6240
6161 ..................................................................................... 6240

6241 GCCCGTTTGGCTTTCGTGGCAAGTGGCCAAGGCTGGTTTGAAGACGAATGATCGTAAGTGGTGTTTTGAAGGCCCTGAGG 6320
6241 ..................................................................................... 6320

6321 AACATGAGATCTTGAATGACAGCGGTGAAACAGTGAAGTGCAGGGCTCCTGGAGGAGCAAAGAAGCCTCTGCGCCCAAGG 6400
6321 ..................................................................................... 6400

6401 TGGTGTGATGAAAGGGTGTCATCTGACCAGAGTGCGCTGTCTGAATTTATTAAGTTTGCTGAAGGTAGGAGGGGAGCTGC 6480
6401 ..................................................................................... 6480

6481 TGAAGTGCTAGTTGTGCTGAGTGAACTCCCTGATTTCCTGGCTAAAAAAGGTGGAGAGGCAATGGATACCATCAGTGTGT 6560
6481 ..................................................................................... 6560

6561 TCCTCCACTCTGAGGAAGGCTCTAGGGCTTACCGCAATGCACTATCAATGATGCCTGAGGCAATGACAATAGTCATGCTG 6640
6561 ..................................................................................... 6640

6641 TTTATACTGGCTGGACTACTGACATCGGGAATGGTCATCTTTTTCATGTCTCCCAAAGGCATCAGTAGAATGTCTATGGC 6720
6641 ..................................................................................... 6720
```

WN 02 x M66 Variant => DNA Alignment

```
6721 GATGGGCACAATGGCCGGCTGTGGATATCTCATGTTCCTTGGAGGCGTCAAACCCACTCACATCTCCTATGTCATGCTCA 6800
6721 ..................................................................................... 6800

6801 TATTCTTTGTCCTGATGGTGGTTGTGATCCCCGAGCCAGGGCAACAAAGGTCCATCCAAGACAACCAAGTGGCATACCTC 6880
6801 ..................................................................................... 6880

6881 ATTATTGGCATCCTGACGCTGGTTTCAGCGGTGGCAGCCAACGAGCTAGGCATGCTGGAGAAAACCAAAGAGGACCTCTT 6960
6881 ..................................................................................... 6960

6961 TGGGAAGAAGAACTTAATTCCATCTAGTGCTTCACCCTGGAGTTGGCCGGATCTTGACCTGAAGCCAGGAGCTGCCTGGA 7040
6961 ..................................................................................... 7040

7041 CAGTGTACGTTGGCATTGTTACAATGCTCTCTCCAATGTTGCACCACTGGATCAAAGTCGAATATGGCAACCTGTCTCTG 7120
7041 ..................................................................................... 7120

7121 TCTGGAATAGCCCAGTCAGCCTCAGTCCTTTCTTTCATGGACAAGGGGATACCATTCATGAAGATGAATATCTCGGTCAT 7200
7121 ..................................................................................... 7200

7201 AATGCTGCTGGTCAGTGGCTGGAATTCAATAACAGTGATGCCTCTGCTCTGTGGCATAGGGTGCGCCATGCTCCACTGGT 7280
7201 ..................................................................................... 7280

7281 CTCTCATTTTACCTGGAATCAAAGCGCAGCAGTCAAAGCTTGCACAGAGAAGGGTGTTCCATGGCGTTGCCAAGAACCCT 7360
7281 ..................................................................................... 7360

7361 GTGGTTGATGGGAATCCAACAGTTGACATTGAGGAAGCTCCTGAAATGCCTGCCCTTTATGAGAAGAAACTGGCTCTATA 7440
7361 ..................................................................................... 7440

7441 TCTCCTTCTTGCTCTCAGCCTAGCTTCTGTTGCCATGTGCAGAACGCCCTTTTCATTGGCTGAAGGCATTGTCCTAGCAT 7520
7441 ..................................................................................... 7520

7521 CAGCTGCCTTAGGGCCGCTCATAGAGGGAAACACCAGCCTTCTTTGGAATGGACCCATGGCTGTCTCCATGACAGGAGTC 7600
7521 ..................................................................................... 7600

7601 ATGAGGGGGAATCACTATGCTTTTGTGGGAGTCATGTACAATCTATGGAAGATGAAAACTGGACGCCGGGGGAGCGCGAA 7680
7601 ..................................................................................... 7680

7681 TGGAAAAACTTTGGGTGAAGTCTGGAAGAGGGAACTGAATCTGTTGGACAAGCGACAGTTTGAGTTGTATAAAAGGACCG 7760
7681 ..................................................................................... 7760

7761 ACATTGTGGAGGTGGATCGTGATACGGCACGCAGGCATTTGGCCGAAGGGAAGGTGGACACCGGGGTGGCGGTCTCCAGG 7840
7761 ..................................................................................... 7840

7841 GGGACCGCAAAGTTAAGGTGGTTCCATGAGCGTGGCTATGTCAAGCTGGAAGGTAGGGTGATTGACCTGGGGGTGTGGCCG 7920
7841 ..................................................................................... 7920

7921 CGGAGGCTGGTGTTACTACGCTGCTGCGCAAAAGGAAGTGAGTGGGGTCAAAGGATTTACTCTTGGAAGAGACGGCCATG 8000
7921 ..................................................................................... 8000

8001 AGAAACCCATGAATGTGCAAAGTCTGGGATGGAACATCATCACCTTCAAGGACAAAACTGATATCCACCGCCTAGAACCA 8080
8001 ..................................................................................... 8080

8081 GTGAAATGTGACACCCTTTTGTGTGACATTGGAGAGTCATCATCGTCATCGGTCACAGAGGGGGAAAGGACCGTGAGAGT 8160
8081 ..................................................................................... 8160

8161 TCTTGATACTGTAGAAAAATGGCTGGCTTGTGGGGTTGACAACTTCTGTGTGAAGGTGTTAGCTCCATACATGCCAGATG 8240
8161 ..................................................................................... 8240

8241 TTCTTGAGAAACTGGAATTGCTCCAAAGGAGGTTTGGCGGAACAGTGATCAGGAACCCTCTCTCCAGGAATTCCACTCAT 8320
8241 ..................................................................................... 8320

8321 GAAATGTACTACGTGTCTGGAGCCCGCAGCAATGTCACATTTACTGTGAACCAAACATCCCGCCTCCTGATGAGGAGAAT 8400
8321 ..................................................................................... 8400

8401 GAGGCGTCCAACTGGAAAAGTGACCCTGGAGGCTGACGTCATCCTCCCAATTGGGACACGCAGTGTTGAGACAGACAAGG 8480
8401 ..................................................................................... 8480
```

WN 02 x M66 Variant => DNA Alignment

```
 8481 GACCCCTGGACAAAGAGGCCATAGAAGAAAGGGTTGAGAGGATAAAATCTGAGTACATGACCTCTTGGTTTTATGACAAT 8560
 8481 ..........................................................................  8560

 8561 GACAACCCCTACAGGACCTGGCACTACTGTGGCTCCTATGTCACAAAAACCTCCGGAAGTGCGGCGAGCATGGTAAATGG 8640
 8561 ..........................................................................  8640

 8641 TGTTATTAAAATTCTGACATATCCATGGGACAGGATAGAGGAGGTCACAAGAATGGCAATGACTGACACAACCCCTTTTG 8720
 8641 ..........................................................................  8720

 8721 GACAGCAAAGAGTGTTTAAAGAAAAAGTTGACACCAGAGCAAAGGATCCACCAGCGGGAACTAGGAAGATCATGAAAGTT 8800
 8721 ..........................................................................  8800

 8801 GTCAACAGGTGGCTGTTCCGCCACCTGGCCAGAGAAAAGAACCCCAGACTGTGCACAAAGGAAGAATTTATTGCAAAAGT 8880
 8801 ..........................................................................  8880

 8881 CCGAAGTCATGCAGCCATTGGAGCTTACCTGGAAGAACAAGAACAGTGGAAGACTGCCAATGAGGCTGTCCAAGACCCAA 8960
 8881 ..........................................................................  8960

 8961 AGTTCTGGGAACTGGTGGATGAAGAAAGGAAGCTGCACCAACAAGGCAGGTGTCGGACTTGTGTGTACAACATGATGGGG 9040
 8961 ..........................................................................  9040

 9041 AAAAGAGAGAAGAAGCTGTCAGAGTTTGGGAAAGCAAAGGGAAGCCGTGCCATATGGTATATGTGGCTGGGAGCGCGGTA 9120
 9041 ..........................................................................  9120

 9121 TCTTGAGTTTGAGGCCCTGGGATTCCTGAATGAGGACCATTGGGCTTCCAGGGAAAACTCAGGAGGAGGAGTGGAAGGCA 9200
 9121 ..........................................................................  9200

 9201 TTGGCTTACAATACCTAGGATATGTGATCAGAGACCTGGCTGCAATGGATGGTGGTGGATTCTACGCGGATGACACCGCT 9280
 9201 ..........................................................................  9280

 9281 GGATGGGACACGCGCATCACAGAGGCAGACCTTGATGATGAACAGGAGATCTTGAACTACATGAGCCCACATCACAAAAA 9360
 9281 ..........................................................................  9360

 9361 ACTGGCACAAGCAGTGATGGAAATGACATACAAGAACAAAGTGGTGAAAGTGTTGAGACCAGCCCCAGGAGGGAAAGCCT 9440
 9361 ..........................................................................  9440

 9441 ACATGGATGTCATAAGTCGACGAGACCAGAGAGGATCCGGGCAGGTAGTGACTTATGCTCTGAACACCATCACCAACTTG 9520
 9441 ..........................................................................  9520

 9521 AAAGTCCAATTGATCAGAATGGCAGAAGCAGAGATGGTGATACATCACCAACATGTTCAAGATTGTGATGAATCAGTTCT 9600
 9521 ..........................................................................  9600

 9601 GACCAGGCTGGAGGCATGGCTCACTGAGCACGGATGTGACAGACTGAAGAGGATGGCGGTGAGTGGAGACGACTGTGTGG 9680
 9601 ..........................................................................  9680

 9681 TCCGGCCCATCGATGACAGGTTCGGCCTGGCCCTGTCCCATCTCAACGCCATGTCCAAGGTTAGAAAGGACATATCTGAA 9760
 9681 ..........................................................................  9760

 9761 TGGCAGCCATCAAAAGGGTGGAATGATTGGGAGAATGTGCCCTTCTGTTCCCACCACTTCCATGAACTACAGCTGAAGGA 9840
 9761 ..........................................................................  9840

 9841 TGGCAGGAGGATTGTGGTGCCTTGCCGAGAACAGGACGAGCTCATTGGGAGAGGAAGGGTGTCTCCAGGAAACGGCTGGA 9920
 9841 ..........................................................................  9920

 9921 TGATCAAGGAAACAGCTTGCCTCAGCAAAGCCTATGCCAACATGTGGTCACTGATGTATTTTCACAAAAGGGACATGAGG 10000
 9921 ..........................................................................  10000

10001 CTACTGTCATTGGCTGTTTCCTCAGCTGTTCCCACCTCATGGGTTCCACAAGGACGCACAACATGGTCGATTCATGGGAA 10080
10001 ..........................................................................  10080

10081 AGGGGAGTGGATGACCACGGAAGACATGCTTGAGGTGTGGAACAGAGTATGGATAACCAACAACCCACACATGCAGGACA 10160
10081 ..........................................................................  10160

10161 AGACAATGGTGAAAAAATGGAGAGATGTCCCTTATCTAACCAAGAGACAAGACAAGCTGTGCGGATCACTGATTGGAATG 10240
10161 ..........................................................................  10240
```

DNA Strider™ 1.3f7 ### Thursday, October 21, 2004 3:10:16 PM

WN02 M Prot. x M66 M Prot. => Protein Alignment

```
Protein  sequence        75  aa   SLTVQTHGESTL ... VVLLLLVAPAYS

Protein  sequence        75  aa   SLTVQTHGESTL ... VVPLLLVAPAYS


   Layout:           Standard
   Method:           Single Block
   Block Length ≤:   6-aa
   Mismatch penalty: Smaller (1)
   Gap penalty:      Medium  (2)
   Weighting:        BLOSUM62

                .         20        .         40        .         60        .
     1 SLTVQTHGESTLANKKGAWMDSTKATRYLVKTESWILRNPGYALVAAVIGWMLGSNTMQRVVFVVLLLLVAPAYS      75
       SLTVQTHGESTLANKKGAWMDSTKATRYLVKTESWILRNPGYALVAAVIGWMLGSNTMQRVVFVV LLLVAPAYS
     1 SLTVQTHGESTLANKKGAWMDSTKATRYLVKTESWILRNPGYALVAAVIGWMLGSNTMQRVVFVVPLLLVAPAYS      75
                .         20        .         40        .         60        .

    % Identity =  98.7 (74/75)
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Claims

1. A recombinant Flavivirus comprising a mutation in the transmembrane domain of the membrane protein of the Flavivirus.

2. The Flavivirus of claim 1, wherein the mutation attenuates the Flavivirus.

3. The Flavivirus of claim 2, wherein the mutation decreases the viscerotropism/viremia of the Flavivirus.

4. The Flavivirus of claim 1, wherein the mutation results in increased stability of the Flavivirus, relative to a corresponding Flavivirus lacking the mutation.

5. The Flavivirus of claim 1, wherein the mutation results in increased virus replication in cells, relative to a corresponding Flavivirus lacking the mutation.

6. The Flavivirus of claim 1, wherein the Flavivirus is a chimeric Flavivirus.

7. The Flavivirus of claim 6, wherein the chimeric Flavivirus comprises the capsid and non-structural proteins of a first Flavivirus and the membrane and/or envelope proteins of a second Flavivirus.

8. The Flavivirus of claim 7, wherein the first Flavivirus is a yellow fever virus.

9. The Flavivirus of claim 8, wherein the yellow fever virus is YF-17D.

10. The Flavivirus of claim 7, wherein the second Flavivirus is a Japanese encephalitis virus.

11. The Flavivirus of claim 7, wherein the second Flavivirus is a West Nile virus.

12. The Flavivirus of claim 7, wherein the second Flavivirus is selected from the group consisting of a dengue virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, and Tick-borne encephalitis virus.

13. The Flavivirus of claim 12, wherein the dengue virus is dengue-1, dengue-2, dengue-3, or dengue-4 virus.

14. The Flavivirus of claim 7, wherein the chimeric Flavivirus comprises the capsid and non-structural proteins of a yellow fever virus and the membrane and envelope proteins of a Japanese encephalitis virus.

15. The Flavivirus of claim 1, 7, or 14, wherein the mutation is a substitution in one or more amino acids corresponding to the region of amino acids 40-75 of the membrane helix within the membrane protein of a Japanese encephalitis virus or a West Nile virus.

16. The Flavivirus of claim 15, wherein the mutation is a substitution of an amino acid corresponding to amino acid 60 of the membrane protein of a Japanese encephalitis virus.

17. The Flavivirus of claim 16, wherein the mutation results in a substitution of arginine with cysteine at amino acid position 60 of the membrane protein.

18. The Flavivirus of claim 15, wherein the mutation is a substitution of an amino acid corresponding to amino acid position 66 of the membrane protein of a West Nile virus.

19. The Flavivirus of claim 18, wherein the mutation results in a substitution of leucine with proline at amino acid position 66 of the membrane protein.

20. The Flavivirus of claim 15, wherein the mutation is in one or more amino acids corresponding to those at positions 60, 61, 62, 63, 64, 65, or 66 of the membrane protein of Japanese encephalitis virus or West Nile virus.

21. The Flavivirus of claim 1, 7, or 14, further comprising a mutation in the ectodomain of the membrane protein.

22. The Flavivirus of claim 21, wherein the ectodomain mutation is in an amino acid selected from the group consisting of amino acids 1-5 of the ectodomain.

23. The Flavivirus of claim 22, wherein the ectodomain mutation is a substitution in amino acid 5 of the ectodomain.

24. The Flavivirus of claim 23, wherein the ectodomain mutation is a substitution of glutamine with proline.

25. The Flavivirus of claim 1, 7 or 14, wherein the Flavivirus further comprises one or more envelope protein mutations in residues corresponding to West Nile virus envelope protein amino acids selected from the group consisting of amino acids 107, 138, 176, 177, 224, 264, 280, 316, and 440.

26. The Flavivirus of claim 25, wherein the Flavivirus comprises envelope protein mutations in residues corresponding to West Nile virus envelope protein amino acids 107, 316, and 440.

27. The Flavivirus of claim 25, wherein the Flavivirus comprises mutations at residues corresponding to West Nile virus position 66 of the membrane protein and positions 107, 316, and 440 of the envelope protein.

28. The Flavivirus of claim 1, 7 or 14, further comprising a mutation in the hydrophobic pocket of the hinge region of the envelope protein of the Flavivirus.

29. The Flavivirus of claim 28, wherein the hinge region mutation is present in an amino acid corresponding to amino acid 204 of the dengue 1 virus envelope protein.

30. The Flavivirus of claim 28, wherein the hinge region mutation is in one or more hinge region amino acids corresponding to yellow fever virus envelope protein amino acids 48-61, 127-131, and 196-283.

31. The Flavivirus of claim 1, 7, or 14, further comprising an attenuating mutation in the 3'-untranslated region of the Flavivirus.

32. The Flavivirus of claim 1, 7, or 14, further comprising an attenuating mutation in the capsid protein of the Flavivirus.

33. A vaccine composition comprising the Flavivirus of claim 1, 7, or 14, and a pharmaceutically acceptable carrier or diluent.

34. A use of the vaccine composition of claim 33 for the manufacture of a medicament for inducing an immune response to a Flavivirus in a patient.

35. The use of claim 34, wherein the patient does not have, but is at risk of developing, infection by the Flavivirus.

36. The use of claim 34, wherein the patient is infected by the Flavivirus.

37. A method of producing a vaccine against a Flavivirus comprising a recombinant Flavivirus, the method comprising introducing a mutation into the transmembrane domain of the membrane protein of the Flavivirus.

38. The method of claim 37, wherein the mutation attenuates the Flavivirus, relative to a corresponding Flavivirus lacking the mutation.

39. The method of claim 37, wherein the mutation results in increased stability of the Flavivirus, relative to a corresponding Flavivirus lacking the mutation.

40. The method of claim 37, wherein the mutation results in increased replication of the Flavivirus, relative to a corresponding Flavivirus lacking the mutation.

41. The method of claim 37, wherein the Flavivirus is a chimeric Flavivirus.

42. A nucleic acid molecule corresponding to the genome of the Flavivirus of claim 1 or the complement thereof.

43. A method of manufacturing the Flavivirus of claim 1, 7, or 14, the method comprising introducing a nucleic acid molecule corresponding to the genome of the Flavivirus into cells and isolating Flavivirus produced in the cells from the cells or the supernatant thereof.

44. The method of claim 43, wherein the cells are Vero cells.

45. The method of claim 43, wherein the cells are cultured in serum free medium.

46. The vaccine composition of claim 33, wherein said vaccine composition comprises a chimeric Flavivirus, and wherein the chimeric Flavivirus comprises the capsid and non-structural proteins of a first Flavivirus and the membrane and/or envelope proteins of a second Flavivirus.

47. The vaccine composition of claim 33 or 46, wherein the mutation is a substitution in one or more amino acids corresponding to the region of amino acids 40-75 of the membrane helix within the membrane protein of a Japanese encephalitis virus or a West Nile virus.

48. The vaccine composition of claim 47, wherein the mutation is a substitution of an amino acid corresponding to amino acid 60 of the membrane protein of a Japanese encephalitis virus.

49. The vaccine composition of claim 48, wherein the mutation results in a substitution of arginine with cysteine at amino acid position 60 of the membrane protein.

50. The use of claim 34, wherein said vaccine composition is the vaccine composition of claim 47.

51. The use of claim 34, wherein said vaccine composition is the vaccine composition of claim 48.

52. The use of claim 34, wherein said vaccine composition is the vaccine composition of claim 49.