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(54) Benævnelse: **VARIANT-FLAVIVIRUS-KAPPESEKVENSER OG ANVENDELSER DERAF**

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DESCRIPTION

Technical Field

[0001] The invention relates to nucleic acid and protein variants of the wild-type E proteins of Flaviviruses (e.g., a dengue or Zika virus) and binding molecules, such as complementary nucleic acids or antigen-binding molecules, e.g., antibodies, specific thereto, as well as to compositions, such as therapeutic, prophylactic or diagnostic compositions, kits, kit-of-parts, methods and uses relating thereto, in particular for diagnosis of Flavivirus infection and for vaccines to immunise against Flavivirus infection.

Background Art

[0002] The Flaviviridae are a family of positive, single-stranded, enveloped RNA viruses. They are found in arthropods, (primarily ticks and mosquitoes), and can infect humans. Members of this family belong to a single genus, Flavivirus, and cause widespread morbidity and mortality throughout the world. Some of the mosquito-transmitted viruses include: Dengue Fever, Zika virus, Yellow Fever, Japanese encephalitis and West Nile viruses. Other Flaviviruses are transmitted by ticks and are responsible of encephalitis and hemorrhagic diseases: Tick-borne Encephalitis (TBE), Kyasanur Forest Disease (KFD) and Alkhurma disease, and Omsk hemorrhagic fever.

[0003] Flaviviruses are small spherical virions encoding ten viral proteins: three structural (capsid, precursor membrane/membrane, and envelope (E)) and seven nonstructural proteins. The E protein has important roles in viral attachment to cells, fusion with endosomal compartments, and modulating host immune responses. The ectodomain of the virus E protein folds into three structurally distinct domains (DI, DII, and DIII) forming head-to-tail homodimers on the surface of the virion. DI is the central domain that organizes the entire E protein structure. DII is formed from two extended loops projecting from DI and lies in a pocket at the DI and DIII interface of the adjacent E protein in the dimer. At the distal end of DII is a glycine-rich, hydrophobic sequence called the fusion loop, which encompasses residues 98-110, and is highly conserved among flaviviruses. This region has been implicated in the pH-dependent type II fusion event; during this process it becomes exposed and reoriented outward, making it available for membrane contact. DIII forms a seven-stranded Ig-like fold, is the most membrane distal domain in the mature virion, and has been suggested to be involved in receptor binding. A stem region links the ectodomain to a two-helix C-terminal transmembrane anchor that is important for virion assembly and fusion.

[0004] Dengue disease is a mosquito-borne viral infection caused by dengue virus (DENV), one of the most important human pathogens worldwide. The infection produces a systemic disease with a broad spectrum of outcomes, ranging from non-symptomatic/mild febrile illness (Dengue Fever, DF) to severe plasma leakage and haemorrhagic manifestations (Dengue Haemorrhagic Fever, DHF) that can further evolve into potentially fatal conditions (Dengue Shock Syndrome, DSS). DENV, is spread by *Aedes* spp. mosquitoes and is widely distributed throughout the tropical and subtropical regions of the world. About 3 billion people, in over 100 countries, are estimated to be

at risk of infection, with over 300 million infections, 500,000 episodes of DHF manifestations and 20,000 deaths reported each year. The spread and impact of Dengue disease has led the World Health Organization to classify it as the "most important mosquito-borne viral disease in the world".

[0005] Four different serotypes of dengue viruses (DENV1, DENV2, DENV3 and DENV4) have been identified to date; each serotype is pathogenic in humans. Infection with any one serotype induces lifelong immunity against that specific serotype, with only transient cross-protection against the three other serotypes. Severe manifestations of dengue infection are associated with secondary infections involving different viral serotypes; this happens through a mechanism known as antibody-dependent enhancement of infection (ADE). In ADE, recognition of viral particles by cross-reacting, but weakly or non-neutralising antibodies, leads to an increased Fc receptor-mediated uptake of immature or incompletely neutralised viruses by monocytes, macrophages and dendritic cells (the primary targets of dengue virus infections in humans) resulting in increased infectivity and deterioration of the patient's clinical condition. ADE is a critical consideration in dengue vaccine development, because an immunogen that does not elicit fully-neutralising antibodies to all four serotypes may contribute to disease, rather than prevent infection. Given the lack of efficient treatment against the infection and the risk to human health, there is a need to develop an efficient vaccine that provides a protective response without the potential to cause antibody-dependent enhancement.

[0006] One dengue vaccine has been licensed, Dengvaxia® (CYD-TDV), developed by Sanofi Pasteur. Approximately five additional dengue vaccine candidates are in clinical development, with two candidates (developed by Butantan and Takeda) expected to begin Phase III trials in early 2016.

[0007] In clinical trials, the Dengvaxia® vaccine was found to increase risk of hospitalization due to dengue haemorrhagic fever (the very disease it is meant to prevent) in young children (<5 years). As a result, Dengvaxia® vaccine has a limited license, *i.e.*, only for persons of 9 years of age and above. Given the antigenic cross-reactivity of Zika and dengue, there is concern that vaccination with Dengvaxia® vaccine and other dengue vaccines under development may promote ADE of Zika virus, increasing the incidence of Guillain-Barre' syndrome in adults and microcephaly in infants, and that vaccines in development against Zika may likewise increase risk of dengue haemorrhagic fever, as does Dengvaxia in some subjects.

[0008] Zika virus is a mosquito-borne flavivirus that was first identified in Uganda in 1947 in monkeys, it was later identified in humans in 1952 in Uganda and the United Republic of Tanzania. Outbreaks of Zika virus disease have been recorded in Africa, the Americas, Asia and the Pacific. From the 1960s to 1980s, human infections were found across Africa and Asia, typically accompanied by mild illness. The symptoms are similar to infections such as dengue, and include fever, skin rashes, conjunctivitis, muscle and joint pain, malaise, and headache. These symptoms are usually mild and last for 2-7 days. However, Zika virus infection may cause complications in some subjects. Zika virus infection during pregnancy has been recognised as a cause of congenital brain abnormalities, including microcephaly. Zika virus is a trigger of Guillain-Barre syndrome. Links between Zika virus and a range of neurological disorders are being investigated.

[0009] Sanofi reported in 2016 its collaboration with the Walter Reed Army Institute of Research

(WRAIR) in the United States and Fiocruz public health center in Brazil to develop a Zika vaccine and reported in 2016 that immunization with a plasmid DNA vaccine or a purified inactivated virus vaccine provided complete protection in susceptible mice against challenge with a strain of Zika virus involved in an outbreak in northeast Brazil (Larocca et al., 2016 Nature 536, 474-478 (25 August 2016)

[0010] However, plasmid DNA vaccination in man requires 'gene gun' or similar technology (e.g., electroporation) for delivery and this approach is not considered to provide a global solution to the problems of dengue and Zika. Also, both the DNA vaccine and inactivated virus vaccine approaches in development contain dengue-Zika cross-reactive epitopes implicated in the causation of ADE.

[0011] After infection, or vaccination, the body's immune system produces neutralizing antibodies that bind to the surface proteins of a virus to block infection. Antibody-dependent enhancement (ADE) occurs when antibodies elicited by one virus can bind to, but do not block (neutralise) the infection of a similar virus.

[0012] ADE is most commonly observed for dengue virus. The 4 known serotypes of dengue virus have distinct, but related surface proteins. Infection with a first dengue virus serotype typically results in mild, or no, symptoms in the infected subject. If the subject is infected subsequently with a second dengue serotype, the immune system will produce antibodies to the first serotype that bind to the second serotype of virus, but will not always block infection and which have the potential to cause ADE. As a result there is antibody-mediated uptake of virus into cells that dengue virus does not normally infect *(i.e.,* cells having receptors for the 'tail' or Fc region of the antibody). This can result in a more severe form of disease such as dengue hemorrhagic fever or dengue shock syndrome. Only young infants develop dengue haemorrhagic fever upon a first exposure to dengue, as a result of transplacentally transmitted maternal anti-dengue antibodies. As such, antibodies are equal partners with virus in (severe) disease causation in adults and infants alike.

[0013] Dengue virus antibodies not only promote ADE of other dengue virus serotypes, but also enhance Zika virus infection. Dejnirattisai et al., (2016) Nature Immunology 17, 1102-1108. "Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with Zika virus". Dejnirattisai *et al.* tested the effect of dengue neutralizing antibodies or serum from dengue virus patients on Zika virus in cell culture. In the absence of antibody, Zika virus poorly infected the cells, but when Zika virus was incubated with dengue serum or neutralizing antibodies, Zika virus robustly infected these cells, indicating the operation of ADE. The physiological relevance of this finding requires confirmation in epidemiological studies, but these findings pose an obvious risk for current vaccine approaches. To date no satisfactory solution to this problem has been conceived or advocated.

[0014] While vaccines in this field may transpire to have net benefit on a population basis, on an individual basis the picture is different. In some subjects, tragically, preventing one disease may increase the severity or risk of mortality from another. Paul LM et al. Clinical & Translational Immunology (2016) 5, e117 "Dengue virus antibodies enhance Zika virus infection" have reported that:
"For decades, human infections with Zika virus (ZIKV), a mosquito-transmitted Flavivirus, were

sporadic, associated with mild disease, and went underreported since symptoms were similar to other acute febrile diseases. Recent reports of severe disease associated with ZIKV have greatly heightened awareness. It is anticipated that ZIKV will continue to spread in the Americas and globally where competent *Aedes* mosquito vectors are found. Dengue virus (DENV), the most common mosquito-transmitted human flavivirus, is both well-established and the source of outbreaks in areas of recent ZIKV introduction. DENV and ZIKV are closely related, resulting in substantial antigenic overlap. Through antibody-dependent enhancement (ADE), anti-DENV antibodies can enhance the infectivity of DENV for certain classes of immune cells, causing increased viral production that correlates with severe disease outcomes. Similarly, ZIKV has been shown to undergo ADE in response to antibodies generated by other flaviviruses. We tested the neutralizing and enhancing potential of well-characterized broadly neutralizing human anti-DENV monoclonal antibodies (HMAbs) and human DENV immune sera against ZIKV using neutralization and ADE assays. We show that anti-DENV HMAbs, cross-react, do not neutralize, and greatly enhance ZIKV infection *in vitro.* DENV immune sera had varying degrees of neutralization against ZIKV and similarly enhanced ZIKV infection. Our results suggest that pre-existing DENV immunity may enhance ZIKV infection *in vivo* and may lead to increased disease severity. Understanding the interplay between ZIKV and DENV will be critical in informing public health responses and will be particularly valuable for ZIKV and DENV vaccine design and implementation strategies."

[0015] Dengue virus antibodies can promote ADE of Zika virus. Zika virus antibodies can promote ADE of dengue virus. Thus, immunization against Zika virus could increase the incidence of dengue hemorrhagic fever or dengue shock syndrome, or foster the development of these conditions in individuals that would not otherwise have developed them, but for immunisation. Given the interval between infections, which can be several years, it will be years before post-marketing surveillance studies are able to inform if, and to what extent, new vaccines predispose to severe dengue disease (haemorrhagic fever, shock syndrome) or severe Zika sequelae, such as Guillain Barre' syndrome or microcephaly.

[0016] Accordingly, there is a clear need for vaccine approaches that are designed purposefully to avoid the problem of antibody-dependent enhancement.

[0017] Specific diagnosis of Flavivirus infections using current serological testing is complicated by the cross-reactivity between antibodies against other clinically-relevant flaviviruses. Cross-reactivity is particularly problematic in areas where different flaviviruses co-circulate or in populations that have been immunized with vaccines to Flaviviruses. The majority of cross-reactive antibodies are raised against the immunodominant flavivirus envelope (E) protein target a conserved epitope in the fusion loop at the distal end of domain II.

[0018] There is a need for a diagnostic approach that can differentiate between closely-related Flaviviruses, to assess if an individual is seronegative and thus has not been exposed to dengue or Zika, or if an individual is seropositive and has been exposed to Zika and / or dengue and for those who are seropositive, to distinguish to which of Zika and / or the four dengue serotypes the individual has been exposed. There is a need for a diagnostic approach that can be used to select subjects for immunization, or assess seroconversion to determine if immunization has raised a protective immune response against dengue or Zika. There is thus a need for diagnostic approaches that enable interrogation of the immune response to distinguish antibodies against the

dengue virus serotypes and against Zika virus.

[0019] WO2016012800 discloses identification and characterisation of cross-reactive neutralising antibodies obtained from patients infected with dengue virus. The acute human antibody response was found to be focused on two major epitopes; a known epitope on the fusion loop (FL FLE), and a second epitope, said to be novel, which was found on intact virions or dimers of envelope protein and which encompassed areas of domains I, II and III. Antibodies reactive with the second epitope, the Envelope Dimer Epitope, or EDE, were reported to fully neutralise virus made in both insect and primary human cells in the low picomolar range. A subunit vaccine comprising a stabilized soluble protein E dimer was therefore proposed as a dengue vaccine. WO2016012800 discloses that a dengue virus envelope glycoprotein E ectodomain (sE; soluble envelope polypeptide/glycoprotein) refers to the 1-395 amino acid fragment of the envelope glycoprotein E of the dengue virus serotypes 1, 2 and 4, and to the 1-393 amino acid fragment of the envelope glycoprotein E of the dengue virus serotype 3. WO2016012800 described the EDE as a stabilised dimer of sE, selected from DENV-1 sE, DENV-2 sE, DENV-3 sE, DENV-4 sE and mutant sE thereof having at least one mutation (substitution) selected among H27F, H27W, L107C, F108C, H244F, H244W, S255C, A259C, T/S262C, T/A265C, L278F, L292F, L294N, A313C (S313C in DEN3) and T315C, which mutations are considered to contribute to increased stability in the dimer configuration. It is disclosed that mutant sE thereof may further comprise at least one mutation (substitution) selected from Q227N, E174N and D329N; preferably all three mutations Q227N, E174N and D329N, which mutations are said to mask non-appropriate immunogenic regions and allow the stabilized recombinant sE dimer of the invention to preferentially elicit neutralizing antibodies directed to all four dengue virus serotypes.

[0020] The sE dimer mutations described are said not to interfere with immunogenicity but to provide a higher dimer affinity, by including cysteine mutations at the dimer contacts to provide stabilization by cross-links, and/or by introduction of new glycosylation sites to allow chemical cross-linking between adjacent sugars on the dimer by click chemistry, and/or by substitution of at least one amino acid residue in the amino acid sequence of at least one sE monomer with at least one bulky side chain amino acid to allow forming cavities at the dimer interface or in domain 1 (D1) / domain 3 (D3) linker of each monomer.

[0021] WO2016012800 discloses that the envelope protein may be engineered such that an improved EDE is generated, an EDE which is incapable of being recognised or raising anti-fusion loop (anti-FL) antibodies was considered to be an improved EDE. It is disclosed that such improvement may be accomplished by one or more mutations, deletions or insertions in the envelope protein, by generating a hybrid protein wherein the specific epitope (without any antigens which would raise anti-FL antibodies) is fused to a scaffold protein, or by engineering the envelope protein by modifying the internal surface of the dimer (projecting to the inside of the virus) with sugars to make it less immunogenic by adding N or O linked glycan sequences.

[0022] Roby et al., (2013, 2014) describe an approach to development of a vaccine candidates for West Nile virus by introduction of large internal deletions within the capsid (C) gene of flavivirus genomes to generate replication-competent RNAs that are unable to be packaged into virions, yet maintain secretion of highly immunogenic subviral particles (SVPs) without generating infectious virus. Such pseudoinfectious C-deleted vaccines are able to replicate and secrete large amounts of

non-infectious immunogenic subviral particles (SVPs) from transfected cells and thus are said to offer the combined benefit of the safety of noninfectious inactivated or subunit vaccines with the robust immune response generated by the replication of live vaccines.

[0023] Roby et al., (2013) generated a construct, pKUNdC/C (KUNdC18-100/CMV-C), with C-deleted CMV-promoter driven cDNA of West Nile virus Kunjin (KUNV) in which alpha helices 1, 2, and 4 were removed in two separate segments and the hydrophilic alpha helix 3 was maintained. In pKUNdC/C C-deleted WNV cDNA was placed under the control of one copy of the cytomegalovirus (CMV) promoter and the C gene was placed under the control of a second copy of the CMV promoter in the same plasmid DNA. The conservation of the larger cytosolic moiety (alpha helix 3) led to a significant improvement in SVP secretion compared to that of constructs with deletions of all alpha helices of C and dC44-59. Additional improvements to SVP secretion were also observed upon the incorporation of an Asn-linked glycosylation motif at N154 of the E protein, a feature of many circulating strains of WNV and recent isolates of KUNV, corresponding to an NYS motif at amino acids 154 to 156 of the E protein. pKUNdC/C was shown to generate single-round infectious particles (SRIPs) capable of delivering self-replicating C-deleted RNA producing SVPs to surrounding cells. However, the amounts of both SRIPs and SVPs produced from pKUNdC/C DNA were relatively low.

[0024] Roby et al., (2014) reported production of a second generation constructs with C-deleted cDNA of West Nile virus Kunjin (KUNV) in which the CMV promoter was replaced by a more powerful elongation factor EF1a promoter and different forms of C were used to attempt to increase SRIP production by optimizing trans-C expression. A construct containing an elongation factor EF1a promoter encoding an extended form of C was demonstrated to produce the highest titres of SRIPs and was immunogenic in mice. SRIP and SVP titres were further improved via incorporation of the N154 glycosylation motif in the envelope protein (corresponding to an NYS motif at amino acids 154 to 156 of the E protein) which enhanced secretion of SVPs.

[0025] Davis et al., (2014) investigated the ability of West Nile virus (WNV) to infect CD209-expressing cells. Mammalian cell-derived West Nile virus preferentially infects cells expressing the C-type lectin CD209L but not cells expressing CD209; by contrast, Dengue virus (DENV) infection is enhanced in cells expressing either attachment factor. DENV and WNV virions have very similar structures. Their surfaces consist of a regular array of 180 envelope (E) protein subunits arranged in an icosahedral lattice (36). The small membrane (M) protein, generated following furin-mediated processing of pre-membrane protein (prM), is also present on the virion surface but is mostly buried in the viral membrane. The major structural differences between DENV and WNV virions stem from the number and location of *N*-linked glycosylation sites in the DENV viral E proteins. Most DENV isolates contain glycosylation sites at residues 67 and 153, although the site at 153 may not always be utilized; WNV E proteins only contain an *N*-linked glycan at asparagine 154, although this is absent in many virus strains. The presence of *N*-glycosylation on the WNV E protein has been linked in some studies to increased neuroinvasiveness in mice and to altered cellular tropism *in vitro.* Davis *et al.* introduced a glycosylation site at position 67 into West Nile virus E. Reporter virus particles pseudotyped with this E protein infected cells using either CD209 or CD209L. Glycosylation sites were introduced at several other positions. The WNV strain NY99 prM-E expression plasmid pCBWN and a derivative of this plasmid lacking the N-linked glycosylation site at E protein residue 154 (NY99-N154Q) were used as templates for the introduction of novel N-

linked glycosylation sites into the WNV E protein by site-directed mutagenesis. The following amino acid changes were introduced into NY99-N154Q: (i) Ala-54 to Thr (A54T) adds an N-linked glycosylation site at Asn-52; (ii) D67N adds a site at Asn-67; (iii) K84T adds a site at Asn-82; (iv) A173N and P174G (AP173NG) add a site at Asn-173; (v) Glu-182 to NGS (E182NGS) adds a site at Asn-182 by mutating Glu-182 to Asn and inserting two amino acids (Gly-Ser) to complete the sequon; (vi) S230N and V232T (STV230NTT) add a site at Asn-230; (vii) V279T adds a site at Asn-277; (viii) T301N and G303S (TYG301NYS) add a site at Asn-301; (ix) T330N adds a site at Asn-330; (x) K370T adds a site at Asn-368; (xi) G389N and Q391T (GEQ389NET) add a site at Asn-389. All sites allowed CD209Lmediated infection, but only a subset promoted CD209 use. As seen for other viruses, mannose-rich glycans on West Nile virus were required for its interactions with CD209, however, mannose-rich glycans were not required for CD209Lmediated infection. Complex glycans, particularly *N*-acetylglucosamine-terminated structures, were able to mediate reporter virus particle interactions with CD209L. Davis *et* al. proposed that that CD209L recognizes glycosylated flaviviruses with broad specificity, whereas CD209 is selective for flaviviruses bearing mannose-rich glycans and thus that the location of the *N*-linked glycosylation sites on a virion determines the types of glycans incorporated, thus controlling viral tropism for CD209-expressing cells.

Statement of Invention

[0026] The invention provides an isolated recombinant analogue of a flavivirus E-protein fusion loop comprising at least one glycosylation site for an N-linked glycan that is not present in a natural flavivirus E-protein fusion loop sequence, wherein the at least one glycosylation site is an N-linked glycosylation sequon (Asn-X-Ser/Thr) and the Asn (N) residue of the sequon may occupy any of positions 98-110 (SEQ ID NO: 1 DRGWGNGCGLFGK) of the natural flavivirus E-protein fusion loop amino acid sequence, wherein X is any amino acid residue except proline and Ser/Thr denotes a serine or threonine residue.

[0027] An isolated recombinant analogue of a flavivirus E-protein fusion loop according to the invention may comprise two glycosylation sites that are not present in a natural flavivirus E-protein fusion loop sequence.

[0028] The invention provides an isolated recombinant analogue of a flavivirus E-protein comprising an analogue of a flavivirus E-protein fusion loop of the invention. In some embodiments the only modifications to the sequence of the isolated recombinant analogue of a flavivirus E-protein are the modifications of the invention in the fusion loop to introduce N-linked glycosylation sequon(s) (Asn-X-Ser/Thr), in other embodiments one or more further modifications may be introduced in flavivirus E-protein at residues outside the fusion loop.

[0029] An analogue of the invention having at least one additional glycan attached thereto is provided. Preferably the at least one additional glycan is an N-linked glycan. Preferably an analogue of the invention is the product of expression of a recombinant DNA or RNA sequence. The at least one additional glycan may be present at one or more native glycosylation sites in the flavivirus E-protein outside the flavivirus E-protein fusion loop.

[0030] An analogue of the invention, may comprise an N-linked glycosylation sequon (Asn-X-Ser/Thr) such that an Asn (N) residue of the sequon occupies any of positions 98-101 and / or 106-110.

[0031] Preferably, in an analogue of the invention, X is any of the following 13 amino acid residues Gly, His, Asn, Gln, Tyr, Val, Ala, Met, Ile, Lys, Arg, Thr or Ser.

[0032] In preferred analogues of the invention, the flavivirus E-protein is a dengue virus E-protein and the Asn (N) residue of a sequon occupies position 101, 108 or both 101 and 108 of the amino-acid sequence of the analogue flavivirus E-protein fusion loop or the flavivirus E-protein is a Zika E-protein and the Asn (N) residue of a sequon occupies position 100 of the amino acid sequence of the analogue flavivirus E-protein fusion loop.

[0033] In a preferred analogue of the invention, the flavivirus is a dengue virus and the amino acid sequence of the analogue flavivirus E-protein fusion loop 98-110 is selected from: SEQ ID NO: 2 DRGNGSGCGLNGS, SEQ ID NO: 3 DRGNGSGCGLFGK and SEQ ID NO: 4 DRGWGNGCGLNGS.

[0034] In another preferred analogue of the invention, the flavivirus is a Zika virus and the amino acid sequence of the analogue flavivirus E-protein fusion loop 98-110 is SEQ ID NO: 5 DRNHTNGCGLFGK.

[0035] The invention further provides an isolated recombinant DNA or RNA sequence comprising a sequence encoding an analogue of a flavivirus E-protein fusion loop according to the invention.

[0036] An isolated recombinant DNA sequence may be a plasmid or a linear DNA-based vaccine. An isolated recombinant DNA sequence of the invention may encode an analogue of a flavivirus E-protein according to the invention under control of a mammalian promoter.

[0037] The invention yet further provides a host cell comprising a DNA or RNA sequence according to the invention. The host cell may be an eukaryotic host cell comprising a DNA sequence according to the invention or a plasmid or linear DNA-based vaccine immunogen according to the invention.

[0038] Preferably, a host cell of the invention is capable of expressing an analogue of the invention. Further preferably, a host cell of the invention is capable of expressing and glycosylating an analogue of the invention.

[0039] The invention provides a method of making an analogue of the invention comprising culturing a host cell according to the invention in conditions suitable for expression of the analogue and isolating the analogue.

[0040] Further provided is a composition comprising an analogue of the invention and a diluent.

[0041] A composition of the invention may be an immunogenic (vaccine) composition capable of inducing an immunological response in a subject inoculated with said composition, the composition comprising an analogue according to the invention together with a pharmaceutically acceptable

diluent, adjuvant and / or carrier.

[0042] A composition of the invention may comprise one or more flavivirus analogues of the invention selected from an analogue of DEN-1, an analogue of DEN-2, an analogue of DEN-3, an analogue of DEN-4 and an analogue of Zika.

[0043] A composition of the invention may comprise four dengue analogues of the invention representing each of the four dengue virus serotypes DEN-1 DEN-2 DEN-3 and DEN-4.

[0044] A composition of the invention may comprise a zika virus analogue of the invention.

[0045] A composition of the invention may comprise four dengue analogues of the invention representing each of the four dengue serotypes DEN-1 DEN-2 DEN-3 and DEN-4 and a zika virus analogue of the invention.

[0046] The invention also provides a binding molecule capable of binding specifically to an analogue of the invention. The binding molecule may be an antibody or a fragment thereof, a domain antibody, a protein scaffold, or an aptamer, provided that it is capable of binding specifically to an analogue of the invention.

[0047] The invention provides an analogue, composition or binding molecule of the invention for use as a medicament.

[0048] Further, the invention provides an analogue, composition or binding molecule of the invention for use as a vaccine.

[0049] Also provided is an analogue, composition or binding molecule of the invention for use as a medicament for the prophylactic or therapeutic treatment of a flavivirus infection or for use in the manufacture of a medicament for the prophylactic or therapeutic treatment of a flavivirus infection.

[0050] The invention provides a method for the protection of a subject against infection by a Flavivirus, comprising administering an analogue, composition of or binding molecule of the invention to said subject.

[0051] In preferred embodiments the flavivirus infections is a dengue virus infection or a Zika virus infection.

[0052] The invention provides an analogue, composition or binding molecule of the invention for use as a diagnostic.

[0053] The invention provides a diagnostic kit comprising an analogue, composition or binding molecule of the invention and a reagent capable of detecting an immunological (antigen-antibody) complex which contains said isolated analogue or binding molecule.

[0054] A diagnostic test kit in accordance with the invention may further comprise one or more control standards and / or a specimen diluent and/or washing buffer.

[0055] In a diagnostic test kit of the invention, the analogue and / or binding molecule specific thereto of the invention may be immobilized on a solid support. The solid support may be a microplate well. In a diagnostic test kit according to the invention, an immunological complex which contains said isolated analogue or binding molecule may be detected by ELISA or by lateral flow.

[0056] The invention provides vaccine approaches that are designed purposefully to avoid the problem of antibody-dependent enhancement.

[0057] The invention provides diagnostic approaches that can differentiate between closely-related Flaviviruses, to assess if an individual is seronegative and thus has not been exposed to dengue or Zika, or if an individual is seropositive and has been exposed to Zika and / or dengue and for those who are seropositive, to distinguish to which of Zika and / or the four dengue serotypes the individual has been exposed. The invention provides diagnostic approaches that can be used to select subjects for immunization, or assess seroconversion to determine if immunization has raised a protective immune response against dengue or Zika. The invention provides diagnostic approaches that enable interrogation of the immune response to distinguish antibodies against the dengue virus serotypes and against Zika virus.

Detailed Description of the Invention

[0058] The invention is be described with reference to various embodiments of different aspects of the invention. It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in one or more embodiments or in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments are specifically embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0059] The invention provides modified Flavivirus nucleic acid and protein sequences in which the natural (native, wild-type) E-protein fusion loop epitope, known to be associated with generation of flavivirus cross-reactive, infection-enhancing antibodies has been modified to comprise one or more (e.g., 2) glycosylation sites for glycosylation of the protein with an N-linked glycan that is not normally present on the native fusion loop epitope. Such modification alters the fusion loop amino acid sequence and the presence of a glycan further disguises the epitope. Thus the modified Flavivirus nucleic acid and protein sequences of the invention are designed to generate a protective response without concomitant generation of flavivirus cross-reactive infection-enhancing antibodies, thereby intending to avoid the problems of antibody-dependent enhancement observed with existing vaccine approaches. The modified Flavivirus nucleic acid and protein sequences of the invention are also designed for diagnostic use, either as antigens for detection of a specific Flavivirus or to generate binding molecules such as antibodies for detection of a specific Flavivirus.

[0060] By antibody we include the meaning of a substantially intact antibody molecule, as well as a chimeric antibody, humanised antibody (wherein at least one amino acid is mutated relative to a non-human antibody , for example a naturally occurring non-human antibody or antibody assembled from non-human antibody sequences), single chain antibody, bi-specific antibody, antibody heavy chain, antibody light chain, homo-dimer or heterodimer of antibody heavy and/or light chains, and antigen binding portions and derivatives of the same. When the compound is a protein, for example an antibody or fragment thereof is administered to a human subject and if the antibody is not a human antibody or fragment thereof, then it can be humanized in order to reduce immunogenicity in human. Methods for producing humanized antibodies or fragments thereof are known in the art.

[0061] A binding molecule of the invention is preferably an antibody or antigen binding portion thereof. The antigen binding portion may be a Fv fragment; a Fab-like fragment (e.g. a Fab fragment, a Fab' fragment, a F(ab)2fragment, Fv or scFv fragments); or a domain antibody. The antibody binding portion may be derived from the linear amino acid sequence present in an intact antibody, or may comprise a set of non-consecutive amino acids, optionally interspersed with other amino acids, for example may comprise particular amino acids that are required for contact with an epitope, but may for example not comprise the amino acids required for the framework of a native antibody, which, in some cases, may be replaced by a heterologous scaffold protein, for example. An antibody according to the present invention is obtainable by a method comprising a step of immunizing a mammal, such as a human, a monkey, a rabbit or a mouse; and/or by an in vitro method, for example comprising a phage display selection step, as will be well known to those skilled in the art.

[0062] The term antibody also includes all classes of antibodies, including IgG, IgA, IgM, IdD and IgE. The term antibody also includes variants, fusions and derivatives of any defined antibodies and antigen binding portions thereof.

[0063] By neutralise we mean reduce the ability of the virus to infect previously uninfected cells. The person skilled in the art will be well aware of suitable techniques to monitor viral neutralising ability.

[0064] Methods for manipulation of nucleic acid sequences to introduce sequence changes as described herein are well known in the art.

Table 1. Alignment of amino acids 98-110 of a group of wild-type sequences of flaviviruses and recombinant analogue sequences of the invention.

1	ZIKV_H/PF/2013	**DRGWGNGCGLFGK(SEQ ID NO: 1)**
2	ZIKV_MR766	**DRGWGNGCGLFGK(SEQ ID NO: 1)**
3	DENV_1_SG/07K3640DK1/2008	**DRGWGNGCGLFGK**(SEQ ID NO: 1)
4	DENV_2_16681	**DRGWGNGCGLFGK**(SEQ ID NO: 1)
5	DENV_3_SG/05K863DK1/2005	**DRGWGNGCGLFGK**(SEQ ID NO: 1)

6	DENV_4_SG/06K2270DK1/2005	**DRGWGNGCGLFGK**(SEQ ID NO: 1)
7	WNV_NY99	**DRGWGNGCGLFGK**(SEQ ID NO: 1)
8	JEV_SA14	**DRGWGNGCGLFGK**(SEQ ID NO: 1)
9	YFV_Asibi	**DRGWGNGCGLFGK**(SEQ ID NO: 1)
10	pCR021 (dengue-1 HX)	DRG**N**GSGCGL**N**G**S**(SEQ ID NO: 2)
11	pCR022 (dengue-2 HX)	DRG**N**G**S**GCGL**N**G**S**(SEQ ID NO: 2)
12	pCR023 (dengue-3 HX)	DRG**N**G**S**GCGL**N**G**S**(SEQ ID NO: 2)
13	pCR024 (dengue-4 HX)	DRG**N**G**S**GCGL**N**G**S**(SEQ ID NO: 2)
14	pCR028 (Zika HX)	DR**NHT**NGCGLFGK(SEQ ID NO: 5)
15	pCR026 (dengue-1 HX)	DRG**N**G**S**GCGLFGK (SEQ ID NO: 3)
16	pCR027 (dengue-1 HX)	DRGWGNGCGL**N**G**S**(SEQ ID NO: 2)
17	pCR025 (Zika)	DRG**N**G**S**GCGL**N**G**S**(SEQ ID NO: 2)
18	pCR029 (Zika)	DRGWGNGCG**N**H**T**K(SEQ ID NO: 6)
19	pCR030 (Zika)	DRG**N**G**S**GCGLFGK(SEQ ID NO: 3)
20	pCR031 (Zika)	DRGWGNGCGL**N**G**S**(SEQ ID NO: 2)

[0065] The fusion loop DRGWGNGCGLFGK (defined as residues 98-110, SEQ ID NO: 1) in the wild type sequences (rows 1 to 9) is shown in bold. The residues changed to make the N-linked glycosylation sequons in the modified analogue HX sequences are shown in bold in rows 10-20The constructs pCRO21-24, 26, and 28 expressed well and were selected for further investigation. In the case of dengue E-proteins, 4 residues were changed to make two glycosylation sites (pCRO21-24). In the case of Zika E-protein, 3 residues were changed to make one glycosylation site (pCRO28).

[0066] The constructs pCRO25, 29, 30 and 31 did not express well in the expression system chosen, thus in some contexts the recombinant analogue sequences of the invention do not comprise the following sequences:

pCRO25 CKRTLVDRGNGSGCGLNGSGSLVTCAKFA (SEQ ID NO: 7)

pCRO29 CKRTLVDRGWGNGCGNHTKGSLVTCAKFA (SEQ ID NO: 8)

pCRO30 CKRTLVDRGNGSGCGLFGKGSLVTCAKFA (SEQ ID NO: 9)

pCRO31 CKRTLVDRGWGNGCGLNGSGSLVTCAKFA (SEQ ID NO: 10).

[0067] In an analogue of the invention, the N-linked glycosylation sequon (Asn-X-Ser/Thr) may be present such that an Asn (N) residue of the sequon occupies any of positions 98-101 and / or 106-110. That is, the N residue may occupy position a position selected from 98, 99, 100, and 101 and / or a position selected from 106, 107, 108, 109 and 110.

[0068] Preferably, in an analogue of the invention, X is any of the following 13 amino acid residues Gly, His, Asn, Gln, Tyr, Val, Ala, Met, Ile, Lys, Arg, Thr or Ser, with Gly or His being particularly preferred. In specific embodiments of the invention described herein for dengue viruses it is preferred that X is Gly and for Zika is preferred that X is His.

[0069] In preferred analogues of the invention, the flavivirus E-protein is a dengue virus E-protein and the Asn (N) residue of a sequon occupies position 101, 108 or both 101 and 108 of the amino-acid sequence of the analogue flavivirus E-protein fusion loop or the flavivirus E-protein is a Zika E-protein and the Asn (N) residue of a sequon occupies position 100 of the amino acid sequence of the analogue flavivirus E-protein fusion loop.

[0070] In a preferred analogue of the invention, the flavivirus is a dengue virus and the amino acid sequence of the analogue flavivirus E-protein fusion loop 98-110 is selected from: DRGNGSGCGLNGS (SEQ ID NO: 2), DRGNGSGCGLFGK (SEQ ID NO: 3) and DRGWGNGCGLNGS (SEQ ID NO: 4).

[0071] In another preferred analogue of the invention, the flavivirus is a Zika virus and the amino acid sequence of the analogue flavivirus E-protein fusion loop 98-110 is DRNHTNGCGLFGK (SEQ ID NO: 5).

[0072] The nucleic acid sequence encoding recombinant analogue E-protein fusion loop protein or encoding recombinant analogue E-protein comprising such fusion loop protein can be generally be expressed following the functional and operable insertion of the DNA sequence into an expression vector containing control sequences and secretory signal sequences.

[0073] A suitable promoter for expression of nucleic acid sequences of the invention is CMV.

[0074] Host cells that may be employed in accordance with the invention include HEK and CHO cell lines. The host may be genetically engineered to produce therapeutic glycoproteins with human-like *N*-linked glycans.

[0075] The immunogenic composition of the invention may be administered with or without adjuvant. Adjuvants can be added directly to the immunogenic composition or can be administered

separately, either concurrently with or shortly after, administration of the vaccine. Such adjuvants include but are not limited to aluminium salts (aluminium hydroxide), oil-in-water emulsion formulations with or without specific stimulating agents such as muramyl peptides, saponin adjuvants, cytokines, detoxified mutants of bacteria toxins such as the cholera toxin, the pertussis toxin, or the E. coli heat-labile toxin.

[0076] The immunogenic composition of the invention may be administered with other immunogens or immunoregulatory agents, for example, immunoglobulins, cytokines, lymphokines and chemokines.

[0077] In specific embodiments described herein the adjuvant used was Alhydrogel®, which is an acceptable adjuvant for human and veterinary use. However it should be apparent to a person skilled in the art that other suitable adjuvants and adjuvantation and formulation strategies are available for either (or both) nucleic acid and protein forms of the antigens. Alhydrogel requires proteins to be negatively charged at neutral or near-neutral pH values (eg. pH 7.4) in order to be maximally effective. This is because Alhydrogel has a net positive charge under such conditions of pH. Aluminium phosphate, conversely has a net negative charge and is generally better for proteins that are positively charged under physiological conditions of pH used for vaccine formulation. If proteins have a near neutral isolectric point they may not bind well to Alhydrogel or aluminium phosphate adjuvants, limiting the adjuvant effect, and would benefit from other adjuvantation strategies.

[0078] For example vaccine adjuvants based on oil-in-water emulsions or liposome suspensions have made considerable progress in licensed vaccine products and in clinical trials recently (Alving, Beck, Matyas, & Rao, 2016). These adjuvant materials exploit either natural or synthetic versions of monophosphoryl lipid-A, with and without other adjuvant materials such as QS21 saponin and CpG adjuvant. Such strategies have allowed the development of a highly efficacious vaccine against shingles and a promising malaria vaccine candidate (after 30 years of research) which is expected to be licensed soon.

[0079] Other promising delivery and adjuvantation strategies have been developed, e.g. Virosomes, which may be suitable for use with the glycosylated exodomain proteins of the present disclosure. Likewise there are promising adjuvant materials and strategies in earlier stages of development such as CD40 agonistic antibodies as stand-alone, conjugate or liposomal vaccine components (Hatzifoti C, Bacon A, Marriott H, Laing P, Heath AW (2008) Liposomal Co-Entrapment of CD40mAb Induces Enhanced IgG Responses against Bacterial Polysaccharide and Protein. PLOS ONE 3(6): e2368). Compositions of the invention may be used in co-delivery strategies for administration of protein and DNA vaccines, such as by liposomal formulation (Laing et al., 2006).

[0080] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, Fritsch, and Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989), Oligonucleotide Synthesis (M. J. Gait Ed., 1984), Animal Cell Culture (R. I. Freshhey, Ed., 1987), the series Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells (J. M. Miller and M. P. Calos eds. 1987), Handbook of Experimental Immunology, (D. M. Weir and C. C.

Blackwell, Eds.), Current Protocols in Molecular Biology (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Siedman, J. A. Smith, and K. Struhl, eds., 1987), and Current Protocols in Immunology (J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach and W. Strober, eds., 1991).

[0081] Standard three and one-letter terminology is used for amino acid residues.

[0082] As used herein, the term "recombinant" refers to the use of genetic engineering methods (cloning, amplification) to produce an analogue, or a binding molecule such as an antibody or an antibody fragment of the present invention.

[0083] The principal problem of dengue vaccine development, wherein the use of vaccines runs the risk (in a finite number of cases) of giving rise to 'antibody dependent enhancement' of dengue infection, making the illness worse rather than preventing it. The application relates quite generally to flavivirus vaccines, because it applies to highly conserved sequences of the envelope protein 'E' of this family of viruses. Enhancement is a feature of natural infection (where antibodies sent to neutralize the virus are subverted to gain access to human myeloid cells), usually upon encounter with a second 'serotype' of virus, resulting in more severe symptoms (Halstead, Rojanasuphot, & Sangkawibha, 1983). Vaccination, while for the most part conferring protection, is also liable on some occasions to predispose a recipient to severe dengue, including dengue haemorrhagic fever (DHF), upon first exposure to a wild dengue virus: *i.e.*, 'iatrogenic' cases of severe dengue or DHF, which would not have occurred but for the vaccine. Furthermore, existing vaccine approaches also have the potential to create a population of vaccinated individuals who develop severe iatrogenic dengue, at some interval after the vaccine (or vaccine course) has been administered (e.g. a decade). This is because, as immunity to dengue wanes, protective antibodies reach a concentration where they 'enhance' rather than prevent infection. Also, the rate of decay of 'immunological memory' (where the immune system recalls encounter with a wild virus or vaccine dose) is not synchronous for the four serotypes of the vaccine, such that immunity to each serotype (at the antibody and memory level) of dengue is lost at different times, successively increasing the risk of severe disease. This gradual failure of immune memory likewise creates a new population of individuals who are now predisposed to severe dengue (when bitten by an infected mosquito), instead of protected, as a result of previous vaccination. The solution is to make a vaccine that has zero or minimal propensity to give rise to 'antibody dependent enhancement', while preserving efficacy, in a manner amenable to incorporation into several of the various vaccine formats now in existence (live vector, DNA vaccine, oral vaccine, subunit vaccine, virus-like particles etc.). The invention of the present application avoids cases of vaccine-induced enhancement of disease by dengue and/or Zika vaccines by creating novel immunogens that fail to produce antibodies that facilitate infection. This is achieved by introducing one or more additional glycosylation sites (e.g. N-linked glycosylation sites) into particular site(s) of recombinantly expressed E-proteins of dengue and Zika viruses that are particularly associated with the generation of infection-enhancing antibodies, thereby cloaking such sites, and preventing them from generating antibodies following vaccination.

[0084] While current vaccines against dengue (licensed and in development) may meanwhile prove to be of substantial 'net' benefit to public health, improved safety is still desirable in order to avoid cases of vaccine-induced dengue (*i.e.*, iatrogenically-caused severe dengue). The likely role of

natural dengue infection in paving the way for pandemic Zika infection has been elaborated recently by Philip K Russell of the Sabin Vaccine Institute (Russell, 2016). While no systematic investigation has been conducted that would determine the risk of dengue vaccination predisposing to Zika virus infection or of dengue vaccination giving rise to Zika infections of enhanced severity, it is a logical extension of Russell's observations to expect such cases. Likewise although dengue-vaccine-induced predisposition to severe dengue has not yet been reported or investigated 'as such', in a recent three-year follow-up study of the Sanofi-Pasteur vaccine there was an increased rate of hospitalisation in children less than nine years of age (Hadinegoro et al., 2015) which could be explained by vaccine-induced enhancement of susceptibility to severe dengue. These new epidemiological developments, and laboratory data (below) indicate that there is a significant risk that vaccines (unless designed to avoid enhancement) will cause, in some instances, enhancement of disease: i.e. dengue vaccination will result in cases of severe dengue that would not otherwise have happened. It is also possible that dengue vaccines could facilitate the spread of Zika virus infection if used on a population-wide basis. The legitimacy of this concern is supported additionally by in vitro experimental data which demonstrates that dengue virus antibodies enhance the infection of human myeloid cells by Zika virus (Paul et al., 2016). Furthermore, it follows that a stand-alone Zika vaccine could give rise to similar antibodies that would (conversely) enhance dengue infection giving rise to cases of severe iatrogenic dengue, by generating anti-Zika antibodies that cross-react with dengue virus, and that facilitate dengue infection. For the purposes of this application, while not wishing to be bound by any particular hypothesis, Zika virus is accorded the status of a 'fifth dengue serotype'. This is because dengue infection (and dengue vaccines) have the potential to facilitate the spread of Zika by generating infection-enhancing antibodies which also react with Zika virus facilitating its infection of bodily cells. In addition to novel immunogens, the present disclosure has an additional safety feature which minimises any tendency for vaccine to enhance dengue or Zika infection (upon being bitten by an infected mosquito), by combining these vaccines in a single dose or course of vaccination, in the form of a pentavalent vaccine representing the four serotypes of dengue, plus Zika virus.

[0085] The invention relates to vaccines to prevent flavivirus infections, in particular to vaccines to prevent dengue and Zika infections. Since the advent of Zika as a pandemic phenomenon, its rapid global spread apparently facilitated by dengue-infection (Russell, 2016), the problem of vaccination (i.e. how to make a vaccine that does not, in some cases, worsen disease) has become more complicated. A new vaccine design is required in order to avoid homologous enhancement (whereby a dengue vaccine would facilitate, in some cases, dengue infection) and cross-enhancement (whereby a dengue vaccine would facilitate, in some cases, Zika infection); and moreover, whereby a Zika vaccine would facilitate, in some cases, dengue infection. Conventional approaches to the antibody enhancement problem, which involve such stratagems as combining all four serotypes of dengue in a single vaccine (Sanofi-Pasteur) or, for example, a subunit approach using N-terminal regions of the E-proteins of dengue (Merck) have recognized the antibody enhancement problem but have not provided a comprehensive solution appropriate to the Zika-pandemic situation. The most advanced dengue vaccine (the licensed Sanofi-Pasteur live attenuated tetravalent dengue vaccine), fails to deal with Zika, and from the epidemiological and in vitro observations above may be capable of promoting cases of Zika virus infection by cross-enhancement (even while having a net benefit community-wide by dint of herd immunity).

[0086] It is important to recognize that the distinction between enhancing epitopes and protective

epitopes of flaviviruses is not 'binary' in character. Generally speaking, almost all anti-dengue-E antibodies (for example) have the potential to be both neutralising and infection-enhancing, the latter property emerging at lower antibody concentrations (Dejnirattisai et al., 2014), e.g. as immunity to a vaccine or an exposure wanes. Moreover, Dejnirattisai et.al. also found that antibodies against the fusion loop of the dengue E-protein (which comprise about half of all antibodies generated convalescently) are markedly worse than antibodies against other sites on the E-protein in terms of their propensity for antibody-dependent enhancement of infection.

[0087] The present disclosure provides a vaccine that deals with the issues of antibody-dependent enhancement and cross-enhancement, by providing immunogens that have reduced capacity to elicit or stimulate infection-enhancing antibodies. In order to ensure that infection-enhancing antibodies are not generated, the present disclosure uses E-proteins with an additional glycan planted in the fusion loop, by virtue of engineering an additional, novel, glycosylation site into the nucleotide and amino acid sequence of recombinantly expressed E-proteins. The 'cloaking' effect of the glycan prevents antibodies being generated against the fusion loop site, while preserving other sites better situated to generate neutralising antibodies. In this way, glycans, which are usually considered an impediment to the generation of neutralising antibodies (e.g. in the case of HIV where they mask much of the protein surface with glycan structures that are substantially identical to those of host glycoproteins) are used to advantageous effect, i.e. in the present disclosure to mask a site on a vaccine immunogen that would otherwise give rise to problematic antibody responses (in this case, infection-enhancing antibodies).

[0088] In the case of dengue, four vaccine antigens are needed, namely the E-proteins of the four serotypes, suitably modified by glycoengineering to mask epitopes involved in antibody dependent enhancement. However, because of the risk of mutual cross-enhancement of dengue and Zika virus infections as a result of infection or vaccination, it is apparent that a Zika component is also desirable, i.e. a 'pentavalent' vaccine covering the four serotypes of dengue 'and' Zika.

[0089] Fortunately, from the point of view of the present vaccine design, the E-protein of Zika virus is highly homologous in terms of its amino acid sequence and three-dimensional structure, to that of the dengue virus E-proteins. The recent cryo-EM 3.8 Angstrom structure of the Zika virion E-protein clearly identifies (by analogy) the Zika E-protein fusion loop location (Kostyuchenko et al., 2016; Sirohi et al., 2016). Indeed Sirohi et. al. catalogue the remarkable degree of homology among diverse flaviviruses with respect to the fusion loop sequence "DRGWGNGCGLFGK" (residues 98-110), which is perfectly preserved among diverse virus isolates of Zika, the four dengue serotypes, West-Nile, Japanese encephalitis and yellow fever viruses (see supplementary figure S2 of Sirohi).

[0090] There are notable differences between dengue and Zika E-proteins, such as a five amino acid insert in the Zika E-protein, and the fact that Zika has a single N-linked glycan rather than two per monomer, but these differences are highly permissive of the present vaccine design. In the present disclosure it is anticipated that the E-protein fusion loop of Zika virus will be a site recognized particularly by infection-enhancing antibodies capable of homologous and heterologous enhancement of infection, i.e. a site against which antibody production during infection or vaccination is not desirable.

[0091] Methods for introducing additional glycosylation sites into proteins by site directed mutagenesis are well known in the art. In particular the creation of Aranesp (darbepoetin alfa), a modified form of the natural hormone erythropoietin, is a good example (Elliott ("EP0640619A1," 2010), (Elliott et al., 2003). It is important in making suitable genetic constructs to ensure that the leader sequence of the protein is incorporated into recombinant plasmid or other vector DNA sequences, in order to direct the nascent polypeptide chain into the endoplasmic reticulum of the host cell, allowing glycosylation and to facilitate protein folding. Various eukaryotic cell systems are suitable for recombinant production - such as Chinese hamster ovary cells (CHO), as well as yeast (e.g., Pichia pastoris) and other vector systems such as baculovirus (which has the added advantage of equipping the viral protein immunogen with an insect glycan, as per the inoculum form of the flavivirus). However, prokaryotic systems such as those based on E. coli are not suitable, because they do not have the cellular apparatus required to effect glycosylation of proteins.

[0092] In the case of Aranesp, the molecule has two additional N-linked glycosylation sites, strategically placed to avoid hindrance of interaction of the glycoengineered molecule with the erythropoietin receptor. The purpose of glycoengineering the earlier erythropoietin-based product in this way was to improve the longevity of the molecule in circulation by increasing its size giving rise to a product that can be administered once instead of thrice weekly (Elliott et al., 2003). Glycoengineering is 're-purposed' in the present disclosure, to cloak a site on a vaccine immunogen that would otherwise have adverse consequences of antibody dependent enhancement of infection.

[0093] Viruses have been demonstrated to exploit the immune-evasion properties of glycans thwarting the generation of neutralising antibodies. In the field of vaccine development (e.g. against HIV glycoprotein gp160/120), glycans have generally been regarded as a problem (rather than an aid to vaccine development), limiting the access of antibodies to the protein surface of a glycoprotein antigen by forming a dense glycocalyx comprised of host glycans, to which the immune system of the host is programmed to be immunologically tolerant. There are notable exceptions that prove the generality of this rule: e.g. where the glycan itself or a minor variant is a target or part thereof, which is the case for rare anti-HIV neutralising antibodies; and in the case of insect-specific glycan epitopes on arboviruses, which are themselves targets in some vaccine designs)(Dalziel, Crispin, Scanlan, Zitzmann, & Dwek, 2014). The present disclosure is different from the prior art in exploiting the stealth qualities of glycans to advantageous effect in a vaccine immunogen. In this novel application a glycan is used to cloak a troublesome site on a vaccine immunogen, preventing antibodies from being generated that would recognise the equivalent uncloaked site on the natural virion. Glycoengineering (unlike deletion or truncation of amino acid sequence elements) allows this cloaking to be achieved while causing minimal interference with the underlying structure of the protein part of the antigen. Preservation of protein structure by employing glycoengineering rather than deletion or truncation protects remote neutralising epitopes that might otherwise be altered to detrimental effect.

[0094] The glycoengineered flavivirus E-proteins of the present disclosure are amenable to incorporation into various forms for the purpose of vaccination. These forms may be protein (i.e. glycoprotein) or nucleic acid in character. They may be represented in a vaccine formulation as a mixture of purified proteins (as a subunit vaccine, e.g. with aluminium hydroxide or aluminium

phosphate as adjuvant), as virus-like particles (Frietze, Peabody, & Chackerian, 2016), or as mammalian-expressible DNA constructs (e.g. plasmid DNA with cytomegalovirus promoter) for administration as DNA vaccines using subunit (Tregoning & Kinnear, 2014) or infectious-attenuated clone approaches as exemplified for the YFD strain of yellow fever virus (Tretyakova et al., 2014). They are also amenable to incorporation into live attenuated virus vectors such as measles vector vaccines as per the Chikungunya vaccine candidate by Themis Bioscience GmbH (Ramsauer et al., 2015). Likewise the glycoengineered flavivirus E-proteins of the present disclosure would be suitable candidates for advanced adjuvant strategies such as 'Co-Delivery' where mammalian-expressible DNA and protein representations of the same immunogen are co-formulated in the selfsame particles (e.g. liposomes) giving dramatic improvements in antibody responses compared to protein or DNA immunogens used in isolation (Laing *et al.*, 2006).

[0095] Since the present glycoengineering approach involves defined changes at multiple base positions in the nucleic acid sequence of the E-protein, then live attenuated vaccines of the present disclosure will have a high level of resistance to reversion by mutation to wild type, which is a known problem in live attenuated approaches (e.g. the Sabin polio vaccine which was replaced by the non-viable Salk version in the USA for this reason): i.e. they will be safer and less likely to give rise to cases of disease by reversion to wild-type or de novo mutation to increased virulence (Hanley, 2011). From the reasoning of Hanley, and given the present disclosure, it is now evident that introduction of further glycosylation sites into viral proteins (*i.e.*, more than is needed to achieve cloaking of infection-enhancing epitopes) is a viable strategy to guard against adverse mutation in live attenuated viral vaccines, and to guard against 'mosquito competence' whereby a live attenuated flavivirus vaccine might be spread, allowing evolution to increased virulence enabled via vector transmission in mosquitoes. Such additional glycosylation sites are best placed at non-neutralising sites of the flaviviral E-protein.

[0096] In the case of flavivirus subunit vaccines of the present disclosure (as distinct from live vector approaches) favoured sites for a second additional glycan would include sequence elements comprising contact surfaces of E with the underlying M-protein of the virion. These highly soluble hyperglycosylated E-proteins allow for monovalent engagement of antigen-specific B-cells, favouring higher affinity neutralising antibodies by creating greater competition for antigen during clonal selection and somatic mutation of antigen-specific B-cells.

Brief Description of Drawings

[0097] The invention will now be described with reference to the accompanying drawing in which:

Figure 1. Design of vaccine immunogens of the invention, to avoid generation of cross-reactive fusion loop antibodies and the elicitation or stimulation of infection-enhancing antibodies.

Figure 1 '**A**' shows the effect of vaccination with a flavivirus vaccine, such as a live attenuated vaccine known in the art comprising the four dengue serotypes DEN-1, DEN-2, DEN-3 and DEN-4. Attenuated vaccine virions are shown as round structures with the E-protein moiety stem projecting therefrom, the fusion loop is depicted as a small spur on the stem of the virion E-protein moiety; antibodies are depicted as Y-shaped molecules, infection-enhancing antibodies are shown in solid

black whereas neutralising antibodies are shown in white outlined in black, '**B**' illustrates a vaccine immunogen design of the invention. The novel immunogen contains an E-protein wherein the fusion loop sequence has been substituted to include a glycosylation site for attachment of a glycan (depicted as a crescent attached to the fusion loop spur, to generate neutralising antibodies against the E-proteins of the vaccine without generating infection-enhancing antibodies. '**C**' shows how infection-enhancing antibodies against the fusion loop of the E-proteins, when bound to the E-protein of a wild-type flavivirus virion, are able to engage with high affinity the Fc-gamma-receptor-IIa (depicted as a white rectangle outlined in black), facilitating infection of myeloid cells that carry the Fc-gamma receptor IIa. '**D**' represents occasional failure of a vaccine to elicit a protective level of antibody response in some subjects (*e.g.*, the immunosuppressed). While not protected against dengue, such immunocompromised subjects (immunized with the vaccine of the present disclosure) are at least not predisposed to dengue by the novel vaccine because they have not mounted an antibody response against the fusion loop. This may be contrasted to a vaccine of conventional design containing an uncloaked fusion loop, where a subject might then be predisposed to severe dengue infection by the conventional vaccine having elicited sub-neutralising concentrations of fusion-loop antibody.

Figure 2. Recombinant expression of glycoengineered forms of dengue and Zika exodomain proteins.

Figure 2a: Coomassie stained gel showing evaluation of expression of dengue and Zika constructs in HEK293 cells, lanes shown as follows:
1: pSF236 transfected cells WT, 2: pCRO21 transfected cells, 3: pSF237 transfected cells WT, 4: pCRO22 transfected cells, 5: pSF238 transfected cells WT, 6: pCRO23 transfected cells, 7: pSF239 transfected cells WT, 8: pCRO24 transfected cells, 9: pSF233 transfected cells WT, 10: pCRO25 transfected cells. 11: pSF236 transfected cells WT, 12: pCRO21 transfected cells, 13: pSF237 transfected cells WT, 14: pCRO22 transfected cells, 15: pSF238 transfected cells WT, 16: pCRO23 transfected cells, 17: pSF239 transfected cells WT, 18: pCRO24 transfected cells, 19: pSF233 transfected cells WT, 20: pCRO25 transfected cells. For lanes 1 to 10, the supernatant concentrate was 1ul / 1.1ml, for lanes 11 to 20 the supernatant concentrate Talon eluate concentration was 26ul / 400ul.

Figure 2b: Anti-his-tag Western blot showing further expression evaluation of dengue-1 and Zika constructs. Lanes 1-8 show cell pellets, lanes 9-16 show raw (filtered) supernatants, lanes 17-24 show Ni-NTA eluates, as follows: 1: pSF236 cell pellet, 2: pCRO26 cell pellet, 3: pCRO27 cell pellet, 4: pSF233 cell pellet 5: pCRO28 cell pellet, 6: pCRO29 cell pellet, 7: pCRO30 cell pellet, 8: pCRO31 cell pellet, 9: pSF236 filtered supernatant, 10: pCRO26 filtered supernatant, 11: pCRO27 filtered supernatant, 12: pSF233 filtered supernatant, 13: pCRO28 filtered supernatant, 14: pCRO29 filtered supernatant, 15: pCRO30 filtered supernatant, 16: pCRO31 filtered supernatant, 17: pSF236 Ni-NTA eluate, 18: pCRO26 Ni-NTA eluate, 19: pCRO27 Ni-NTA eluate, 20: pSF233 NI-NTA eluate, 21: pCRO28 Ni-NTA eluate, 22: pCRO29 Ni-NTA eluate, 23: pCRO30 Ni-NTA eluate, 24: pCRO31 Ni-NTA eluate. Three arrows indicate detected hyperglycosylated exodomain forms.

Figure 2c shows a Western blot of the hyperglycosylated forms pCRO21, pCRO22, pCRO23, pCRO24 for dengue serotypes 1-4 (D1, D2, D3 and D4) respectively and pCRO28 for Zika. The left lane of each pair shows the wild type (wt), whereas the right lane of each pair shows the hyperglycosylated form of the dengue or Zika E-protein exodomain. +2 indicates two additional

glycosylation sites / glycans, +1 indicates one additional glycosylation site / glycan.

Figure 2d shows Coomassie blue stained gels of the purified hyperglycosylated E exodomain proteins D1, D2, D3, D4 and Zika, which correspond to plasmids pCRO21, pCRO22, pCRO23, pCRO24 and pCRO28, respectively, in the sequence listings. The scale to the left is the migration position of molecular weight markers in '000s.

Figure 3. Characterisation of glycans present on the glycoengineered dengue 2 and Zika exodomain proteins and degree of occupancy of sequence-programmed N-linked-glycosylation-sites

Figure 3a shows an SDS-PAGE analysis of dengue and Zika samples prior to and after PNGase digestion.

Figure 3b shows analysis of glycans released from dengue-2 and Zika compared to reference standards by HPAEC-PAD.

Figure 3c shows dengue-2 tryptic cleavage sites and peptide fragments.

Figure 3d shows Zika tryptic cleavage sites and peptide fragments.

Figure 3e shows Zika Endo-Lys-C cleavage sites and peptide fragments.

Figure 3f shows tryptic digestion of dengue-2 with and without PNGase F digestion.

Figure 3g shows tryptic digestion of Zika with and without PNGase digestion.

Figure 3h shows endo-Lys-C digestion of Zika with and without PNGase digestion.

Figure 4. Immunogenicity of select glycoengineered dengue proteins 1, 2, 3 and 4 and Zika in mice measured by direct ELISA.
The x-axis shows the number of days after immunisation and the y-axis shows the IgG antibody titre. Three doses were given on days 0, 14 and 21. Dosages are indicated in Table 9. Antibody responses were measured in individual mice against all five antigens as wild-type VLPs on the ELISA solid phase as indicted: top row left Den 1 VLP antigen, top row right Den 2 VLP antigen, middle row left Den 3 VLP antigen, middle row right Den 4 VLP antigen, bottom row left Zika VLP antigen. *Immunogens* (as distinct from antigens uses for assay above) were Penta-DNA (a combination of each of the Den1-4 and Zika DNAs of the invention) shown as an open circle, Penta-Prot (a combination of each of the Den1-4 and Zika proteins of the invention) is shown as an filled square, Monovalent Zika is shown as a filled triangle, Penta VLP (a combination of each of the Den1-4 and Zika VLPs of the invention) is shown as a filled inverted triangle. PBS control is shown as an open inverted triangle.

Figure 5. Avoidance of recognition of the glycoengineered proteins by fusion loop antibodies and retention of neutralizing epitopes.
In order to further characterize the hyperglycosylated antigens of the present disclosure, comparing them to wild-type equivalent antigens, an ELISA assay was established to measure antibody binding to diverse wild-type and recombinant exodomains (as distinct from the VLP antigens of Fig. 4). Unlike the ELISA used in Fig 4, which used only wild-type VLPs as antigens this assay used only exodomain-type antigens (recombinant wild-type and recombinant hyperglycosylated forms 'HX' of

the invention). In order to ensure the same orientation of each of these materially diverse (non-glycosylated bacterial, insect-glycosylated and human-glycosylated) species, they were anchored to the solid phase by a rabbit anti-His-tag monoclonal antibody, recognizing their C-terminal His tags. Coated plates were blocked and exposed to a constant concentration of the various His-tagged proteins in a 'post-coating' step and were then probed with monoclonal antibodies at various concentrations (Fig. 5a, for 4G2) or at a constant concentration (Fig. 5b,c). Various dengue and Zika antigens and probe antibodies were tested in Fig. 5b,c, including a human polyclonal anti-Zika convalescent serum sample. Probe antibodies were followed by incubation with a rabbit anti-mouse IgG Fc - horseradish peroxidase (or rabbit-anti-human IgG Fc - horseradish peroxidase) conjugate (as appropriate) and tetramethylbenzidine substrate. A mouse monoclonal anti-human-CD4 antibody served as a control for the mouse monoclonal antibodies.

Figure 5a represents fusion-loop antibody 4G2 (x-axis, ng/ml), which was raised against dengue-2 serotype but is highly cross-reactive among flaviviruses, binding to solid phase wild-type dengue serotype-2 or dengue serotype-4 wild type exodomain antigens, or their hyperglycosylated counterparts containing two additional programmed sequons in the fusion loop ('HX' for hyperglycosylated exodomain). (Asterisks denote absorbance values higher than the read-capability of the ELISA reader), Y Axis shows absorbance at 450nm. Points are mean of duplicate determinations.

Figure 5b is a photograph of an ELISA plate result of the present assay design, wherein various exodomains were screened for binding to antibodies, including a set of murine monoclonal antibodies, (left to right columns 1 and 2: 4G2 (cross-reative fusion-loop antibody), columns 3 and 4: Aalto Bioreagents anti-Zika antibody AZ1176-0302156-Lot3889; columns 5 and 6: Z48 anti-Zika antibody, wells 7 and 8: Z67 anti-Zika antibody (these are described as ZV48 and ZV67 Zika-neutralizing antibodies by Zhao et al, Cell 2016 and were obtained from The Native Antigen Company ZV67=MAB12125 and ZV48=MAB12124), wells 9 and 10: anti-human-CD4 control Millipore 024-10D6.B3 2322501; wells 11 and 12: Zika human convalescent serum). Exodomains (all having His-6 C-terminal tag) were as follows (top to bottom): 'Aalto insect' = Sf9 insect-cell produced wild-type recombinant Zika exodomain from Aalto Bioreagents, Dublin, Ireland; Prospec Zika = bacterially produced recombinant wild-type exodomain from Prospec, Israel; NAC WT den-2 = HEK293-produced human wild-type dengue-2 exodomain (based on residues 280-675 of NCBI ACA48859.1 followed by a glycine-serine linker of 7 or 8 amino acids in length followed by the His6 tag); 'Excivion HX den-1 (human) cloaked' represents the expressed product of plasmid pCRO21 from HEK 293 cells having two N-glycosylation sequons programmed into the fusion loop; likewise for Excivion HX den-2 through den-4 , representing plasmids pCRO22, pCRO23 and pCRO24 respectively. 'Excivion HX Zika human (cloaked)' represents the protein product of plasmid pCRO28 expressed in HEK293 cells, having a single glycosylation programmed into the fusion loop.

Figure 5c shows the absorbance values represented as Excel data bars as % values of the maximum absorbance (which was 3.0 absorbance units), demonstrating the quality of replicates (duplicates). Fig 5c is a graphical representation of the data in Fig 5b and has the same layout as Fig 5b.

Figure 5d shows the ELISA plate depicted in Figure 5b in greater detail.

Figure 6. Avoidance of generation of fusion-loop antibodies by the glycoengineered proteins.
A further ELISA assay was developed, different to those used in Fig 4 and Fig 5, to detect

antibodies in polyclonal sera from immunized mice, against the fusion loop. This was a competitive binding assay in which biotin-labelled 4G2 was used as a label, and unlabeled 4G2 was used as a standard. Top row left, unconjugated 4G2, x-axis concentration of 4G2 ng/mL; top row middle, Penta DNA, Group 1, Day 42, x-axis dilution of serum; top row right Penta Prot Group 2, Day 42, x-axis dilution of serum; bottom row left Mono Zika, Group 3 Day 42, X-axis dilution of serum; bottom row middle Penta VLPs, Group 4 day 42, x-axis dilution of serum; bottom row right PBS, Group 5 Day 42, x-axis dilution of serum. In each instance the y-axis was %biotinylated (Bt)-4G2 bound.

Figure 7. Generation of neutralising antibodies by the glycoengineered proteins (PRNT).

Figure 7a shows Dengue PRNT responses for Sample groups 1 to 5 measured in pooled sera: dose response curves against DENV, Top row left Penta DNA (Neutralisation of DENV by Group 1 pool); top row middle Penta Prot (Neutralisation of DENV by Group 2 pool); top row right Mono Zika (Neutralisation of DENV by Group 3 pool); bottom row left Penta VLPs (Neutralisation of DENV by Group 4 pool); Bottom row middle PBS (Neutralisation of DENV by Group 5 pool). In each instance the x-axis is dilution factor and the y-axis shows percentage neutralisation.

Figure 7b shows PRNT responses for Sample groups 1 to 5 measured in pooled sera: dose response curves against ZIKV, Top row left Penta DNA (Neutralisation of ZIKV by Group 1 pool); top row middle Penta Prot (Neutralisation of ZIKV by Group 2 pool); top row right Mono Zika (Neutralisation of ZIKV by Group 3 pool); bottom row left Penta VLPs (Neutralisation of ZIKV by Group 4 pool); Bottom row middle PBS (Neutralisation of ZIKV by Group 5 pool). In each instance the x-axis is dilution factor and the y-axis shows percentage neutralisation.

Figure 8. Reaction of convalescent dengue and Zika sera with immobilized Zika and dengue wild-type (WT) and hyperglycosylated (HX) exodomain proteins

[0098] Upper panel shows ELISA reactivity of antibodies in a dengue convalescent serum with immobilized Zika and dengue wild-type (WT) and hyperglycosylated (HX) exodomain proteins oriented on the solid phase by capture with a rabbit anti-His-tag monoclonal antibody, in the presence (grey bars, right of each pair) and absence (black bars, left of each pair) of competing mouse monoconal flavivirus fusion loop antibody 4G2 (an anti-dengue-serotype-2 cross-reactive monoclonal antibody) at a concentration of 10 ug/ml during serum incubation. Human sera were tested at a constant concentration of 1/1000.

[0099] Lower panel shows ELISA reactivity of antibodies in a Zika convalescent serum with immobilized Zika and Dengue wild-type (WT) and hyperglycosylated (HX) exodomain proteins in the presence (grey bars) and absence (black bars) of competing mouse monoclonal flavivirus fusion loop antibody 4G2. Conditions and labelling are the same as for the upper panel. Error bars are standard error of duplicate determinations.

Examples

Example 1 Design of new vaccine immunogens designed to avoid the elicitation or stimulation of infection-enhancing antibodies.

[0100] Figure 1, '**A**' shows the effect of vaccination with a flavivirus vaccine, such as a live attenuated vaccine known in the art comprising the four dengue serotypes DEN-1, DEN-2, DEN-3 and DEN-4. The vaccine generates a mixture of antibodies capable of virus neutralisation and other antibodies capable of antibody-dependent enhancement of infection. Antibodies capable of virus neutralisation include those that recognise sites on the receptor-interacting surface of the virion E-protein, *i.e.*, that surface that binds to the DCSIGN lectin/receptor. (For simplicity of illustration, only the DCSIGN receptor is shown, noting that there are other receptors for dengue and flaviviruses generally). '**C**' shows how infection-enhancing antibodies against the fusion loop of the E-proteins, when bound to the E-protein of the virion, are able to engage with high affinity the Fc-gamma-receptor-IIa, facilitating infection of myeloid cells. Several types of Fc-gamma receptors have been implicated in this phenomenon, even (paradoxically) including the low-affinity receptor Fc-gamma-receptor-IIb, which is normally inhibitory to myeloid cells and B-cells (Bournazos S, Signaling by Antibodies... Ann. Rev. Immunol 2017, 35:285-311). The result of vaccination with a live attenuated vaccine (an example of a vaccine known in the art) is the net effect of two opposing populations of antibody, one set that neutralises dengue virions, and a further set that is capable of infection enhancement. In most subjects of vaccination, neutralising antibodies overcome the effect of the infection-enhancing antibodies, such that the net effect of vaccination is protection against the four dengue serotypes. However, in subjects who do not mount a balanced response to the four serotypes, or who are immunosuppressed e.g., due to measles or HIV infection, flavivirus-infection-enhancing antibodies prevail rendering such subjects predisposed to, rather than protected against, severe infection with dengue and more prone to infection with other flaviviruses. Further, infection-enhancing antibodies in some healthy (non-immunosuppressed) dengue-vaccinated subjects cross-react with Zika virus. Those dengue-immunised subjects are now predisposed to Zika infection upon first being bitten by a Zika-infected mosquito '**C**'. Conversely, '**B**' illustrates a vaccine immunogen designed in accordance with the invention. The novel immunogen, containing an E-protein wherein the fusion loop sequence has been modified and has been designed to be substituted with a glycan with the aim to generate neutralising antibodies against the E-proteins of the vaccine without generating infection-enhancing antibodies. '**D**' represents occasional failure of the vaccine of the invention to elicit a protective level of antibody response in some subjects (*e.g.*, the immunosuppressed), however, unlike other vaccine designs known in the art, the vaccine of the invention is designed to not render immunosuppressed subjects susceptible to enhanced infection with dengue or Zika viruses. Immunogens and vaccines of the present design are thereby designed to be safer on an individual subject basis and moreover to lack the potential to facilitate the epidemic spread of Zika by creating a population of subjects that have Zika-infection-enhancing antibodies, in the absence of neutralising antibodies. (WT = wild type).

Example 2 (Fig. 2) Recombinant expression of glycoengineered (hyperglycosylated) forms of dengue and Zika exodomain proteins.

[0101] Plasmid inserts encoding various novel recombinant forms of the natural wild type (WT) exodomain sequences representative of the four dengue serotypes and of Zika and containing an *E. coli* origin of replication and a cytomegalovirus (CMV) promoter, as well as a hexahistidine C-

terminal tag, were made by *de novo* gene synthesis (Thermofisher, GeneArt). Where two glycosylation sequons were inserted in the DNA sequence, the sequence was changed 'manually' to avoid the creation of direct DNA sequence repeats that might otherwise allow undesirable homologous recombination events.

[0102] Plasmid expression vectors pCRO21 (SEQ ID NO: 13), pCRO22 (SEQ ID NO: 14), pCRO23 (SEQ ID NO: 15), pCRO24 (SEQ ID NO: 16) and pCRO28 (SEQ ID NO: 17), coding for the mutated exodomain of the Envelope proteins of DENV1, DENV2, DENV3, DENV4 and ZIKV, respectively, were ultimately selected and produced by The Native Antigen Company, Oxford, as follows: expression cassettes were synthesized *de novo* to contain a 5' NotI site followed by a consensus Kozak sequence followed by the coding sequence for the first 17 amino acids of the influenza-A virus haemagglutinin protein acting as secretion signal. The Envelope protein coding sequences used, (numbering relative to the polyprotein), were 280-675 (NCBI ACA48859.1), 281-676 (NCBI ADK37484.1), 281-673 (NCBI AIH13925.1), 280-675 (NCBI ANK35835.1) and 291-696 (NCBI ARB07957.1), respectively. [Elsewhere, for ease of reference, numbering is expressed according to residue number in the E-protein, with W at 101 of the fusion loop as a reference point]. Each construct contained coding sequences for a glycine-serine linker 7 to 8 amino acids in length followed by a 6x His-tag and a stop codon. The stop codon is followed by a NheI site in each expression cassette. The mammalian expression vector pSF-CMV (Oxford Genetics, Oxford) was digested with NotI and NheI, and the 4.2kb fragment was ligated to the 1,3kb NotI and NheI fragments of the expression cassette harbouring maintenance vectors (pUC57). In each case, one or two *additional* sequons of the general formula (NXS/T) was introduced into the fusion loop of the E-protein exodomain, capable (theoretically) of encoding a functional N-linked glycosylation site. The wild-type dengue proteins naturally already have two glycosylation sites, and Zika one. None of the natural glycans are found in the fusion loop.

[0103] For small-scale preparation 15ml aliquots of HEK293FT cells at 3e6/ml were individually transfected with pCRO21, pCRO22, pCRO23, pCRO24 or pCRO25 (SEQ ID NO: 18), 4 control transfections were performed using pSF233, pSF236, pSF237, pSF238 or pSF239. After a day, 15ml of rescue medium was added to each transfection. At day 3 after transfection each of the 10 transfections was treated the same way as follows: 30ml of suspension was spun at 4,000g for 7 minutes. The resulting supernatant was filtered using a 0.22um disc filter. The pellet was resuspended in 1ml of PBS. The filtered supernatant was then concentrated using a Vivaspin20 (30,000Da cutoff) as per manufacturer's instructions. Concentrate volumes ranged from 0.6ml to 1.2ml. All concentrates were brought up to 1.2ml with PBS. The concentrated supernatants were subjected to Talon purification as per manufacturer's instructions using Talon HiTrap Spin (GE). Buffers for Talon capture were: Equilibration Buffer: 50mM phosphate pH7.8, 300mM NaCl; Wash Buffer: 50mM phosphate pH78, 300mM NaCl, 5mM imidazole; Elution Buffer: 50mM phosphate pH7.8, 300mM NaCl, 150mM imidazole.

[0104] Characterisation of the resulting proteins by coomassie-blue staining (Figure 2a, Figure 2d) and by western blot (Figure 2b, Figure 2c) of SDS electrophoresis gels is shown in Figure 2.

[0105] Figure 2c shows a Western blot with anti-His-tag monoclonal antibody of chosen constructs pCRO21 (D1), pCRO22 (D2), pCRO23 (D3), pCRO24 (D4) (for dengue serotypes 1-4 respectively) and pCRO28 for Zika, which gave rise to secreted hyperglycosylated proteins. Molecular weight

increments due to glycosylation are apparent, higher for the +2 glycan dengue constructs than for the Zika +1 glycan construct, demonstrating the practical attainment of select theoretically designed constructs as expressible proteins. Wild type forms are shown on the left of each pair.

[0106] Figure 2d shows Coomassie blue stained gels of the purified proteins, hyperglycosylated E protein exodomains from the four dengue virus strains D1, D2, D3, D4 and Zika after cobalt chelate (TALON) chromatography using cobalt chelate. Hyperglycosylated exodomains D1, D2, D3, D4 and Zika correspond to plasmids pCRO21, pCRO22, pCRO23, pCRO24 and pCRO28, respectively.

[0107] For scale-up production, the novel hyperglycosylated proteins were expressed recombinantly in human embryonic kidney cells (HEK 293) by transient transfection with linear polyethyleneimine (PEI), and purified by metal chelate affinity chromatography with a cobalt chelate (TALON®, Clontech/GE), as described as follows for the dengue-1 hyperglycosylated construct based on pCRO21. 20x 1L of HEK293 cells were transfected with DENV1_Eexo_2xglyco expression vector pCRO21. 3 days post transfection, the supernatant was harvested by centrifugation, and the cleared supernatant was 0.2um filtered and concentrated to ~200ml by tangential flow filtration (TFF). Immobilised metal affinity chromatography (IMAC) was performed on the TFF retentate using 5ml HiTRAP Talon pre-packed column (GE) according to manufacturer's instructions using 20mM sodium phosphate pH7.8 based buffer systems. DENV1_Eexo_2xglyco protein containing fractions were pooled and dialysed against 20mM TRIS-HCl pH7.8 10mM NaCl. Ion exchange chromatography was performed using a pre-packed 5ml HiTrap Q HP column according to manufacturer's instructions. DENV1_Eexo_2xglyco were pooled and dialysed against DPBS pH7.4. The dialysed solution was 0.22um filtered and vialled under sterile conditions. BCA assay and SDS-PAGE were performed according to manufacturer's instructions (Bio-Rad).

[0108] Note that three of the hyperglycosylated constructs express at levels much higher than wild type (these are the hyperglycosylated dengue serotypes 2, 3 and 4 corresponding to plasmids pCRO22, pCRO23 and pCRO24). Zika plasmid, pCRO25 did not give rise to detectable secreted protein (Figure 2a, lane 20), although significant amounts of cell-associated protein were found (not shown).

[0109] Therefore a further round of constructs was made (see Figure 2b) seeking to improve levels of expression of dengue-1 and Zika hyperglycosylated forms. In this instance nickel chelate chromatography was used for purification. Further constructs of dengue (pCRO26 (SEQ ID NO: 19), and pCRO27 (SEQ ID NO: 20)) and of Zika (pCRO28 (SEQ ID NO: 17), pCRO29 (SEQ ID NO: 21), pCRO30 (SEQ ID NO: 22) and pCRO31 (SEQ ID NO: 23)) were expressed and purified. Favourable expression of the plasmid construct pCRO28 was demonstrated by anti-His-tag Western blot (Figure 2 c) and coomassie staining (Figure 2 d).

[0110] The hyperglycosylated forms chosen were pCRO21, pCRO22, pCRO23, pCRO24 (for dengue serotypes 1-4 respectively) and pCRO28 for Zika. Hyperglycosylated exodomains D1, D2, D3, D4 and Zika correspond to plasmids pCRO21, pCRO22, pCRO23, pCRO24 and pCRO28, respectively (SEQ ID NO: 24, 25, 26, 27 and 28 respectively). Molecular weight increments due to glycosylation are apparent, higher for the +2 dengue constructs than for the Zika +1 construct.

[0111] In all, eleven plasmid constructs were made and tested for protein expression and five were selected for further investigation, based on equivalent or (in most cases) superior levels of expression compared to wild type (pCRO21, pCRO22, pCRO23, pCRO24 representing the four serotypes of dengue, and pCRO28 representing Zika).

[0112] Surprisingly, given the extremely hydrophobic nature of the fusion loop (which features the residues W, F and L exposed at the tip of the E protein in close juxtaposition at its distal end in three dimensional space) in the case of dengue, all four representative serotypes tolerated substitution of two glycans (which are hydrophilic, and radically transform the topography of this part of the protein to an extent that mere amino-acid substitutions cannot) with no penalty to levels of expression (*i.e.*, all expressed as well as the wild type sequence, in some cases markedly better). An objective had been set of 'no less than wild type' for levels of expression in order to ensure that the proteins were not misfolded which would have resulted in eradication from the endoplasmic reticulum via the ERAD channel for proteasomal degradation. Examples of the dengue serotype-1 sequence with a single glycan in the fusion loop were also made, but it did not express any better than wild type or the species with two glycans. In the case of Zika, attempts to generate variants with two glycosylation sites into the fusion loop (following the method established for dengue) were not successful, resulting in less secretion of the recombinant protein into the culture medium than for wild type.

[0113] In the case of the Zika E-protein exodomain we therefore explored the generation of variants with a single glycan at various sites in the fusion loop. Substitution of the tryptophan (W101), as for one of the dengue sequons, with an asparagine (the N of the sequon at 101 in place of W), resulted in a level of expression of the construct that was less than for wild type. Likewise, insertion of a glycan at F108 (i.e. the N of the sequon at 108, in place of F), resulted in a level of expression of the construct that was less than for wild type. We concluded that the Zika fusion loop was less tolerant to glycan insertion, and sought a more conservative way to allow it.

[0114] Having established, in the case of Zika, that neither the W101 nor the following F of the fusion loop could be replaced with the N of an N-linked glycosylation sequon, an alternative strategy was developed, which was not modeled on the approach taken for dengue. We sought to place a single glycan as near as possible to the end of the fusion loop (based on the 3D structure PDB 5IRE). Rather than go through the process of systematically making and testing the hundreds of possible variants that might allow glycan insertion (which would have been arduous by gene synthesis or by library technologies), we contrived a hypothetical solution and tested it. We contrived to straddle the W at the apex of the fusion loop with an N-linked glycosylation sequon. However, we reasoned that may have been infeasible by insertion of the classical NXS/T sequon, because W is not tolerated at the X position of a sequon. However, although W is not tolerated in the 'X' position in the centre of a sequon, H (histidine, a relatively conserved replacement for W, having a hydrophobic-aromatic/cationic dual character) can be tolerated in the X-position. We therefore substituted the 100 position with an N, used a H in place of the W for the X-position, and used a T (which we find works better with H than S), to make a single sequon that read 'NHT' (i.e. residues 100, 101, 102, using the E-protein numbering convention rather than the polyprotein numbering convention). The resulting protein, made from plasmid pCRO28, was found to express as well as wild type, and gave greater yield on purification than wild type, indicating no impediment to expression. The other variants of Zika that we explored gave rise to low level or no secreted

protein in the expression systems used.

Example 3 (Figure 3) Characterisation of glycans present on the glycoengineered dengue serotype-2 and Zika proteins.

[0115] Glycan compositional analysis (GlycoThera, Germany) was performed on two of the selected proteins from Example 2, the dengue-2 serotype product of pCRO22 (representative of the selected dengue constructs that were all designed to carry two glycans in the fusion loop) and that of Zika (the product of pCRO28, designed to carry one glycan in the fusion loop) obtained from transfections of HEK 293.

[0116] The results of SDS-PAGE analysis of dengue and Zika samples prior to and after digestion with polypeptide N-glycosidase F (PNGase, Prozyme Inc.) are shown in Figure 3a. The samples were reduced in 50 mM DTT for 5 min at 95°C prior to SDS-PAGE analysis (15% polyacrylamide gel after coomassie blue staining) Lane 1: CV94 (pCRO22 protein, dengue-2) prior to PNGase digestion; Lane 2: CV94 after PNGase digestion; Lane 3: CV95 (pCRO28 protein, Zika) prior to PNGase digestion; Lane 4: CV95 after PNGase digestion; Lane 5: molecular weight standard. In this case the degree of decrease in apparent molecular weight (as distinct from the increment in Fig. 2c relative to WT) conforms to theoretical expectation based on the number of additional glycans introduced into the sequence: i.e. dengue-2 has lost four glycans in this digestion (two natural, and two introduced by sequence programming of additional sequons), whereas Zika has lost two glycans (one natural, and one introduced by sequence programming of one additional sequon). Enzymatic digestion with PNGase was conducted according to Tarentino and Plummer, Methods in Enzymology, 1994; 230; 44-57.

[0117] Glycans were released from the hyperglycosylated protein products and quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and normal-phase HPLC with fluorescence detection of 2-AB-labelled N-glycans, along with specific exoglycosidase treatment (Figure 3b). Table 2 summarizes the results of this analysis.

Table 2

Sample	DENV2_ENV_2xGlyco recombinant Antigen; Lot #20161026	Zika_ENV_recombinant Antigen; Lot #20161213
Structure	mol (%)	mol (%)
neutral	16.9	17.0
monosialylated	30.7	36.9
disialylated	26.6	32.0
trisialylated	15.0	8.4
tetrasialylated	9.5	5.1
pentasialylated / sulphated	1.3	0.6
sum	100.0	100.0

[0118] Quantitative HPAEC-PAD analysis of native oligosaccharides was performed on an ICS 5000+ ion chromatography system of the Thermo Fisher Scientific Inc. (Waltham, MA, USA; GlycoThera device-ID: HPAEC-7) using high resolution CarboPac PA200 columns. Injection of appropriate oligosaccharide reference standards was included in the analytical sequence.

[0119] N-glycans were detected via electrochemical detection. The data were collected and the chromatograms were acquired by using Chromeleon Chromatography Management System Version 6.8. Native N-glycans were analyzed via HPAEC-PAD revealing mainly neutral, monosialylated, disialylated and trisialylated oligosaccharides in both preparations according to GlycoThera's reference oligosaccharide standards. (Fig. 3b, Table 3).

[0120] Desialylated N-glycans were analyzed via NP-HPLC after 2-AB labelling revealing predominantly complex-type N-glycans with significant permutational diversity, having proximal *α* 1,6-linked fucose in both samples (CV94=dengue-2, and CV95=Zika) according to GlycoThera's reference oligosaccharide standards. HPAEC-PAD mapping of native N-glycans released from dengue and Zika preparations CV94 (dengue 2 pCRO22 protein) and CV95 (pCRO28 protein) Zika (as shown in Table 2) revealed the presence of predominantly neutral (16.9% and 17.0%, respectively), monosialylated (30.7% and 36.9%, respectively), disialylated (26.6% and 32.0%, respectively) and trisialylated (15.0% and 8.4%, respectively) oligosaccharides in both samples. Significant amounts of tetrasialylated N-glycans (9.5% and 5.1%, respectively) as well as low proportions of pentasialylated / sulphated oligosaccharides (1.3% and 0.6%, respectively) were found in dengue and Zika samples CV94 and CV95; phosphorylated N-glycan structures such as oligomannosidic Man5-6GlcNAc2 glycan chains with one phosphate residue were not detected in either of the samples analyzed.

Table 3. N-glycan mapping of 2-AB labelled desialylated N-glycans, according to standard procedures at GlycoThera, from Dengue and Zika preparations CV94 and CV95 after sialidase treatment using normal-phase HPLC with fluorescence detection revealed the following compositions for the two proteins.

Sample code		**CV94**	**CV95**
Sample code		**DENV2_ENV_2x Glyco recombinant**	**Zika_ENV_recombinant Antigen; Lot #20161213**
#	**N-glycan structure**	**mol (%)**	**mol (%)**
	complex-type N-glycans	**61.4**	**56.6**
1	diantennary w/o 2 β-Gal w/o 1 GlcNAc with α1,6-Fuc	0.1	0.2
2	diantennary w/o 2 β-Gal with α1,6-Fuc	0.9	1.2
3	diantennary w/o 1 β-Gal with α1,6-Fuc	3.1	4.4
4	diantennary w/o 1 β-Gal w/o α1,6-Fuc	0.4	0.8
5	diantennary with α1,6-Fuc	8.1	8.8

Sample code		**CV94**	**CV95**
Sample code		**DENV2_ENV_2x Glyco recombinant**	**Zika_ENV_recombinant Antigen; Lot #20161213**
#	**N-glycan structure**	**mol (%)**	**mol (%)**
6	diantennary with α1,6-Fuc with 1x α1,3-Fuc	5.0	6.1
7	triantennary w/o 3 β-Gal with α1,6-Fuc	0.6	0.4
8	triantennary w/o 2 β-Gal with α1,6-Fuc	1.6	2.9
9	triantennary w/o 1 β-Gal with α1,6-Fuc	3.9	7.5
10	triantennary with α1,6-Fuc	8.8	7.3
11	tetraantennary w/o 4 β-Gal with α1,6-Fuc	1.0	1.9
12	tetraantennary w/o 3 β-Gal with α1,6-Fuc	1.4	2.7
13	tetraantennary w/o 2 β-Gal with α1,6-Fuc	3.8	6.0
14	tetraantennary w/o 1 β-Gal with α1,6-Fuc	4.9	3.3
15	tetraantennary with α1,6-Fuc	15.8	2.6
16	tetraantennary with one LacNAc repeat	2.0	0.5
	oligomannosidic N-glycans	**0.1**	**0.8**
17	Man5GlcNAc2	0.1	0.8
	hybrid-type N-glycans	**n.d.***	**n.d.***
	not identified	38.5	42.6
X1	-	0.1	0.1
X2	-	0.4	1.5
X3	-	1.0	2.3
X4	-	3.9	8.8
X5	-	4.0	8.2
X6	-	2.5	6.5
X7	-	1.1	1.1
X8	-	2.4	3.7
X9	-	7.4	4.4
X10	-	12.9	5.0

Sample code		CV94	CV95
Sample code		DENV2_ENV_2x Glyco recombinant	Zika_ENV_recombinant Antigen; Lot #20161213
#	N-glycan structure	mol (%)	mol (%)
X11	-	2.8	1.0
	sum	100.0	100.0
* n.d. = not detected.			

Site Occupancy Analysis of the glycans:

[0121] Site occupancy was determined by LC-MS measurement of tryptic peptides. The analysis was based on the LC-MS measurement of tryptic or Endo Lys-C generated peptides liberated from proteins de-N-glycosylated enzymatically by PNGase F. Since PNGaseF is a glycoamidase, the asparagine (N) becomes converted to an aspartic acid residue (D). Quantification was done by creation of extracted ion chromatograms (EICs). The EICs were generated using the theoretical m/z values of differently charged target peptides within a mass window of +/- m/z of 0.01. In order to compare the peptide intensity with the specifically modified counterpart generated by de-N-glycosylation, the area of the peak of the EIC was used. The ratio / extent of modification was then calculated as follows: extent of modification = [area under EIC of modified peptide] / ([area under EIC of modified peptide] + [area under EIC of unmodified peptide]).

[0122] Sequence numbering is by protein rather than the polyprotein sequence numbering convention, with W101 (at the very tip of the fusion loop) as a useful reference point. Sites are numbered according to their appearance in the linear sequence starting at the N-terminus, such that in dengue (pCRO22, GlycoThera sample number CV94) there were two additional sequons comprising sites 2 and 3. The Occupancy of the natural WT N-glycosylation sites was confirmed to be 100% and 99% for site 1 and site 4, respectively. The added N-glycosylation sites 2 and 3 (in the fusion loop) are located on one tryptic peptide (T15) and the occupancy was 38% (both sites) and additional 51% where only one of the two sites were N-glycosylated. In all 89% of the fusion loops had at least one glycan.

[0123] In the case of Zika, the occupancy of the N-glycosylation sites was confirmed to be 99.5% and 100% for the added 'site1' (residue 100, fusion loop) and site 2 (residue 154 the glycan naturally present), respectively. Site occupancy of the programmed glycosylation sequons was deduced from PNGase digestion and its effects on the mass of tryptic peptide fragments (whereby the amide NH_2 group of the asparagine side chain is lost and converted to a hydroxyl group). (In the following sequences programmed sequons are in bold). In the hyperglycosylated dengue 2 exodomain the relevant tryptic peptide was T15, i.e., the 15^{th} tryptic peptide (G**N**$_{101}$**GS**GCGL**N**$_{108}$**GS**GGIVTCAMFTCK$_{122}$ (SEQ ID NO: 35) - containing the substituted N residues at 101 and 108. In the hyperglycosylated Zika exodomain (with a single introduced glycosylation sequon 'NHT') the relevant peptide was T10 (**N**$_{100}$**HT**NGCGLFGK$_{110}$ (SEQ ID NO:

36)).

[0124] These findings of efficient introduction of large and complex glycans into the fusion loop of dengue and Zika exodomain proteins strengthened our expectation that these proteins would neither bind to the fusion loop, nor elicit fusion-loop antibodies, giving confidence that B-cells or antibodies capable of recognising the wild type versions of the fusion loop would not engage with the glycosylated forms of the invention. This scenario is markedly different from mere introduction of mutations into the fusion loop, because by imposing one or more large additional glycan structures into the fusion loop, the resulting variant fusion loop cannot bind antibodies or B-cell receptors or generate fusion loop antibodies reactive with the wild type versions of the fusion loop. This was fully confirmed in later examples. This strategy may also be contrasted to deleting domains I and II from the structure of the protein, as these domains also contribute neutralising epitopes and T-cell epitopes useful for anamnestic immune responses upon encounter with flaviviruses in the wild, while preconditioning the immune system in such a way as to avoid the dangerous dominance of the fusion loop in immune responses to natural virus infections or to other vaccines.

Table 4: list of m/z values used for creating Extracted-Ion-Chromatograms (EIC) for N-glycosylation-site occupancy for dengue-2

ID	**Amino Acid Range**	**Amino acid sequence**	**Theor. mass in Da**	**m/z values used for EIC $[M+ n H]^{n+}$**
Site 1				
T10	[65-73]	L65T**N**67TTTESR73 (SEQ ID NO: 37)	1022.511	1022.511;
T10	[65-73]	L65T**D**67TTTESR73(SEQ ID NO: 38)	1023.495	1023.495;
Site 2+3				
T15	[100-122]	G100**N**101GSGCGL**N**108GSGGIVTCAMFTCK122 (SEQ ID NO: 39)	2304.983	1152.995; 768.999
T15 1x de-N	[100-122]	G100**D**101GSGCGL**N**108GSGGIVTCAMFTCK122 (SEQ ID NO: 40) *OR*	2305.967	1153.487; 769.327
		G100**N**101GSGCGL**D**108GSGGIVTCAMFTCK122 (SEQ ID NO: 41)		
T15 2x de-N	[100-122]	G100**D**101GSGCGL**D**108GSGGIVTCAMFTCK122 (SEQ ID NO: 42)	2306.951	1153.979; 769.655
Site 4				
T18	[129-157]	V129VQPENLEYTIVITPHSGEEHAVG**N**153DTGK157 (SEQ ID NO: 43)	3133.544	1567.276; 1045.186; 784.142; 627.515
T18 de-N	[129-157]	V129VQPENLEYTIVITPHSGEEHAVG**D**153DTGK157 (SEQ ID NO: 44)	3134.528	1567.768; 1045.514; 784.388; 627.712

Table 5: list of m/z values used for creating Extracted-Ion-Chromatograms (EIC) for N-

glycosylation-site occupancy for Zika

ID	Amino Acid Range	Amino acid sequence	Theor. mass in Da	m/z values used for EIC $[M+ n H]^{n+}$
Site 1				
L4	[94-110]	R94TLVDR99**N**100HTNGCGLFGK110 (SEQ ID NO: 45)	1944.982	1944.98972.99648.992; 5; 9;
L4 de-N	[94-110]	R94TLVDR99**D**100HTNGCGLFGK110 (SEQ ID NO: 46)	1945.966	1945.96973.48649.326; 7; 7;
Site 2				
T16	[139-164]	I_{139}MLSVHGSQHSGMIV**N**$_{154}$DTGHETDENR$_{164}$ (SEQ ID NO: 47)	2864.305	1432.65955.44716.836; 0; 2;
T16 de-N	[139-164]	I_{139}MLSVHGSQHSGMIV**D**$_{154}$DTGHETDENR$_{164}$ (SEQ ID NO: 48)	2865.289	1433.14955.76717.078; 8; 8;

Table 6: site occupancy (% occupation) for dengue-2 (sites 2 and 3 are in the fusion loop)

Rate of N_glycosylation site occupancy [%]					
Sample	**GT-code**	**N-glycosylation site [peptide]**			
		Site 1 N_{67} [T10]	**Site 2+3 N_{101}, N_{108} [T15]**	**Site 2 or 3 N_{101} or N_{108}**	**Site 4 N_{153} [T15]**
DENV2_ENV	CV94	100	38	51	99
(collectively, 89% of molecules have a glycan or two in the fusion loop. N101 replaced W101 of the WT sequence; N108 replaced F108 of the wild type sequence)					

Table 7: site occupancy (% occupation) for Zika (site 1 is in the fusion loop)

Rate of N-glycosylation site occupancy [%]			
Sample	**GT-code**	**N-glycosylation site [peptide]**	
		Site 1 N_{100} [L4]	**Site 2 N_{154} [T16]**
Zika_ENV	CV95	99.5	100
(99.5% of molecules have a single glycan in the fusion loop; N100 replaced G100 of the WT sequence)			

Example 4 (Fig. 4) Immunogenicity of select glycoengineered dengue proteins 1, 2, 3 and 4 and Zika in direct ELISA.

[0125] Female Balb-c mice were immunized with PBS (negative control) and various dengue and Zika formulations of the hyperglycosylated exodomain proteins on Alhydrogel, alone (Zika mono) and in combination (Penta-) and as naked DNA (DNA). Alhydrogel formulations of proteins were injected subcutaneously (s.c.) in a total volume of 200 ul and naked DNA (comprising plasmids pCRO21, pCRO22, pCRO23 and pCRO24 of dengue plus pCRO28 representing Zika) was injected intramuscularly (i.m.) in a total volume of 50 ul for pentavalent DNA (representing 5 micrograms of each plasmid immunogen). Pentavalent protein combinations contained 5 ug amounts per dose of each hyperglycosylated exodomain, and monovalent (Zika) contained 10 ug per dose. Mice were dosed three times, once at each of day 0, day 14 and day 21. The legend at the bottom right of figure 4 denotes the composition of each immunogen. The title of each panel denotes the antigen used on the solid phase ELISA plate. (Wild type recombinant VLPs were used both as immunogens, Group 4, and as antigens in Figure 4). Mice were bled retro-orbitally at the intervals indicated and serum was collected for ELISA and PRNT assays.

[0126] The Balb-c Mice were immunized with DNA and protein representations of the glycoengineered exodomains and with the corresponding VLPs (i.e. VLPs representing the wild type sequences) from The Native Antigen Company Ltd, Oxford, UK (with no extra glycans, and exposed fusion loops) as positive control. These VLPs (see Table 8, used as both immunogens and also as test antigens in the ELISA tests of Figure 4) also contain multiple additional epitopes not present in the exodomains, notably epitopes of the pre-membrane protein prM.

Table 8.

Group (n=5) female Balb-c mice	**Immunogen**	**Route of immun-ization**	**Doe**	**Injectate volume**	**Alhydrogel* adjuvant** (2% w/v aqueous alhydrogel suspension)(ul)
1	Pentavalent glycoengineered DNA ('Penta-DNA' in figures)	i.m., in 10mM Tris-HCl pH 7.4	50ug of each plasmid (250 ug total)	50 ul	None
2	Pentavalent glycoengineered proteins (Penta-Prot)	s.c.	5 ug of each protein (25 ug in total)	200 ul	50
3	Monovalent Zika glycoengineered protein (Zika-mono)	s.c.	10 ug of Zika protein	80 ul	20
4	Pentavalent wild type VLP (Penta VLP)	s.c.	5 ug of each VLP (25ug in total)	200 ul	50

Group (n=5) female Balb-c mice	**Immunogen**	**Route of immun-ization**	**Doe**	**Injectate volume**	**Alhydrogel* adjuvant** (2% w/v aqueous alhydrogel suspension)(ul)
5	PBS	s.c.	0	200 ul	none

[0127] There was little antibody response to naked DNA representing the five exodomains - as expected in the absence of delivery assistance from liposomal formulation, gene-gun or electroporation technology. Antibody responses to naked DNA were evident against dengue 1, 2 and 3 native VLPs, and not against Zika and dengue 4 VLPs. However these results served to demonstrate the potential utility of these DNA encoded antigens (all of them) with appropriate delivery systems. The assay is naturally more sensitive to detect immune responses to VLPs, due to the presence of additional epitopes (noted above), such that, as expected, antibody responses to the VLP antigens were uniform and very strong in the VLP-immunised 'Group 4'. However, so too were responses to the novel glycoengineered exodomain proteins of the present invention, which gave strong, balanced immune responses against all five components (dengue serotypes 1,2,3 and 4 plus Zika) with the pentavalent immunogen formulation. Responses were uniformly high to the exodomain immunogens (pentavalent protein and monovalent Zika) and there were no non-responders. Also, the response to Zika in the monovalent-Zika-hyperglycosylated-exodomain-immunized group (10µg dose) was modestly higher than that in the pentavalent protein group where the same exodomain was used at half the dose. This finding indicates a favorable lack of competition among the serotypes in the generation of type specific immune responses (this is a known problem with live attenuated flavivirus vaccine approaches, such as Dengvaxia, where immune responses to dengue serotype 2 are problematically low).

[0128] For direct ELISA (Figure 4) to measure murine antibodies against dengue and Zika viruses Nunc™ Flat 96-Well Microplates, Thermoscientific, Cat. No. 269620, were coated with VLPs (from The Native Antigen Company (Oxford)) at a concentration of 0.5 µg/ml in bicarbonate-carbonate buffer (pH 9.4 - 9.6) containing sodium bicarbonate at 4.43g/l and sodium carbonate at 1.59g/l, at 100µl/well for 2h at room temperature. Plates were aspirated and blocked with 2% neutral BSA (SigmaAldrich A7906) in Dulbecco's phosphate buffered saline (PBS, ThermoFisher-Gibco 14190136) (PBS-BSA). The blocking buffer was used as diluent for the testing of mouse sera diluted at concentrations of 1/100 and 1/10,000 (duplicates at each concentration). Plates were washed with PBS containing 0.05% Tween-20 detergent (Sigma-Aldrich) (PBS-Tween) after each incubation (blocking, diluted serum incubation, conjugate incubation) by filling and emptying the wells five times with PBS-Tween. After serum incubation and washing, a secondary antibody conjugate was applied in PBS-BSA (goat anti-mouse IgG HRP conjugate BioRad 103005) at a dilution of 1:4000. After washing the plate a final time, substrate for horseradish peroxidase (HRP) was added (3,3',5,5'-tetramethylbenzidine, TMB, Sigma-Aldrich T00440), and stopped with 0.16M sulfuric acid after 20min incubation at room temperature. Incubations were conducted on a mixer (Grant Bio, PMS-1000 at 500rpm approx.). Absorbance of the stopped reaction was read at 450nm.

[0129] Antibody responses were calibrated against fusion loop antibody 4G2 (The Native Antigen

Company Ltd, Oxford) with dengue VLP representing serotype 2 on the solid phase at 0.5 micrograms per ml coating concentration. Units of antibody measurement "IgG antibody titre" are micrograms per ml 4G2-equivalent in undiluted serum, determined by interpolation of the standard curve using a four-component polynomial regression fit (AssayFit, IVD Tools). At day 42, antibody responses reached 10^4-10^5 for the hyperglycosylated exodomain immunogens (a notional 10 mg per ml - 100 mg per ml in neat serum). These concentrations (taken literally) are unattainably high since the IgG concentration of mouse serum is only 2-5 mg per ml, and probably reflect the higher affinity or avidity of the antibodies generated compared to the antibody, 4G2, used for standardization, or may reflect better epitope exposure (4G2's fusion loop epitope being semi-crytpic in the structure of VLPs and virions). Nevertheless the 4G2 calibration serves a useful purpose allowing the assay to be run from time to time, controlling for such variables as batch to batch variation in the conjugate - (an anti-IgG-Fc horseradish peroxidase conjugate made from polyclonal antibodies which vary by batch). This is more reliable than quoting antibody 'titres' based on a threshold absorbance value which are very conjugate-batch and antigen-batch dependent, and may vary further among conjugates sourced by different manufacturers.

[0130] A further aspect of these observations is that the antibodies generated are of the IgG class demonstrating class-switching (even at day 14) from IgM, for all of the protein immunogens. This is an essential component of the B-cell memory response, important for the development of vaccines. A further aspect of these findings is that the antibodies generated by exodomain protein immunogens (and to some extent the DNA immunogens) strongly recognize the native form of the VLP antigens, which also lack His tags, ruling out the possibility of false positives due to anti-His-tag responses. This proves that both the dengue and Zika exodomain materials represent native epitopes of the exodomain proteins that are immunogenic in generating anti-viral (VLP) antibodies. These results suggest that other nucleic acid encoded forms of the hyperglycosylated exodomain species, e.g., liposomal RNA or lipoplex RNA, would also generate desirable antibody responses against virions (VLPs) and viruses.

[0131] There was specificity in the immune response to the Zika monovalent hyperglycosylated exodomain, which generated higher antibody titres against the homologous Zika VLP than to other VLPs, despite the known cross-reactivity of these various viruses with antibodies. This is a favourable result since type-specific anti-Zika antibodies are known to have better neutralizing activity generally than dengue-cross-reactive ones. Also, as seen in the antibody-responses to the Zika-monovalent hyperglycosylated exodomain at the later time points (after two or three doses), there was a degree of cross-reactivity against dengue strains that developed over time, raising the potential for generation of beneficial cross-reactive neutralizing responses, *excluding the fusion loop epitope* (which was not recognized by antibodies generated by hyperglycosylated exodomain species as demonstrated in the data that follows in later examples).

Example 5 (Fig. 5) Avoidance of recognition of the glycoengineered proteins by fusion loop antibodies, and retention of neutralizing epitopes.

[0132] An ELISA test (of Fig. 5) was devised employing oriented capture of His-6-tagged exodomain proteins on the solid phase (the VLPs of Fig. 4 do not have His-tags). Unless otherwise specified, conditions were the same as for the ELISA test of Example 4 and Fig. 4. 8-well strip

ELISA plates (Dynex) were coated with rabbit monoclonal anti-His-6 tag (Anti-6X His tag® antibody [HIS.H8] (ab18184) Abcam) for 1h at room temperature and then overnight at a concentration of 1 µg/ml in bicarbonate-carbonate coating buffer. Plates were washed and then exposed to Starting Block (ThermoFisher 37538) 30min at room temperature, and then to the various exodomain proteins, all having a C-terminal hexahistidine tag, at a concentration of 0.5µg/ml, for 2h at 37 degrees then at 4 degrees overnight. Antibodies were added to appropriate wells in 0.4% BSA in PBS-Tween and incubated for 2h at 37 degrees. Next a secondary antibody conjugate (rabbit-anti-mouse-HRP IgG H&L, Abcam ab97046), for mouse antibodies, was applied in 0.4% BSA in PBS-Tween, at a dilution of 1/10,000. For human serum, the dilution factor was 1/1000 in PBS-Tween 0.4% BSA followed by goat anti-human IgG Fc (HRP) preadsorbed (Abcam ab98624) at 1/20,000. Secondary antibody HRP conjugates were incubated for 2h at 37 degrees. The plate was washed between exposure to successive reagents. Finally TMB substrate was added and stopped after 10 min at room temperature.

[0133] Antigens were as follows: wild type dengue exodomains representing dengue serotypes 2 and 4 were from The Native Antigen Company (DENV2-ENV, DENV4-ENV); 'HX' designated exodomains (hyperglycosylated exodomains) were the selected set of Excivion exodomains of the present disclosure (pCRO21-24 for dengue, pCRO28 for Zika). Prospec Zika was a non-glycosylated bacterial exodomain from Prospec of Israel (zkv-007-a), and Aalto Zika was an insect (Sf9 cell) derived Zika exodomain (AZ6312- Lot3909). Mouse monoclonal antibodies against Zika virus exodomain were as follows: Aalto Bioreagents AZ1176-0302156-Lot3889; Z48 and Z67 were neutralizing antibodies described by Zhao et al, Cell 2016 (The Native Antigen Company ZV67 MAB12125 and ZV48 MAB12124). Antibody 4G2 is an anti-dengue-serotype-2 antibody recognizing the fusion loop (The Native Antigen Company AbFLAVENV-4G2).

[0134] Fig 5a demonstrates the sensitive detection of wild type exodomains of dengue 2 and 4 by antibody 4G2, giving a signal significantly above background even at very low concentrations (250 pg/ml). In contrast, the hyperglycosylated exodomains gave no detectable signal at any of the concentrations tested (5a). This side-by-side comparison of the wild-type and fusion-loop-glycosylated (HX) exodomains demonstrates that the latter fail to react with this classical fusion loop antibody (which is highly dependent on Leucine 107, Stiasny K et al., J Virol 2006 80:19 9557-68, intolerant of D,T or F at that position), even despite the presence of 11% of non-glycosylated (albeit mutated) fusion loop in the dengue-2 HX exodomain used (refer to example 3 for glycosylation site occupancy data). This demonstrates that the mutations employed, even without the glycans, are sufficient to prevent the binding of this particular fusion loop antibody (4G2). However, given the clonal diversity of human antibodies, ultimately it will be preferable to employ the glycosylated forms as an additional layer of surety that fusion loop antibodies capable of recognizing wild type fusion loops of flaviviruses will not be generated in man with these novel immunogens when used as vaccines.

[0135] The data of Fig 5b&c also demonstrate that, in the case of Zika, the HX version of the exodomain reacts with all three Zika monoclonal antibodies, including the two neutralizing epitopes ZV48 (Z48) and ZV67 (Z67). This demonstrates that the Zika HX exodomain has retained these neutralizing epitopes, plus the Aaalto antibody epitope, despite the drastic changes wrought to the structure of the fusion loop by glycan insertion. Moreover, this Zika HX exodomain fails to react with 4G2, as do the four dengue HX exodomains, confirming that this epitope has been effectively

cloaked in all five HX proteins.

[0136] The data of Fig 5b&c, with respect to the Zika human convalescent serum tested are also diagnostically informative. This serum was a gift from Mark Page of NIBSC selected for its high PRNT activity against Zika and its high levels of Zika NS1 antibody. The data of Fig 5b&c demonstrate that this Zika convalescent serum strongly recognizes, indeed prefers the dengue-2 wt exodomain over other antigens in the test. This observation demonstrates the diagnostic utility of the HX series of proteins, and indicates that this patient had previously also been exposed to another flavivirus other than Zika. In fact it suggests that that other flavivirus was not dengue because the Zika convalescent serum (unlike the dengue convalescent serum) fails to react with the hyperglycosylated exodomain forms of dengue. The fusion loop antibodies in the Zika convalescent serum must therefore have originated from exposure to a third flavivirus, such as yellow fever (by vaccination or infection) or West Nile virus, both of which are prevalent in Trinidad where this serum was collected.

[0137] A further aspect of the data of Fig 5b&c are that the Zika HX antigen has the capacity to selectively inform the presence of neutralizing antibodies, since the 4G2 fusion loop epitope has been effectively cloaked, while neutralizing epitopes noted above, have been retained.

[0138] The HX Zika exodomain protein and likely therefore the dengue HX exodomain proteins will therefore have the capacity to inform the development and deployment of Zika and dengue vaccines. In the case of the latter, the HX antigens of the test will be useful in identifying persons that are naïve to dengue and who might be spared vaccination with the currently licensed DengVaxia® anti-dengue vaccine, in order to reduce the risk of predisposition to subsequent dengue haemorrhagic fever (whereby the vaccine acts as a silent primary dengue infection). Such test may extend the utility of DengVaxia to younger persons (currently it is only licensed to children greater than 9 years of age), or to naïve persons in non-endemic territories such as Europe and the USA (e.g. for use in traveller populations in whom DengVaxia vaccination is not currently advocated).

Example 6 (Fig 6) Avoidance of generation of fusion-loop antibodies by the glycoengineered proteins.

[0139] An ELISA test was established to measure the binding of polyclonal antibodies against the fusion loop (represented in this example by dengue serotype-3 VLP on solid phase ELISA plates).

[0140] A competition ELISA was set up using biotinylated 4G2 (Integrated Biotherapeutics) which was detected using streptavidin-horseradish peroxidase conjugate. Dengue serotype 3 VLP (The Native Antigen Company) which reacts with 4G2 slightly better than the immunizing serotype dengue-2 VLP was used as antigen coated at 0.5 ug per ml on the solid phase. Pooled sera (from the groups of Fig. 4) or unlabeled 4G2 (as standard) were titrated at various dilutions (from 1/10 as the top concentration of the serum pools) to determine their capacity to compete with biotinylated 4G2 for binding to the fusion loop. Similar standard curves were generated (not shown) using Zika VLP and dengue-2 VLP wild type recombinant materials as antigen, underscoring the generality of this phenomenon (cross-reactivity of fusion loop antibodies) across the flaviviruses of interest.

[0141] In this assay (Figure 6) the ability of unlabeled 4G2 to compete for binding to solid phase antigen was demonstrated using biotinylated 4G2 and streptavidin-HRP conjugate (Kirkegaard and Perry KPL KPL 14-30-00 at 1/3000). Unless otherwise specified, conditions were as for Example 4. First, a sample of 4G2 was biotinylated according to manufacturer's instructions using the BioRad EZ-link NHS-PEG4 biotinyation kit (21455) using a molar ratio of reactants of 30:1. Unlabelled antibody and biotinylated antibody were allowed to compete in an overnight room temperature incubation for binding to solid phase antigen. Antigen-coated plates were exposed in parallel to dilutions of standard antibody (four or five-fold serial dilutions of 4G2, unlabeled). Biotinylated antibody was used at a concentration of 100 ng/ml.

[0142] Figure 6 demonstrates that antibodies raised against pentavalent VLPs on Alhydrogel, containing VLPs of all four dengue serotypes plus Zika, generate abundant fusion loop antibodies. It can be calculated from these data (assuming similar affinities of 4G2 and raised antibodies) that the VLP-immunised sera contain approximately 100 micrograms per ml fusion loop antibody, which is the maximum amount generally for viral antibodies in a polyclonal antiserum. In contrast, none of the other groups generate significant amounts of fusion loop antibodies whose binding is mutually exclusive with 4G2. In particularly the pentavalent (HX) exodomain proteins of the present disclosure do not generate fusion loop antibodies as assessed in this test, and neither does the monovalent Zika (HX) protein, despite generating very substantial antibody responses to the VLP antigens used in the competition ELISA test. In the case of Zika, inhibition was detectable only at the highest concentration tested, indicating a >1000 fold advantage in avoidance of fusion loop antibodies compared to VLP immunogens, if this single point at 1/10 serum dilution is (for the sake of argument) deemed to be significant.

[0143] The data of Figure 6 demonstrate that a dengue vaccine (or a Zika vaccine) of the invention would not prime for antibody responses to the conserved fusion loop. This is in contrast with natural primary dengue infections that prime for subsequent haemorrhagic fever upon encounter with a second serotype of dengue. Such antibody responses to natural primary dengue infections are poorly neutralizing or non-neutralizing at physiological concentrations of antibody and are particularly implicated in the causation of antibody-dependent enhancement of dengue infection and disease by allowing antibody-complexed virions to enter and infect myeloid cells via Fc-receptors, while failing to prevent them infecting other host cells.

Example 7 (Figure 7) Generation of neutralising antibodies by the glycoengineered dengue and Zika proteins.

[0144] Serum pools from Example 4 were tested for their ability to neutralize dengue serotype 2 and Zika viruses using Vero cells in plaque reduction neutralization tests (PRNT).

[0145] In the case of dengue, the dengue serotype 2 strain used to infect the Vero cells (D2Y98P) was a different serotype-2 strain (non-homologous) from the sequence of the immunizing dengue 2 strain of the VLPs and exodomains. In the groups expected (from Example 4) to generate dengue neutralizing antibodies (namely pentavalent protein and pentavalent VLPs, Groups 2 & 4) there was potent neutralization of the 'off target' dengue test virus. In the case of Zika there was significant

(albeit partial) neutralization as expected from the results of Example 4, in groups shown to contain antibodies that recognized native Zika VLPs (namely pentavalent protein and pentavalent VLPs, Groups 2, 3 & 4). Due to limitations on sample volume, the maximum concentration of serum that was tested was 1/50, such that in interpreting these results this factor needs to be taken into consideration (i.e. that there would be higher neutralizing capability in the blood of the immunized animals).

Table 9. Immunogenicity Study Design

Group (n=5)	Vaccine*	Vaccine Schedule	Dosage	Bleeds	Readout
1	Pentavalent glycoengineered DNA	On days 0, 14, & 21 via IM route	250µg total DNA (50µg of each)	Test bleed for serum on Days 14 & 21. Terminal bleed on Day 42.	Measurement of antibodies against ZIKV & DENV 1-4 via ELISA
2	Pentavalent glycoengineered proteins on Alhydrogel		25µg total protein (5µg each)		
3	Monovalent Zika glycoengineered protein on Alhydrogel		10µg protein		
4	Pentavalent wild type VLP on Alhydrogel		25µg total VLPs (5µg each)		
5	PBS		-		

[0146] PRNT Assay was performed as follows. Five mouse serum samples were pooled by taking an equal volume of individual samples in each group (sample description in next slide) and were then tested against ZIKV and DENV, respectively. Twelve two-fold serial dilutions of each serum sample in duplicates starting at 1:50 were prepared for the two-hour inoculation with virus. The serum-virus mix was then added to Vero cells seeded in 24-well culture plates and incubated at 37°C in a humidified 5% CO_2 atmosphere. The Vero cells were fixed on 3 days post incubation (dpi) for ZIKV PRNT and 4 dpi for DENV PRNT. Viral plaque was determined by crystal violet staining.

[0147] Potent inhibition of infection by dengue was observed in the group immunized with hyperglycosylated exodomain proteins of the present disclosure (Penta-prot). Zika immunized animals generated antibodies that did not prevent dengue infection of Vero cells, illustrating the type-specific nature of antibodies generated by these novel immunogens. These Zika antibodies (from the Zika monovalent group and from the pentavalent proteins group) were significantly protective of infection of Vero cells by Zika virus. As expected, PBS-sham-immunised animals did not give rise to protective antibodies, nor did pentavalent DNA administered intramuscularly. This latter result may have been due to the low concentrations of antibodies generated by naked DNA, as expected from intramuscular injection (as distinct from gene-gun or electroporation strategies, or strategies incorporating encoded proteins as molecular adjuvants).

[0148] The results of Example 6 (generation of neutralizing antibodies) combined with those of Example 5 (lack of recognition by or generation of fusion loop antibodies) by the hyperglycosylated Exodomain proteins of the invention strongly suggest that these proteins can form the basis of a protective vaccine for dengue or Zika viruses (or, in combination, for both viruses) without the generation of fusion loop antibodies, which are particularly implicated in antibody-dependent enhancement of infection.

Example 8 (Figure 8) Reaction of convalescent dengue or Zika serum with immobilized Zika and dengue wild-type (WT) and hyperglycosylated (HX) exodomain proteins

[0149] The ELISA reactivity of antibodies in a dengue convalescent serum with immobilized Zika and dengue wild-type (WT) and hyperglycosylated (HX) exodomain proteins oriented on the solid phase by capture with a rabbit anti-His-tag monoclonal antibody (Figure 8, upper panel), in the presence (grey bars, right of each pair) and absence (black bars, left of each pair) of competing mouse monoconal flavivirus fusion loop antibody 4G2 (an anti-dengue-serotype-2 monoclonal antibody) at a concentration of 10µg/ml during serum incubation. Human sera were tested at a constant concentration of 1/1000.

[0150] The ELISA reactivity of antibodies in a Zika convalescent serum with immobilized Zika and Dengue wild-type (WT) and hyperglycosylated (HX) exodomain proteins (Figure 8, lower panel) in the presence (grey bars) and absence (black bars) of competing mouse monoclonal flavivirus fusion loop antibody 4G2. Conditions and labelling are the same as for the upper panel. Error bars are standard error.

[0151] The results show that:

1. 1) the HX Zika antigen of the invention is not susceptible to the off-target recognition of WT Zika exodomain by the convalescent dengue serum.
2. 2) The off-target recognition of WT Zika exodomain (Aalto) by dengue serum is a fusion-loop directed phenomenon because it is abolished by 4G2 (anti-fusion loop monoclonal antibody) in solution phase at a concentration that causes 80% inhibition against VLPs (10 micrograms per ml). (The antigen on the solid phase in this instance is exodomain rather than VLP).
3. 3) The 'Zika' convalescent serum does not recognize any of three Zika exodomains, but it strongly recognizes WT dengue 2 and WT dengue 4. In the Example 6 the HX Zika antigen of the invention and Aalto's Zika exodomains exhibit reaction with conformation-dependent anti-Zika neutralising antibodies). This demonstrates that this particular Zika serum (positive for Zika plaque neutralisation and Zika NS1 antibodies) is from a subject also exposed to another flavivirus. Because the Zika convalescent serum (unlike the dengue convalescent serum) does not recognize the fusion-loop-cloaked exodomains, it can be concluded that this other flavivirus is not dengue.
4. 4) The off-target recognition of WT dengue-2 and dengue-4 exodomains by the human Zika convalescent serum is not seen with the HX-cloaked dengue exodomains of the invention. This suggests that it is fusion loop directed and would show false positive in other flavivirus diagnostic tests that do not use glycan-cloaked proteins in accordance with the invention.
5. 5) The off-target recognition of WT dengue-2 and dengue-4 exodomains by the human Zika

convalescent serum is blocked completely by 4G2 showing that it is a fusion loop directed phenomenon.

6. 6) The dengue convalescent serum recognizes WT 2 & 4 indiscriminately, but clearly prefers the d2 exodomain out of the set of 4. This demonstrates that the fusion loop antigens of the invention have superior selectivity (compared to their wild type equiavalent forms) to discriminate between dengue serotypes, due to the glycan cloaking of the fusion loop.

Sequence Listing Free Text

[0152]

SEQ ID NO: 1 DRGWGNGCGLFGK

SEQ ID NO: 2 DRG**N**G**S**GCGL**N**G**S**,

SEQ ID NO: 3 DRG**N**G**S**GCGLFGK

SEQ ID NO: 4 DRGWGNGCGL**N**G**S**

SEQ ID NO: 5 DR**NHT**NGCGLFGK.

SEQ ID NO: 6 DRGWGNGCG**N**H**T**K

SEQ ID NO: 7 pCRO25 fragment CKRTLVDRGNGSGCGLNGSGSLVTCAKFA

SEQ ID NO: 8 pCRO29 fragment CKRTLVDRGWGNGCGNHTKGSLVTCAKFA

SEQ ID NO: 9 pCRO30 fragment CKRTLVDRGNGSGCGLFGKGSLVTCAKFA

SEQ ID NO: 10 pCRO31 fragment CKRTLVDRGWGNGCGLNGSGSLVTCAKFA

SEQ ID NO: 11 DRGWGNNCTLFGK

SEQ ID NO: 12 DRGWGNNCSLFGK

pCRO21 (SEQ ID NO: 13)
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pCRO22 (SEQ ID NO: 14)
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     4621   AACTCGGTCG   CCGCATACAC   TATTCTCAGA   ATGACTTGGT   TGAGTATTCA
CCAGTCACAG
     4681   AAAAGCATCT   TACGGATGGC   ATGACAGTAA   GAGAATTATG   CAGTGCTGCC
ATAACCATGA
     4741   GTGATAACAC   TGCGCCCAAC   TTACTTCTGA   CAACGATTGG   AGGACCGAAG
GAGCTAACCG
     4801   CTTTTTTGCA   CAACATGGGG   GATCATGTAA   CTCGCCTTGA   TCGTTGGGAA
CCGGAGCTGA
     4861   ATGAAGCCAT   ACCAAACGAC   GAGCGTGACA   CCACGATGCC   TGTAGCAATG
GCAACAACCT
     4921   TGCGTAAACT   ATTAACTGGC   GAACTACTTA   CTCTAGCTTC   CCGGCAACAG
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     4981   GGATGGAGGC   GGATAAAGTT   GCAGGACCAC   TTCTGCGCTC   GGCCCTTCCG
GCTGGCTGGT
     5041   TTATTGCTGA   TAAATCTGGA   GCCGGTGAGC   GTGGGTCTCG   CGGTATCATT
GCAGCACTGG
     5101   GCCCAGATGG   TAAGCCCTCC   CGTATCGTAG   TTATCTACAC   GACGGGGAGT
CAGGCAACTA
     5161   TGGATGAACG   AAATAGACAG   ATCGCTGAGA   TAGGTGCCTC   ACTGATTAAG
CATTGGTAAC

     5221   CGATTCTAGG   TGCATTGGCG   CAGAAAAAAA   TGCCTGATGC   GACGCTGCGC
GTCTTATACT
     5281   CCCACATATG   CCAGATTCAG   CAACGGATAC   GGCTTCCCCA   ACTTGCCCAC
TTCCATACGT
     5341   GTCCTCCTTA   CCAGAAATTT   ATCCTTAAGA   TCCCGAATCG   TTTAAACTCG
ACTCTGGCTC
     5401   TATCGAATCT   CCGTCGTTTC   GAGCTTACGC   GAACAGCCGT   GGCGCTCATT
TGCTCGTCGG
     5461 GCATCGAATC TCGTCAGCTA TCGTCAGCTT ACCTTTTTGG CA
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pCRO30 (SEQ ID NO: 22)
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       61   GGTCATAACA   GCAGCTTCAG   CTACCTCTCA   ATTCAAAAAA   CCCCTCAAGA
CCCGTTTAGA
      121   GGCCCCAAGG   GGTTATGCTA   TCAATCGTTG   CGTTACACAC   ACAAAAAACC
AACACACATC
      181   CATCTTCGAT   GGATAGCGAT   TTTATTATCT   AACTGCTGAT   CGAGTGTAGC
CAGATCTAGT
      241   AATCAATTAC   GGGGTCATTA   GTTCATAGCC   CATATATGGA   GTTCCGCGTT
ACATAACTTA
      301   CGGTAAATGG   CCCGCCTGGC   TGACCGCCCA   ACGACCCCCG   CCCATTGACG
TCAATAATGA
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TCAATAATGA
361 CGTATGTTCC CATAGTAACG CCAATAGGGA CTTTCCATTG ACGTCAATGG
GTGGAGTATT
421 TACGGTAAAC TGCCCACTTG GCAGTACATC AAGTGTATCA TATGCCAAGT
ACGCCCCCTA
481 TTGACGTCAA TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG
ACCTTATGGG
541 ACTTTCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG
CTGATGCGGT
601 TTTGGCAGTA CATCAATGGG CGTGGATAGC GGTTTGACTC ACGGGGATTT
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661 ACCCCATTGA CGTCAATGGG AGTTTGTTTT GGCACCAAAA TCAACGGGAC
TTTCCAAAAT
721 GTCGTAACAA CTCCGCCCCA TTGACGCAAA TGGGCGGTAG GCGTGTACGG
TGGGAGGTCT
781 ATATAAGCAG AGCTGGTTTA GTGAACCGTC AGATCAGATC TTTGTCGATC
CTACCATCCA
841 CTCGACACAC CCGCCAGCgg ccgccaccat gaaggccaat ctactggtgt
tgctgtgtgc
901 ccttgcggcg gcagatgccA TCAGGTGCAT TGGAGTCAGC AACAGGGACT
TCGTCGAAGG
961 CATGTCCGGC GGCACCTGGG TGGATGTGGT GCTCGAACAC GGCGGATGCG
TGACCGTCAT
1021 GGCCCAGGAC AAGCCTACCG TCGATATTGA GCTGGTGACC ACCACAGTGA
GCAACATGGC
1081 CGAAGTGAGA AGCTACTGCT ATGAGGCCTC CATCAGCGAT ATGGCTTCCG
ATTCCAGATG
1141 CCCCACACAG GGAGAGGCTT ATCTGGACAA ACAGTCCGAC ACCCAGTACG
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GCAGCCTCGT
1261 GACATGTGCC AAGTTCGCCT GCAGCAAAAA GATGACCGGC AAGTCCATCC
AGCCCGAGAA
1321 CCTGGAATAC AGGATCATGC TGTCCGTGCA TGGATCCCAG CACTCCGGCA
TGATCGTCAA
1381 CGATACCGGC CACGAGACCG ACGAGAACAG GGCTAAAGTG GAGATCACCC
CCAACAGCCC
1441 TAGAGCCGAA GCTACACTGG GCGGCTTCGG AAGCCTGGGC CTGGATTGCG
AACCCAGGAC

1501 CGGCCTGGAT TTCAGCGACC TGTATTACCT GACCATGAAC AATAAGCACT
GGCTGGTGCA
1561 CAACCAATCG TTCCACGACA TCCCCCTGCC TTGGCATGCT GGCGCCGATA
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1621 TCACTGGAAC AATAAGGAAG CCCTGGTCGA GTTTAAGGAC GCCCACGCCA
AAAGACAGAC
1681 CGTGGTGGTG CTGGGAAGCC AGGAGGGAGC TGTCCACACA GCCCTGGCCG
GAGCTCTGGA
1741 AGCCGAGATG GATGGCGCCA AGGGCAGGCT GAGCTCCGGC CACCTGAAAT
GCAGGCTCAA
1801 GATGGACAAG CTGAGGCTGA AGGGCGTGAG CTACAGCCTG TGCACCGCCG
CTTTCACCTT
1861 TACCAAGATC CCTGCCGAGA CACTGCACGG CACCGTCACC GTGGAGGTGC
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1921 AACCGATGGA CCTTGCAAAG TGCCTGCCCA GATGGCTGTG GATATGCAGA
CCCTCACACC
1981 CGTCGGCAGG CTGATCACCG CCAATCCCGT CATTACCGAG TCCACCGAGA
ACAGCAAGAT
2041 GATGCTcGAG CTCGATCCCC CCTTTGGCGA CAGCTACATT GTGATCGGCG
TGGGCGAGAA
2101 GAAGATCACC CACCATTGGC ACAGAAGCGG CTCCACAggg ggtagcggtg
gtagcggagg
2161 tagccatcac caccatcacc actgagctag CTTGACTGAC TGAGATACAG
CGTACCTTCA
2221 GCTCACAGAC ATGATAAGAT ACATTGATGA GTTTGGACAA ACCACAACTA
GAATGCAGTG
2281 AAAAAAATGC TTTATTIGTG AAATTTGTGA TGCTATTGCT TTATTTGTAA
CCATTATAAG
2341 CTGCAATAAA CAAGTTAACA ACAACAATTG CATTCATTTT ATGTTTCAGG
TTCAGGGGGA
2401 GGTGTGGGAG GTTTTTTAAA GCAAGTAAAA CCTCTACAAA TGTGGTATTG
GCCCATCTCT
2461 ATCGGTATCG TAGCATAACC CCTTGGGGCC TCTAAACGGG TCTTGAGGGG
TTTTTTGTGC
2521 CCCTCGGGCC GGATTGCCAT CTACCGGCAT TGCCGCAGAA AAAAATCCCT
GATGCGACGC
2581 TGCGCGTCTT ATACTCCCAC ATATGCCAGA TTCAGCAACG GATACGGCTT

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2641 CCCACTTCCA TACGTGTCCT CCTTACCAGA AATTTATCCT IAAGGTCGTC
AGCTATCCTG
2701 CAGGCGATCT CTCGATTTCG ATCAAGACAT TCCTTTAATG GTCTTTTCTG
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TACCTTGGGC
2821 TATCGAAACT TAATTAACCA GTCAAGTCAG CTACTTGGCG AGATCGACTT
GTCTGGGTTT
2881 CGACTACGCT CAGAATTGCG TCAGTCAAGT TCGATCTGGT CCTTGCTATT
GCACCCCTTC
2941 TCCGATTACG AGTTTCATTT AAATCAIGTG AGCAAAAGGC CAGCAAAAGG
CCAGGAACCG
3001 TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG
AGCATCACAA
3061 AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA CTATAAAGAT
ACCAGGCGTT
3121 TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCCGACC CTGCCGCTTA
CCGGATACCT
3181 GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAT AGCTCACGCT
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3241 CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG CACGAACCCC
CCGTTCAGCC
3301 CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC AACCCGGTAA
GACACGACTT

3361 ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA GCGAGGTATG
TAGGCGGTGC
3421 TACAGAGTTC TTGAAGTGGT GGCCTAACTA CGGCTACACT AGAAGAACAG
TATTTGGTAT
3481 CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT GGTAGCTCTT
GATCCGGCAA
3541 ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG CAGCAGATTA
CGCGCAGAAA
3601 AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG TCTGACGCTC
AGTCGAACGA
3661 AAACTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA AGGATCTTCA
CCTAGATCCT
3721 TTTAAATTAA AAATGAAGTT TTAAATCAAT CTAAAGTATA TATGAGTAAA
CTTGGTCTGA
3781 CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG ATCTGTCTAT
TTCGTTCATC
3841 CATAGTTGCA TTTAAAATTC CGAACTCTCC AAGGCCCTCG TCGGAAAATC
TTCAAACCTT
3901 TCGTCCGATC CATCTTGCAG GCTACCTCTC GAACGAACTA TCGCAAGTCT
CTTGGCCGGC
3961 CTTGCGCCTT GGCTATTGCT TGGCAGCGCC TATCGCCAGG TATTACTCCA
ATCCCGAATA
4021 TCCGAGATCG GGATCACCCG AGAGAAGTTC AACCTACATC CTCAATCCCG
ATCTATCCGA
4081 GATCCGACGA ATATCGAAAT CGGGGCGCGC CTGGTGTACC GAGAACGATC
CTCTCAGTGC
4141 GAGTCTCGAC GATCCATATC GTTGCTTGGC AGTCAGCCAG TCGGAATCCA
GCTTGGGACC
4201 CAGGAAGTCC AATCGTCAGA TATTGTACTC AAGCCTGGTC ACGGCAGCGT
ACCGATCTGT
4261 TTAAACCTAG ATATTGATAG TCTGATCGGT CAACGTATAA TCGAGTCCTA
GCTTTTGCAA
4321 ACATCTATCA AGAGACAGGA TCAGCAGGAG GCTTTCGCAT GAGTATTCAA
CATTTCCGTG
4381 TCGCCCTTAT TCCCITTTTI GCGGCATTTT GCCTTCCTGT TTTTGCTCAC
CCAGAAACGC
4441 TGGTGAAAGT AAAAGATGCI GAAGATCAGT TGGGTGCGCG AGTGGGTTAC
ATCGAACTGG
4501 ATCTCAACAG CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGCTTT
CCAATGATGA
4561 GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC
GGGCAAGAGC
4621 AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT TGAGTATTCA
CCAGTCACAG
4681 AAAAGCATCT TACGGATGGC ATGACAGTAA GAGAATTATG CAGTGCTGCC
ATAACCATGA
4741 GTGATAACAC TGCGGCCAAC TTACTTCTGA CAACGATTGG AGGACCGAAG
GAGCTAACCG
4801 CTTTTTTGCA CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA
CCGGAGCTGA
4861 ATGAAGCCAT ACCAAACGAC GAGCGIGACA CCACGATGCC TGTAGCAATG
GCAACAACCT
4921 TGCGTAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC CCGGCAACAG

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     4981   GGATGGAGGC   GGATAAAGTT   GCAGGACCAC   TTCTGCGCTC   GGCCCTICCG
GCTGGCTGGT
     5041   TTATTGCTGA   TAAATCTGGA   GCCGGTGAGC   GTGGGTCTCG   CGGTATCATT
GCAGCACTGG
     5101   GGCCAGATGG   TAAGCCCTCC   CGTATCGTAG   TTATCTACAC   GACGGGGAGT
CAGGCAACTA
     5161   TGGATGAACG   AAATAGACAG   ATCGCTGAGA   TAGGTGCCTC   ACTGATTAAG
CATTGGTAAC

     5221   CGATTCTAGG   TGCATTGGCG   CAGAAAAAAA   TGCCTGATGC   GACGCTGCGC
GTCTTATACT
     5281   CCCACATATG   CCAGATTCAG   CAACGGATAC   GGCTTCCCCA   ACTTGCCCAC
TTCCATACGT
     5341   GTCCTCCTTA   CCAGAAATTT   ATCCTTAAGA   TCCCGAATCG   TTTAAACTCG
ACTCTGGCTC
     5401   TATCGAATCT   CCGTCGTTTC   GAGCTTACGC   GAACAGCCGT   GGCGCTCATT
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     5461 GCATCGAATC TCGTCAGCTA TCGTCAGCTT ACCTTTTTGG CA
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pCRO31 (SEQ ID NO: 23)
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       61   GGTCATAACA   GCAGCTTCAG   CTACCTCTCA   ATTCAAAAAA   CCCCTCAAGA
CCCGTTTAGA
      121   GGCCCCAAGG   GGTTATGCTA   TCAATCGTTG   CGTTACACAC   ACAAAAAACC
AACACACATC
      181   CATCTTCGAT   GGATAGCGAT   TTTATTATCT   AACTGCTGAT   CGAGTGTAGC
CAGATCTAGT
      241   AATCAATTAC   GGGGTCATTA   GTTCATAGCC   CATATATGGA   GTTCCGCGTT
ACATAACTTA
      301   CGGTAAATGG   CCCGCCTGGC   TGACCGCCCA   ACGACCCCCG   CCCATTGACG
TCAATAATGA
      361   CGTATGTTCC   CATAGTAACG   CCAATAGGGA   CTTTCCATTG   ACGTCAATGG
GTGGAGTATT
      421   TACGGTAAAC   TGCCCACTTG   GCAGTACATC   AAGTGTATCA   TATGCCAAGT
ACGCCCCCTA
      481   TTGACGTCAA   TGACGGTAAA   TGGCCCGCCT   GGCATTATGC   CCAGTACATG
ACCTTATGGG
      541   ACTTTCCTAC   TTGGCAGTAC   ATCTACGTAT   TAGTCATCGC   TATTACCATG
CTGATGCGGT
      601   TTTGGCAGTA   CATCAATGGG   CGTGGATAGC   GGTTTGACTC   ACGGGGATTT
CCAAGTCTCC
      661   ACCCCATTGA   CGTCAATGGG   AGTTTGTTTT   GGCACCAAAA   TCAACGGGAC
TTTCCAAAAT
      721   GTCGTAACAA   CTCCGCCCCA   TTGACGCAAA   TGGGCGGTAG   GCGTGTACGG
TGGGAGGTCT
      781   ATATAAGCAG   AGCTGGTTTA   GTGAACCGTC   AGATCAGATC   TTTGTCGATC
CTACCATCCA
      841   CTCGACACAC   CCGCCAGCgg   ccgccaccat   gaaggccaat   ctactggtgt
tgctgtgtgc
      901   ccttgcggcg   gcagatgccA   TCAGGTGCAT   TGGAGTCAGC   AACAGGGACT
TCGTCGAAGG
      961   CATGTCCGGC   GGCACCTGGG   TGGATGTGGT   GCTCGAACAC   GGCGGATGCG
TGACCGTCAT
     1021   GGCCCAGGAC   AAGCCTACCG   TCGATATTGA   GCTGGTGACC   ACCACAGTGA
GCAACATGGC
     1081   CGAAGTGAGA   AGCTACTGCT   ATGAGGCCTC   CATCAGCGAT   ATGGCTTCCG
ATTCCAGATG
     1141   CCCCACACAG   GGAGAGGCTT   ATCTGGACAA   ACAGTCCGAC   ACCCAGTACG
TCTGCAAAAG
     1201   AACCCTGGTG   GACAGAGGCT   GGGGAAACGG   ATGCGGCCTG   aacggatccG
GCAGCCTCGT
     1261   GACATGTGCC   AAGTTCGCCT   GCAGCAAAAA   GATGACCGGC   AAGTCCATCC
AGCCCGAGAA
     1321   CCTGGAATAC   AGGATCATGC   TGTCCGTGCA   TGGATCCCAG   CACTCCGGCA
TGATCGTCAA
     1381   CGATACCGGC   CACGAGACCG   ACGAGAACAG   GGCTAAAGTG   GAGATCACCC
CCAACAGCCC
     1441   TAGAGCCGAA   GCTACACTGG   GCGGCTTCGG   AAGCCTGGGC   CTGGATTGCG
AACCCAGGAC
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1561 CAAGGAATGG TTCCACGACA TCCCCCTGCC TTGGCATGCT GGCGCCGATA
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1621 TCACTGGAAC AATAAGGAAG CCCTGGTCGA GTTTAAGGAC GCCCACGCCA
AAAGACAGAC
1681 CGTGGTGGTG CTGGGAAGCC AGGAGGGAGC TGTCCACACA GCCCTGGCCG
GAGCTCTGGA
1741 AGCCGAGATG GATGGCGCCA AGGGCAGGCT GAGCTCCGGC CACCTGAAAT
GCAGGCTCAA
1801 GATGGACAAG CTGAGGCTGA AGGGCGTGAG CTACAGCCTG TGCACCGCCG
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1981 CGTCGGCAGG CTGATCACCG CCAATCCCGT CATTACCGAG TCCACCGAGA
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2041 GATGCTcGAG CTCGATCCCC CCTTTGGCGA CAGCTACATT GTGATCGGCG
TGGGCGAGAA
2101 GAAGATCACC CACCATTGGC ACAGAAGCGG CTCCACAggg ggtagcggtg
gtagcggagg
2161 tagccatcac caccatcacc actgagctag CTTGACTGAC TGAGATACAG
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2221 GCTCACAGAC ATGATAAGAT ACATTGATGA GTTTGGACAA ACCACAACTA
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2281 AAAAAAATGC TTTATTTGTG AAATTTGTGA TGCTATTGCT TTATTTGTAA
CCATTATAAG
2341 CTGCAATAAA CAAGTTAACA ACAACAATTG CATTCATTTT ATGTTTCAGG
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2401 GGTGTGGGAG GTTTTTTAAA GCAAGTAAAA CCTCTACAAA TGTGGTATTC
GCCCATCTCT
2461 ATCGGTATCG TAGCATAACC CCTTGGGGCC TCTAAACGGG TCTTGAGGGG
TTTTTTGTGC
2521 CCCTCGGGCC GGATTGCTAT CTACCGGCAT TGCCGCAGAA AAAAATGCCT
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2581 TGCGCGTCTT ATACTCCCAC ATATGCCAGA TTCAGCAACG GATACGGCTT
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2641 CCCACTTCCA TACGTGTCCT CCTTACCAGA AATTTATCCT TAAGGTCGTC
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2701 CAGGCGATCT CTCGATTTCG ATCAAGACAT TCCTTTAATG GTCTTTTCTG
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2761 GGGGTCAGAA GTAGTTCATC AAACTTTCTT CCCTCCCTAA TCTCATTGGT
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2821 TATCGAAACT TAATTAACCA GTCAAGTCAG CTACTTGGCG AGATCGACTT
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2881 CGACTACGCT CAGAATTCCC TCAGTCAAGT TCGATCTGGT CCTTGCTATT
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2941 TCCGATTACG AGTTTCATTT AAATCATGTG AGCAAAAGGC CAGCAAAAGG
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3001 TAAAAACGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG
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3061 AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA CTATAAAGAT
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3121 TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCCGACC CTGCCGCTTA
CCGGATACCT
3181 GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAT AGCTCACGCT
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3241 CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG CACGAACCCC
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3301 CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC AACCCGGTAA
GACACGACTT

3361 ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA GCGAGGTATG
TAGGCGGTGC
3421 TACAGAGTTC TTGAAGTGGT GGCCTAACTA CGGCTACACT AGAAGAACAG
TATTTGGTAT
3481 CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT GGTAGCTCTT
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3541 ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG CAGCAGATTA
CGCGCAGAAA
3601 AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG TCTGACGCTC
AGTGGAACGA
3661 AAACTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA AGGATCTTCA
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3721 TTTAAATTAA AAATGAAGTT TTAAATCAAT CTAAAGTATA TATGAGTAAA
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3781 CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG ATCTGTCTAT

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     3901   TCGTCCGATC   CATCTTGCAG   GCTACCTCTC   GAACGAACTA   TCGCAAGTCT
CTTGGCCGGC
     3961   CTTGCGCCTT   GGCTATTGCT   TGGCAGCGCC   TATCGCCAGG   TATTACTCCA
ATCCCGAATA
     4021   TCCGAGATCG   GGATCACCCG   AGAGAAGTTC   AACCTACATC   CTCAATCCCG
ATCTATCCGA
     4081   GATCCGAGGA   ATATCGAAAT   CGGGGCGCGC   CTGGTGTACC   GAGAACGATC
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     4141   GAGTCTCGAC   GATCCATATC   GTTGCTTGGC   AGTCAGCCAG   TCGGAATCCA
GCTTGGGACC
     4201   CAGGAAGTCC   AATCGTCAGA   TATTGTACTC   AAGCCTGGTC   ACGGCAGCGT
ACCGATCTGT
     4261   TTAAACCTAG   ATATTGATAG   TCTGATCGGT   CAACGTATAA   TCGAGTCCTA
GCTTTTGCAA
     4321   ACATCTATCA   AGAGACAGGA   TCAGCAGGAG   GCTTTCGCAT   GAGTATTCAA
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     4381   TCGCCCTTAT   TCCCTTTTTT   GCGGCATTTT   GCCTTCCTGT   TTTTCCTCAC
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     4441   TGGTGAAAGT   AAAAGATGCT   GAAGATCAGT   TGGGTGCGCG   AGTGGGTTAC
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     4501   ATCTCAACAG   CGGTAAGATC   CTTGAGAGTT   TTCGCCCCGA   AGAACGCTTT
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     4561   GCACTTTTAA   AGTTCTGCTA   TGTGGCGCGG   TATTATCCCG   TATTGACGCC
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     4621   AACTCGGTCG   CCGCATACAC   TATTCTCAGA   ATGACTTGGT   TGAGTATTCA
CCAGTCACAC
     4681   AAAAGCATCT   TACGGATGGC   ATGACAGTAA   GAGAATTATG   CAGTGCTGCC
ATAACCATGA
     4741   GTGATAACAC   TGCGGCCAAC   TTACTTCTGA   CAACGATTGG   AGGACCGAAG
CAGCTAACCG
     4801   CTTTTTTGCA   CAACATGGGG   GATCAIGTAA   CTCGCCTTGA   TCGTTGGGAA
CCGGAGCTGA
     4861   ATGAAGCCAT   ACCAAACGAC   GAGCGIGACA   CCACGATGCC   TGTAGCAATG
GCAACAACCT
     4921   TGCGTAAACT   ATTAACTGGC   GAACTACTTA   CTCTAGCTTC   CCGGCAACAG
TTGATAGACT
     4981   GGATGGAGGC   GGATAAAGTT   GCAGGACCAC   TTCTGCGCTC   GGCCCTICCG
GCTGGCTGGT
     5041   TTATTGCTGA   TAAATCTGGA   GCCGGTGAGC   GTGGGTCTCG   CGGTATCATT
GCAGCACTGG
     5101   GGCCAGATGG   TAAGCCCTCC   CGTATCGTAG   TTATCTACAC   GACGGGGAGT
CAGGCAACTA
     5161   TGGATGAACG   AAATAGACAG   ATCGCTGAGA   TAGGTGCCTC   ACTGATTAAG
CATTGGTAAC

     5221   CGATTCTAGG   TGCATTGGCG   CAGAAAAAAA   TGCCTGATGC   GACGCTGCGC
GTCTTATACT
     5281   CCCACATATG   CCAGATTCAG   CAACGGATAC   GGCTTCCCCA   ACTTGCCCAC
TTCCATACGT
     5341   GTCCTCCTTA   CCAGAAATTT   ATCCTTAAGA   TCCCGAATCG   TTTAAACTCG
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     5401   TATCGAATCT   CCGTCGTTTC   GAGCTTACGC   GAACAGCCGT   GGCGCTCATT
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     5461 GCATCGAATC TCGTCAGCTA TCGTCAGCTT ACCTTTTTGG CA
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Hyperglycosylated exodomain D1 (from pCRO21) (SEQ ID NO: 24)

Hyperglycosylated exodomain D2 (from pCRO22) (SEQ ID NO: 25)

Hyperglycosylated exodomain D3 (from pCRO23) (SEQ ID NO: 26)

Hyperglycosylated exodomain D4 (from pCRO24) (SEQ ID NO: 27)

Hyperglycosylated exodomain Zika(from pCRO28) (SEQ ID NO: 28)

SEQ ID NO: 24 >DENV1_Eexo = **pCRO21**

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PTQGEATLVEEQDSNFVCRRTFVDRG**N**G**S**GCGL**N**G**S**GSLLTCAKFKCVTKLEGKIVQYENLKYSVIVTVHTGDQ
HQVGNETTEHGTIATITPQAPTSEIQLTDYGALTLDCSPRTGLDFNEMVLLTMKEKSWLVHKQWFLDLPLPWTS
GASTSQETWNRQDLLVTFKTAHAKKQEVVVLGSQEGAMHTALTGATEIQTSGTTTIFAGHLKCRLKMDKLTLKG
MSYVMCTGSFKLEKEVAETQHGTVLVQVKYEGTDAPCKIPFSAQDEKGVTQNGRLITANPIVTDKEKPVNIETE

PPFGESYIVVGAGEKALKLSWFKKGSTGGGSHHHHHH

SEQ ID NO: 25 >DENV2_Eexo = **pCRO22**
MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYCIEAKLTNTTTESRC
PTQGEPSLNEEQDKRFVCKHSMVDRG**NGS**GCGL**NGS**GGIVTCAMFTCKKNMEGKVVQPENLEYTIVITPHSGEE
HAVGNDTGKHGKEIKITPQSSITEAELTGYGTVTMECSPRTGLDFNEMVLLQMENKAWLVHRQWFLDLPLPWLP
GADTQGSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKG
MSYSMCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLITVNPIVTEKDSPVNIEAE
PPFGDSYIIIGVEPGQLKLNWFKKGSSGGGSHHHHHH

SEQ ID NO: 26 >DENV3_Eexo **= pCRO23**
MRCVGVGNRDFVEGLSGATWVDVVLEHGGCVTTMAKNKPTLDIELQKTEATQLATLRKLCIEGKITNITTDSRC
PTQGEAVLPEEQDQNYVCKHTYVDRG**NGS**GCGL**NGS**GSLVTCAKFQCLEPIEGKVVQYENLKYTVIITVHTGDQ
HQVGNETQGVTAEITPQASTTEAILPEYGTLGLECSPRTGLDFNEMILLTMKNKAWMVHRQWFFDLPLPWASGA
TTETPTWNRKELLVTFKNAHAKKQEVVVLGSQEGAMHTALTGATEIQNSGGTSIFAGHLKCRLKMDKLELKGMS
YAMCTNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHNGRLITANPVVTKKEEPVNIEAEPP
FGESNIVIGIGDNALKINWYKKGSSGGGSHHHHHH

SEQ ID NO: 27 >DENV4_Eexo **= pCRO24**
MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTAKEVALLRTYCIEASISNITTATRC
PTQGEPYLKEEQDQQYICRRDVVDRG**NGS**GCGL**NGS**GGVVTCAKFSCSGKITGNLVQIENLEYTVVVTVHNGDT
HAVGNDTSNHGVTAMITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMKMKKKTWLVHKQWFLDLPLPWTA
GADTSEVHWNYKERMVTFKVPHAKRQDVTVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKG
MSYTMCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPLAENTNSVTNIELE
PPFGDSYIVIGVGNSALTLHWFRKGSSGGGSHHHHHH

SEQ ID NO: 28 >ZIKV_Eexo **= pCRO25**
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRC
PTQGEAYLDKQSDTQYVCKRTLVDRG**NGS**GCGL**NGS**GSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQH
SGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIP
LPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMD
KLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTE
NSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTGGSGGSGGSHHHHHH

SEQ ID NO: 29 >DENV1_Eexo 2.1 (single sequon W101N;N103S) **[= insert for pCRO26 plasmid]**
MRCVGIGNRDFVEGLSGATWVDVVLEHGSCVTTMAKDKPTLDIELLKTEVTNPAVLRKLCIEAKISNTTTDSRC
PTQGEATLVEEQDSNFVCRRTFVDRG**NGS**GCGLFGKGSLLTCAKFKCVTKLEGKIVQYENLKYSVIVTVHTGDQ
HQVGNETTEHGTIATITPQAPTSEIQLTDYGALTLDCSPRTGLDFNEMVLLTMKEKSWLVHKQWFLDLPLPWTS
GASTSQETWNRQDLLVTFKTAHAKKQEVVVLGSQEGAMHTALTGATEIQTSGTTTIFAGHLKCRLKMDKLTLKG
MSYVMCTGSFKLEKEVAETQHGTVLVQVKYEGTDAPCKIPFSAQDEKGVTQNGRLITANPIVTDKEKPVNIETE
PPFGESYIVVGAGEKALKLSWFKKGSTGGGSHHHHH

SEQ ID NO: 30 >DENV1_Eexo 2.2 (single sequon F108N;K110S) **[= insert for pCRO27 plasmid]**
MRCVGIGNRDFVEGLSGATWVDVVLEHGSCVTTMAKDKPTLDIELLKTEVTNPAVLRKLCIEAKISNTTTDSRC
PTQGEATLVEEQDSNFVCRRTFVDRGWGNGCGL**NGS**GSLLTCAKFKCVTKLEGKIVQYENLKYSVIVTVHTGDQ

HQVGNETTEHGTIATITPQAPTSEIQLTDYGALTLDCSPRTGLDFNEMVLLTMKEKSWLVHKQWFLDLPLPWTS
GASTSQETWNRQDLLVTFKTAHAKKQEVVVLGSQEGAMHTALTGATEIQTSGTTTIFAGHLKCRLKMDKLTLKG
MSYVMCTGSFKLEKEVAETQHGTVLVQVKYEGTDAPCKIPFSAQDEKGVTQNGRLITANPIVTDKEKPVNIETE
PPFGESYIVVGAGEKALKLSWFKKGSTGGGSHHHHH

SEQ ID NO: 31 >ZIKV_Eexo 2.1 (single sequon G100N;W101H;G102T) **[= insert for pCRO28 plasmid]**
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRC
PTQGEAYLDKQSDTQYVCKRTLVDR**NHT**NGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQH
SGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIP
LPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMD
KLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTE
NSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTGGSGGSGGSHHHHHH

SEQ ID NO: 32 >ZIKV_Eexo 2.2 (single sequon L107N;F108H;G109T) **[= insert for pCRO29 plasmid]**
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRC
PTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCG**NHT**KGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQH
SGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIP
LPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMD
KLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTE
NSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTGGSGGSGGSHHHHHH

NSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTGGSGGSGGSHHHHHH

SEQ ID NO: 33 >ZIKV_Eexo 2.3 (single sequon W101N;N103S) **[= insert for pCRO30 plasmid]**
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRC
PTQGEAYLDKQSDTQYVCKRTLVDRG**NGS**GCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQH
SGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIP
LPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMD
KLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTE
NSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTGGSGGSGGSHHHHHH

SEQ ID NO: 34 >ZIKV_Eexo 2.4 (single sequon F108N;K110S) **[= insert for pCRO31 plasmid]**
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRC
PTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGL**NGS**GSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQH
SGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIP
LPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMD
KLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTE
NSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTGGSGGSGGSHHHHHH

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Patentkrav

1. Isoleret rekombinant analog af en flavivirus-E-protein-fusionssløjfe ifølge SEQ ID NO: 1, hvilken analog omfatter mindst ét glycosyleringssted for en N-bundet glycan, som ikke er til stede i en naturlig flavivirus-E-protein-fusionssløjfesekvens ifølge SEQ ID NO: 1, hvor det mindst ene glycosyleringssted er en N-bundet glycosylerings-sequon Asn-X-Ser/Thr, der er substitueret således, at Asn (N)-resten i sequon'en forefindes i en hvilken som helst af positionerne 98-110 DRGWGNGCGLFGK i den naturlige flavivirus-E-protein-fusionssløjfe-aminosyresekvens ifølge SEQ ID NO: 1, hvor X er en hvilken som helst aminosyrerest bortset fra prolin, og Ser/Thr angiver en serin- eller threoninrest.

2. Isoleret rekombinant analog af en flavivirus-E-protein-fusionssløjfe ifølge krav 1, hvilken analog omfatter to glycosyleringssteder, som ikke er til stede i en naturlig flavivirus-E-protein-fusionssløjfesekvens (SEQ ID NO: 1).

3. Isoleret rekombinant analog af et flavivirus-E-protein, som omfatter analogen af en flavivirus-E-protein-fusionssløjfe ifølge krav 1 eller krav 2.

4. Analog ifølge et hvilket som helst af de foregående krav, som har mindst én N-bundet glycan bundet til sig.

5. Analog ifølge et hvilket som helst af de foregående krav, som er produktet af ekspression af en rekombinant DNA- eller RNA-sekvens.

6. Analog ifølge et hvilket som helst af de foregående krav, som omfatter en N-bundet glycosylerings-sequon Asn-X-Ser/Thr, således at en Asn (N)-rest i sequon'en forefindes i en hvilken som helst af positionerne 98-101 og/eller 106-110.

7. Analog ifølge et hvilket som helst af de foregående krav, hvor X er en hvilken som helst af de følgende 13

aminosyrerester Gly, His, Asn, Gln, Tyr, Val, Ala, Met, Ile, Lys, Arg, Thr eller Ser.

8. Analog ifølge et hvilket som helst af de foregående krav, hvor flavivirus-E-proteinet er et dengue-virus-E-protein, og Asn (N)-resten i en sequon forefindes i position 101, 108 eller både 101 og 108 i aminosyresekvensen for flavivirus-E-protein-fusionssløjfen, eller flavivirus-E-proteinet er et zika-E-protein, og Asn (N)-resten i en sequon forefindes i position 100 i aminosyresekvensen for flavivirus-E-protein-fusionssløjfen.

9. Analog ifølge et hvilket som helst af de foregående krav, hvor flavivirusset er et dengue-virus, og aminosyresekvensen for analog-flavivirus-E-protein-fusionssløjfen 98-110 er valgt blandt: DRGNGSGCGLNGS, DRGNGSGCGLFGK og DRGWGNGCGLNGS, eller hvor flavivirusset er et zika-virus, og aminosyresekvensen for analog-flavivirus-E-protein-fusionssløjfen 98-110 er DRNHTNGCGLFGK.

10. Isoleret rekombinant DNA- eller RNA-sekvens, som omfatter:
(a) en sekvens, som koder for en analog af en flavivirus-E-protein-fusionssløjfe ifølge et hvilket som helst af kravene 1-9,
(b) en isoleret rekombinant DNA-sekvens ifølge krav 10 (a), som er en vaccine på plasmidbasis eller lineær DNA-basis, eller
(c) en isoleret rekombinant DNA-sekvens ifølge krav 10 (a) eller 10 (b), som koder for en analog af en flavivirus-E-protein-fusionssløjfe ifølge et hvilket som helst af kravene 1-9 under en pattedyrpromotors kontrol.

11. Værtscelle:
(a) som omfatter en DNA- eller RNA-sekvens ifølge krav 10,
(b) som er en eukaryot værtscelle, der omfatter en DNA-sekvens ifølge krav 10 (a) eller et vaccineimmunogen på plasmidbasis eller lineær DNA-basis ifølge krav 10 (b) eller en isoleret

rekombinant DNA-sekvens ifølge 10 (c), eller
(c) som er en værtscelle ifølge krav 11 (a) eller 11 (b), der er i stand til at:
(i) udtrykke en analog ifølge et hvilket som helst af kravene 1-9 eller
(ii) udtrykke og glycosylere en analog ifølge et hvilket som helst af kravene 1-9.

12. Fremgangsmåde til fremstilling af en analog ifølge et hvilket som helst af kravene 1-9, hvilken fremgangsmåde omfatter dyrkning af en værtscelle ifølge krav 11 under betingelser, som er egnede til ekspression af analogen, og isolering af analogen.

13. Sammensætning:
(a) som omfatter en analog ifølge et hvilket som helst af kravene 1-9 og et fortyndingsmiddel, eller
(b) som er en immunogen (vaccine-) sammensætning, der er i stand til at fremkalde et immunologisk respons hos et individ, der har fået indgivet sammensætningen, hvilken sammensætning omfatter en analog ifølge et hvilket som helst af kravene 1-9 sammen med et farmaceutisk acceptabelt fortyndingsmiddel, en farmaceutisk acceptabel adjuvans og/eller en farmaceutisk acceptabel bærer.

14. Sammensætning ifølge krav 13, som omfatter:
(i) en eller flere flavivirus-analoger valgt blandt en analog af DEN-1, en analog af DEN-2, en analog af DEN-3, en analog af DEN-4 og en analog af zika,
(ii) fire dengue-analoger, som repræsenterer hver af de fire dengue-virus-serotyper DEN-1, DEN-2, DEN-3 og DEN-4,
(iii) en zika-virus-analog eller
(iv) fire dengue-analoger, som repræsenterer hver af de fire dengue-serotyper DEN-1, DEN-2, DEN-3 og DEN-4, og en zika-virus-analog.

15. Bindingsmolekyle, som er i stand til at binde sig specifikt til en analog ifølge et hvilket som helst af kravene

1-9, hvor bindingsmolekylet er valgt blandt et antistof eller et fragment deraf, et domæneantistof, en proteingrundstruktur og en aptamer.

16. Analog, sammensætning eller bindingsmolekyle ifølge et hvilket som helst af kravene 1-9, 13, 14 eller 15 til anvendelse:
(a) som lægemiddel,
(b) som vaccine,
(c) ved profylaktisk eller terapeutisk behandling af en flavivirus-infektion,
(d) til fremstilling af et lægemiddel til profylaktisk eller terapeutisk behandling af en flavivirus-infektion,
(e) til beskyttelse af et individ mod infektion med et flavivirus,
(f) som diagnostikum.

17. Diagnostisk kit, som omfatter en analog, en sammensætning eller et bindingsmolekyle ifølge et hvilket som helst af kravene 1-9, 13, 14 eller 15 og et reagens, der er i stand til at påvise et immunologisk (antigen-antistof-) kompleks, som indeholder den isolerede analog eller det isolerede bindingsmolekyle, hvilket kit eventuelt endvidere omfatter en eller flere kontrolstandarder og/eller et prøvefortyndingsmiddel og/eller en vaskebuffer; eventuelt hvor analogen og/eller bindingsmolekylet er immobiliseret på en fast bærer, f.eks. en mikropladebrønd; eventuelt hvor et immunologisk kompleks, som indeholder den isolerede analog eller det isolerede bindingsmolekyle, påvises ved hjælp af ELISA eller lateral strømning.

DRAWINGS

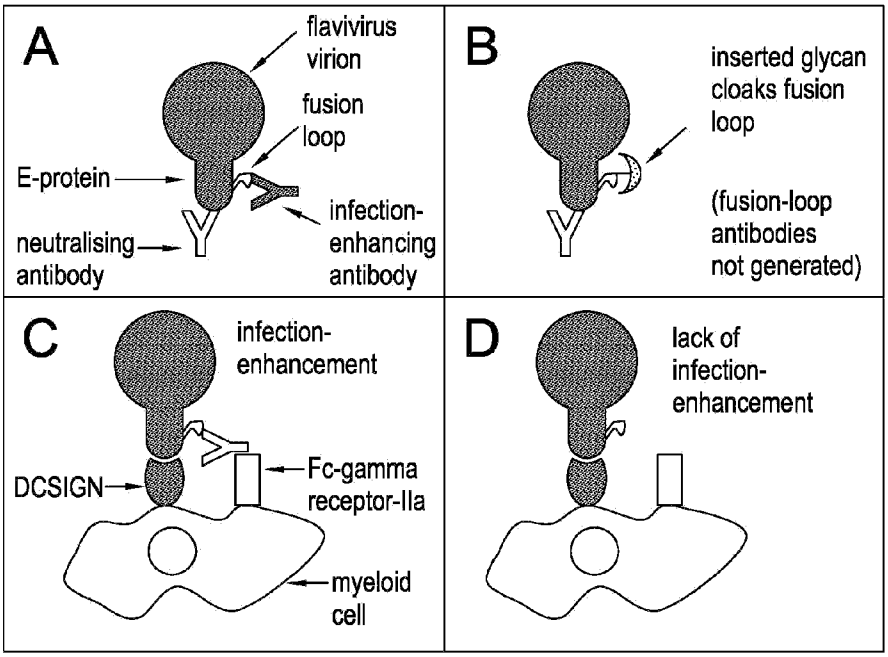


Fig. 1

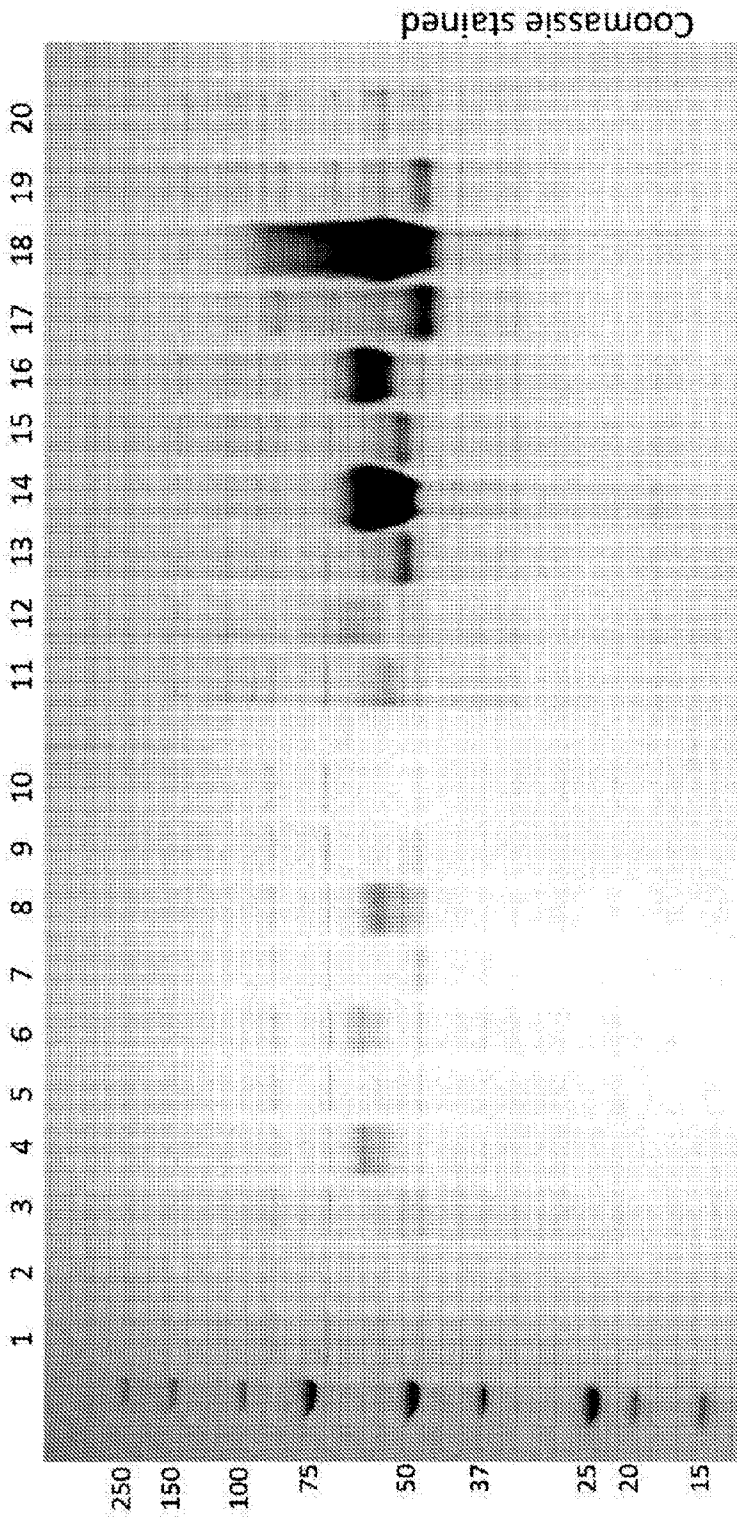

Coomassie stained
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
250
150
100
75
50
37
25
20
15

Fig. 2a

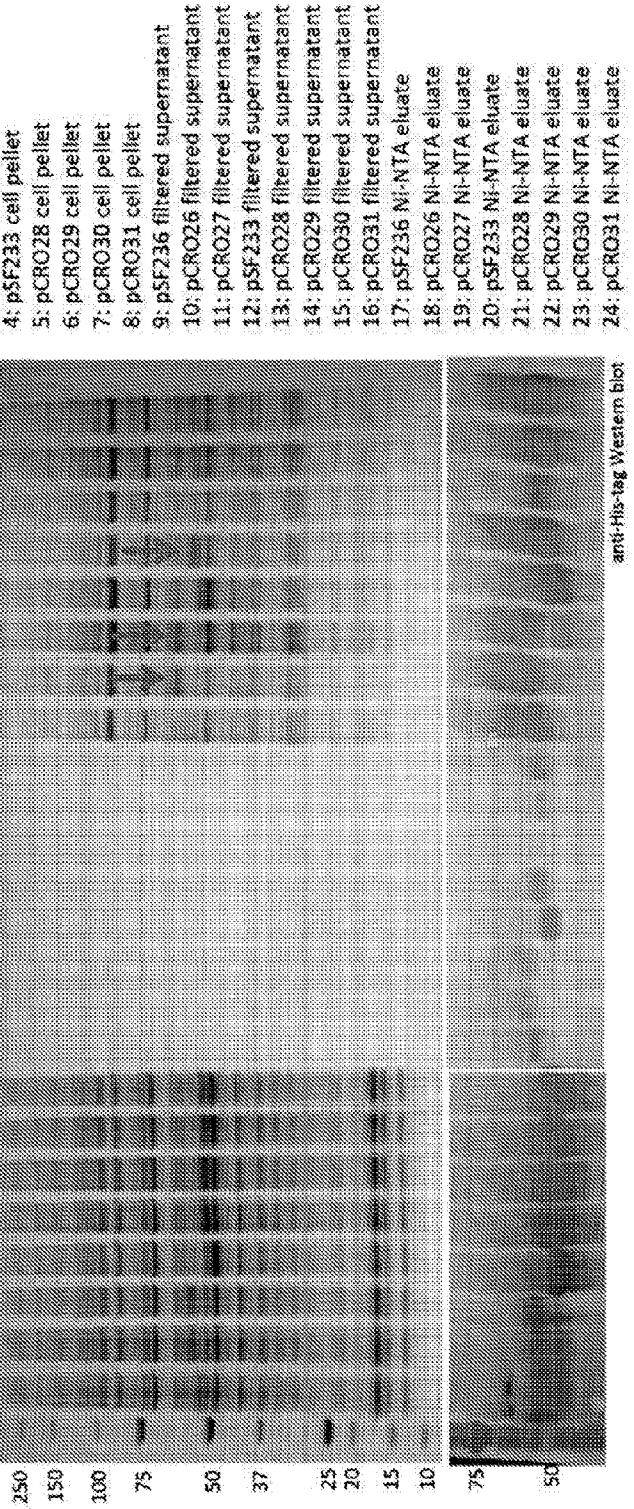
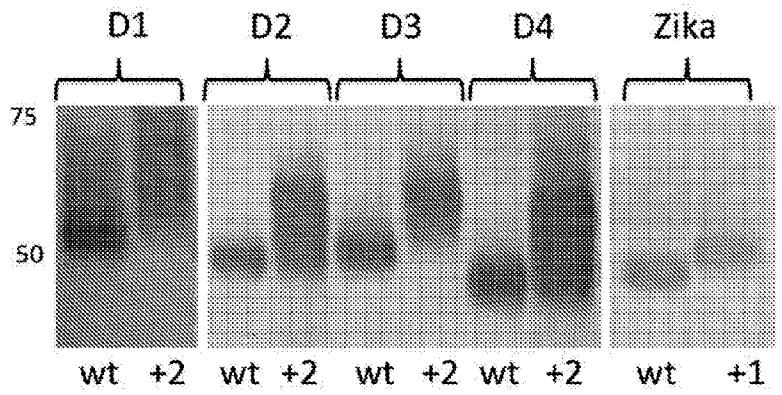
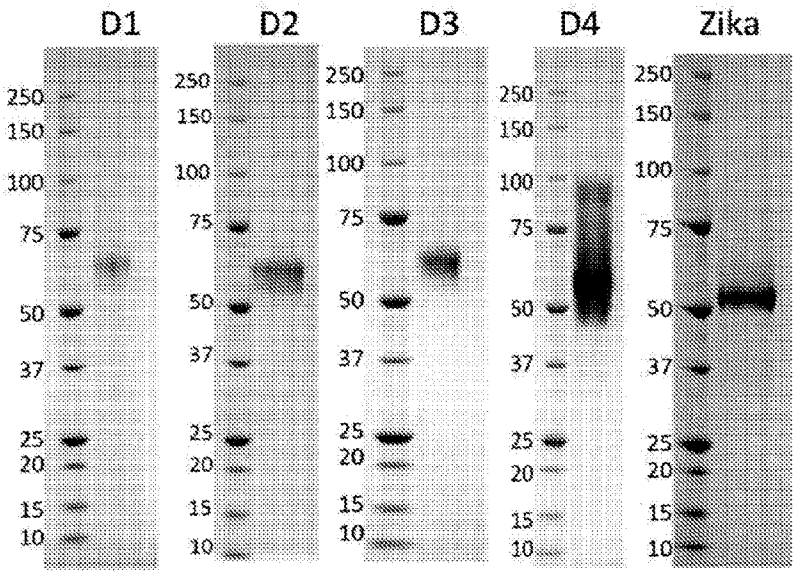

Cell pellets
Raw supernatants
Ni-NTA eluates
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
250
150
100
75
50
37
25
20
15
10
75
50
1: pSF236 cell pellet
2: pCRO26 cell pellet
3: pCRO27 cell pellet
4: pSF233 cell pellet
5: pCRO28 cell pellet
6: pCRO29 cell pellet
7: pCRO30 cell pellet
8: pCRO31 cell pellet
9: pSF236 filtered supernatant
10: pCRO26 filtered supernatant
11: pCRO27 filtered supernatant
12: pSF233 filtered supernatant
13: pCRO28 filtered supernatant
14: pCRO29 filtered supernatant
15: pCRO30 filtered supernatant
16: pCRO31 filtered supernatant
17: pSF236 Ni-NTA eluate
18: pCRO26 Ni-NTA eluate
19: pCRO27 Ni-NTA eluate
20: pSF233 Ni-NTA eluate
21: pCRO28 Ni-NTA eluate
22: pCRO29 Ni-NTA eluate
23: pCRO30 Ni-NTA eluate
24: pCRO31 Ni-NTA eluate
anti-His-tag Western blot

Fig. 2b



Western blot of wild type (left of each pair) and hyperglycosylated forms of dengue and Zika E-protein exodomains, +2 = plus two additional glycans, +1 = plus one additional glycan

Fig. 2c



Purified hyperglycosylated E-protein exodomains from the four dengue virus strains and Zika

Fig. 2d

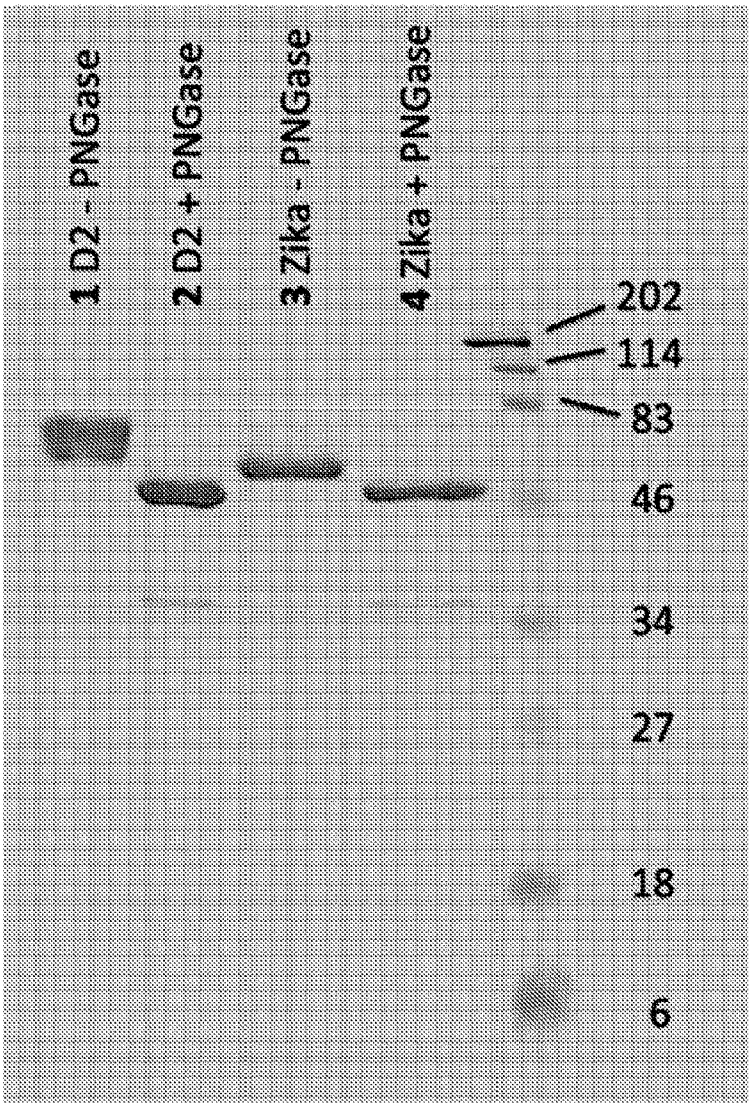
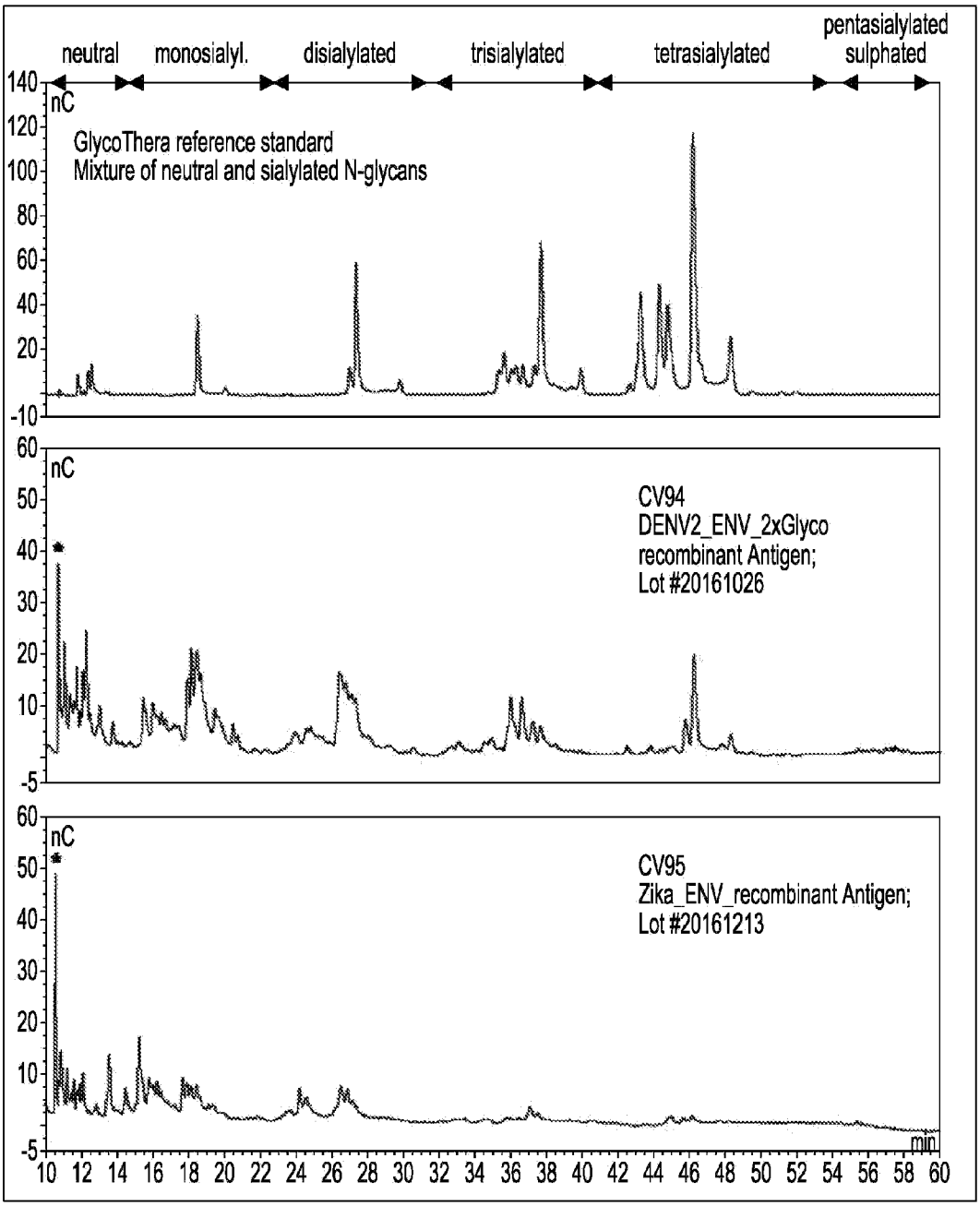

1 D2 - PNGase
2 D2 + PNGase
3 Zika - PNGase
4 Zika + PNGase
202
114
83
46
34
27
18
6

Fig. 3a



* - denotes non-carbohydrate-attributable signal

Fig. 3b

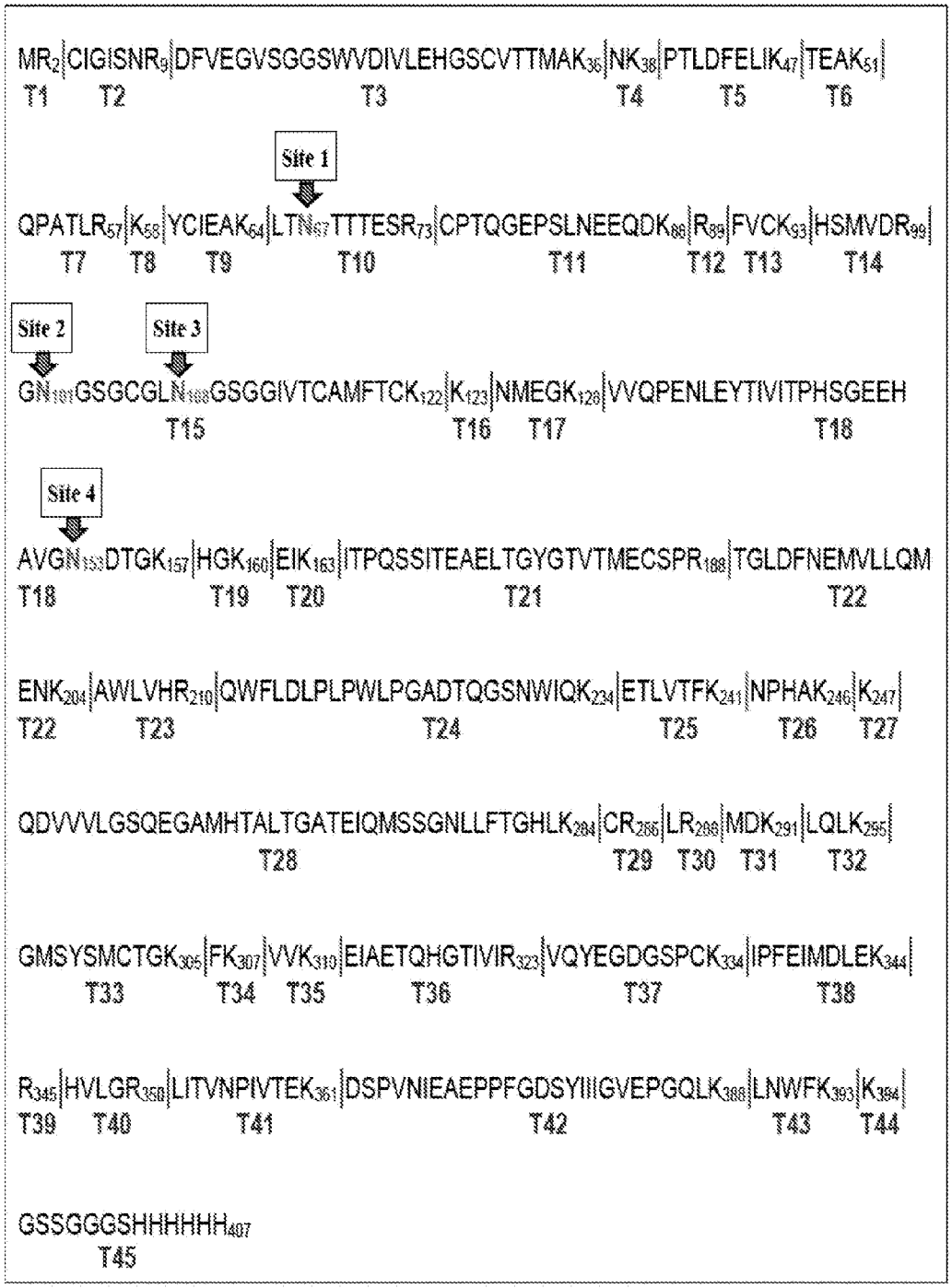

MR2|CIGISNR9|DFVEGVSGGSWVDIVLEHGSCVTTMAK36|NK38|PTLDFELIK47|TEAK51|
T1 T2 T3 T4 T5 T6
Site 1
QPATLR57|K58|YCIEAK64|LTN67TTTESR73|CPTQGEPSLNEEQDK88|R89|FVCK93|HSMVDR99|
T7 T8 T9 T10 T11 T12 T13 T14
Site 2
Site 3
GN101GSGCGLN108GSGGIVTCAMFTCK122|K123|NMEGK128|VVQPENLEYTIVITPHSGEEH
T15 T16 T17 T18
Site 4
AVGN153DTGK157|HGK160|EIK163|ITPQSSITEAELTGYGTVTMECSPR188|TGLDFNEMVLLQM
T18 T19 T20 T21 T22
ENK204|AWLVHR210|QWFLDLPLPWLPGADTQGSNWIQK234|ETLVTFK241|NPHAK246|K247|
T22 T23 T24 T25 T26 T27
QDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLK284|CR286|LR288|MDK291|LQLK295|
T28 T29 T30 T31 T32
GMSYSMCTGK305|FK307|VVK310|EIAETQHGTIVIR323|VQYEGDGSPCK334|IPFEIMDLEK344|
T33 T34 T35 T36 T37 T38
R345|HVLGR350|LITVNPIVTEK361|DSPVNIEAEPPFGDSYIIIGVEPGQLK388|LNWFK393|K394|
T39 T40 T41 T42 T43 T44
GSSGGGSHHHHHH407
T45

Fig. 3c


IR2|CIGVSNR9|DFVEGMSGGTWVDVVLEHGGCVTVMAQDK38|PTVDIELVTTTVSNMAEVR67|
T1 T2 T3 T4
Site 1
SYCYEASISDMASDSR73|CPTQGEAYLDK84|QSDTQYVCK93|R94|TLVDR99|N100HTNGCGLFG
T5 T6 T7 T8 T9 T10
Site 2
K110|GSLVTCAK118|FACSK123|K124|MTGK128|SIQPENLEYR138|IMLSVHGSQHSGMIVN154DTGH
T10 T11 T12 T13 T14 T15 T16
ETDENR164|AK166|VEITPNSPR175|AEATLGGFGSLGLDCEPR193|TGLDFSDLYYLTMNNK209|
T16 T17 T18 T19 T20
HWLVHK215|EWFHDIPLPWHAGADTGTPHWNNK239|EALVEFK246|DAHAK251|R252|QTVVVLGS
T21 T22 T23 T24 T25 T26
QEGAVHTALAGALEAEMDGAK281|GR283|LSSGHLK290|CR292|LK294|MDK297|LR299|LK301|
T26 T27 T28 T29 T30 T31 T32 T33
GVSYSLCTAAFTFTK316|IPAETLHGTVTVEVQYAGTDGPCK348|VPAQMAVDMQTLTPVGR357|
T34 T35 T36
LITANPVITESTENSK373|MMLELDPPFGDSYIVIGVGEK394|K395|ITHHWHR402|SGSTGGSGGS
T37 T38 T39 T40 T41
GGSHHHHHH421
T41

Fig. 3d

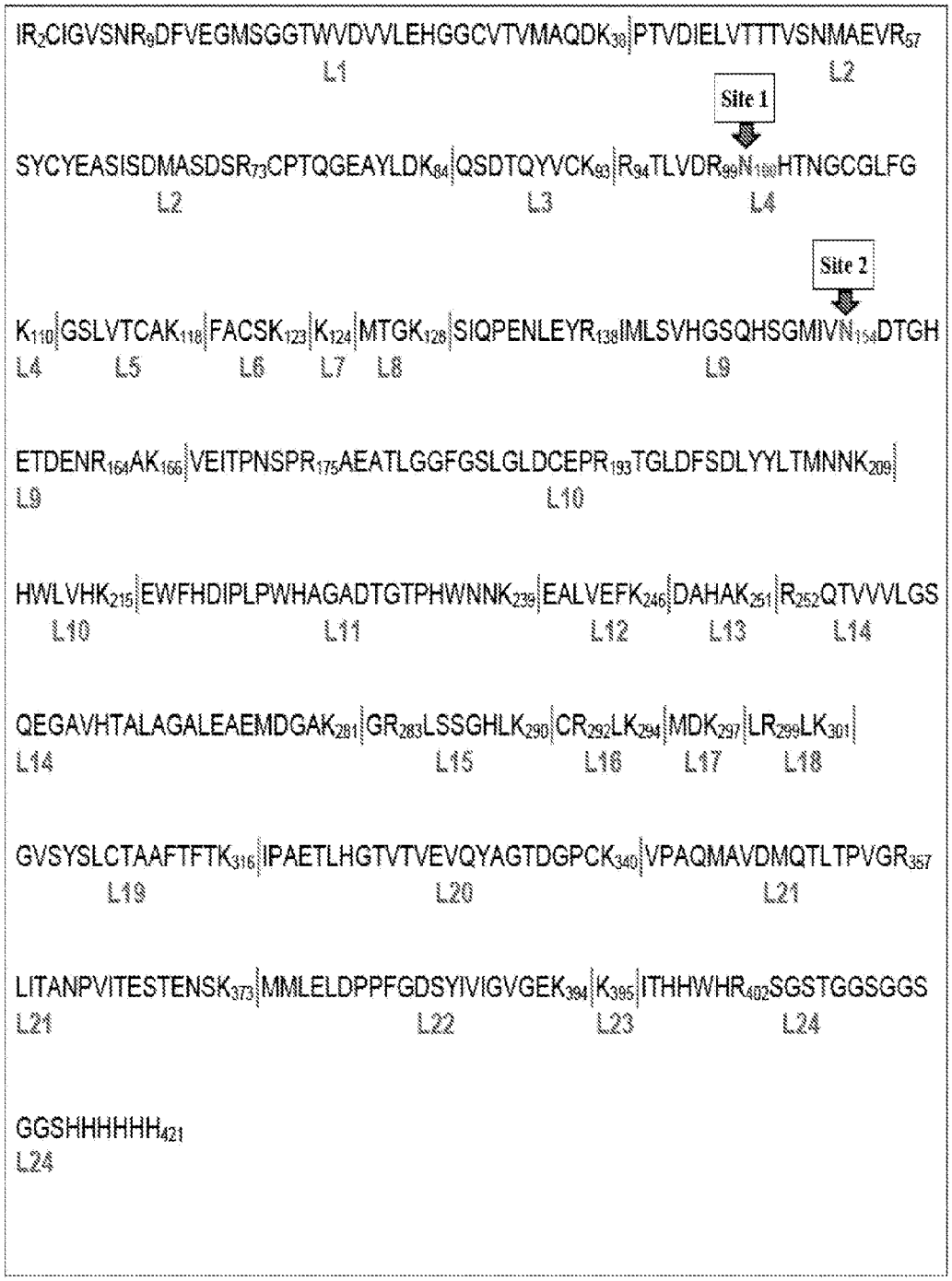

IR2CIGVSNR9DFVEGMSGGTWVDVVLEHGGCVTVMAQDK38|PTVDIELVTTTVSNMAEVR57
L1
L2
Site 1
SYCYEASISDMASDSR73CPTQGEAYLDK84|QSDTQYVCK93|R94TLVDR99N100HTNGCGLFG
L2
L3
L4
Site 2
K110|GSLVTCAK118|FACSK123|K124|MTGK128|SIQPENLEYR138IMLSVHGSQHSGMIVN154DTGH
L4
L5
L6
L7
L8
L9
ETDENR164AK166|VEITPNSPR175AEATLGGFGSLGLDCEPR193TGLDFSDLYYLTMNNK209|
L9
L10
HWLVHK215|EWFHDIPLPWHAGADTGTPHWNNK239|EALVEFK246|DAHAK251|R252QTVVVLGS
L10
L11
L12
L13
L14
QEGAVHTALAGALEAEMDGAK281|GR283LSSGHLK290|CR292LK294|MDK297|LR299LK301|
L14
L15
L16
L17
L18
GVSYSLCTAAFTFTK316|IPAETLHGTVTVEVQYAGTDGPCK340|VPAQMAVDMQTLTPVGR357
L19
L20
L21
LITANPVITESTENSK373|MMLELDPPFGDSYIVIGVGEK394|K395|ITHHWHR402SGSTGGSGGS
L21
L22
L23
L24
GGSHHHHHH421
L24

Fig. 3e

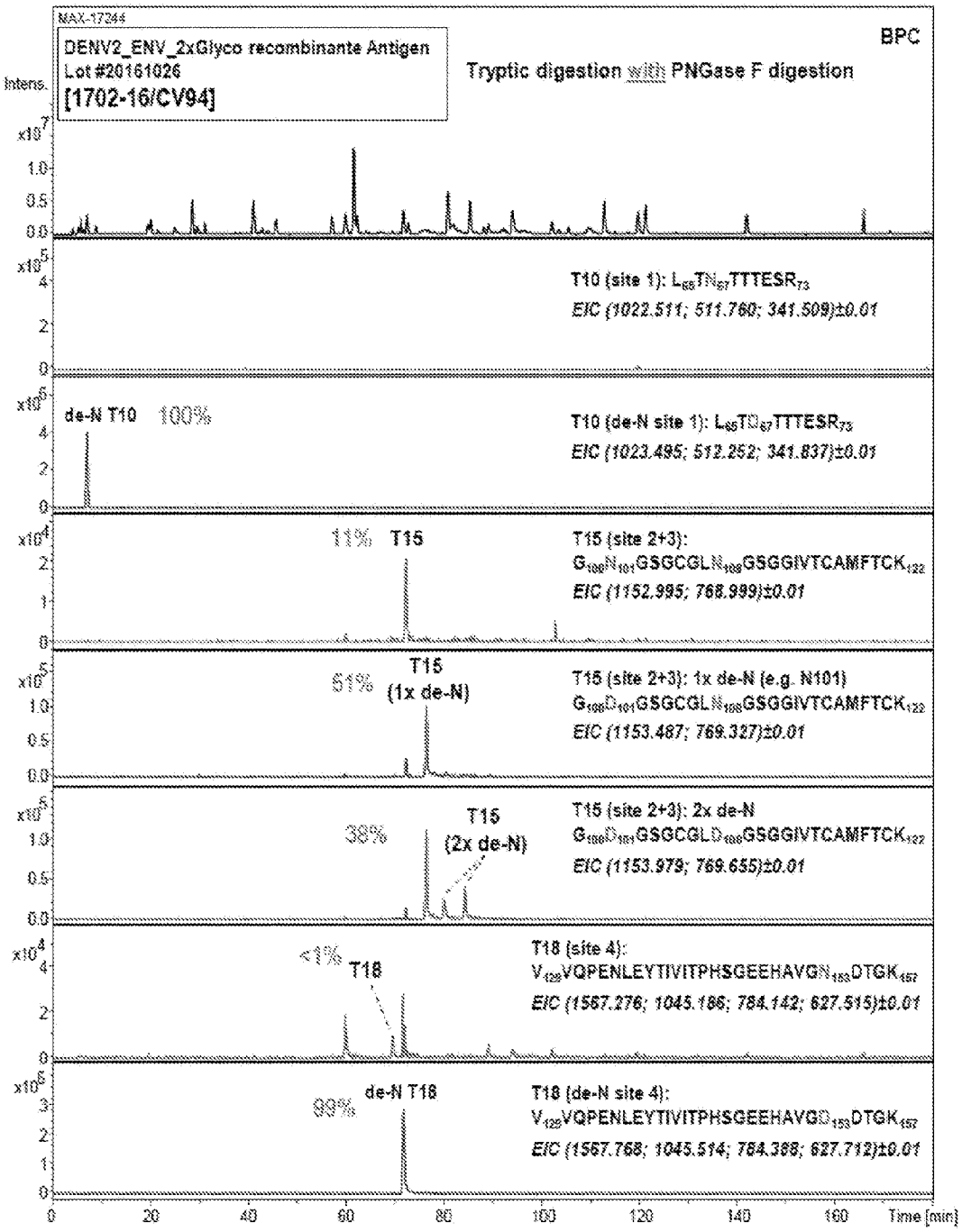

MAX-17244
DENV2_ENV_2xGlyco recombinante Antigen
Lot #20161026
[1702-16/CV94]
BPC
Tryptic digestion with PNGase F digestion
Intens.
T10 (site 1): L68TN67TTTESR73
EIC (1022.511; 511.760; 341.509)±0.01
de-N T10 100%
T10 (de-N site 1): L68TD67TTTESR73
EIC (1023.495; 512.252; 341.837)±0.01
11% T15
T15 (site 2+3):
G100N101GSGCGLN108GSGGIVTCAMFTCK122
EIC (1152.995; 768.999)±0.01
51% T15 (1x de-N)
T15 (site 2+3): 1x de-N (e.g. N101)
G100D101GSGCGLN108GSGGIVTCAMFTCK122
EIC (1153.487; 769.327)±0.01
38% T15 (2x de-N)
T15 (site 2+3): 2x de-N
G100D101GSGCGLD108GSGGIVTCAMFTCK122
EIC (1153.979; 769.655)±0.01
<1% T18
T18 (site 4):
V129VQPENLEYTIVITPHSGEEHAVGN153DTGK157
EIC (1567.276; 1045.186; 784.142; 627.515)±0.01
99% de-N T18
T18 (de-N site 4):
V129VQPENLEYTIVITPHSGEEHAVGD153DTGK157
EIC (1567.768; 1045.514; 784.388; 627.712)±0.01
Time [min]

Fig. 3f

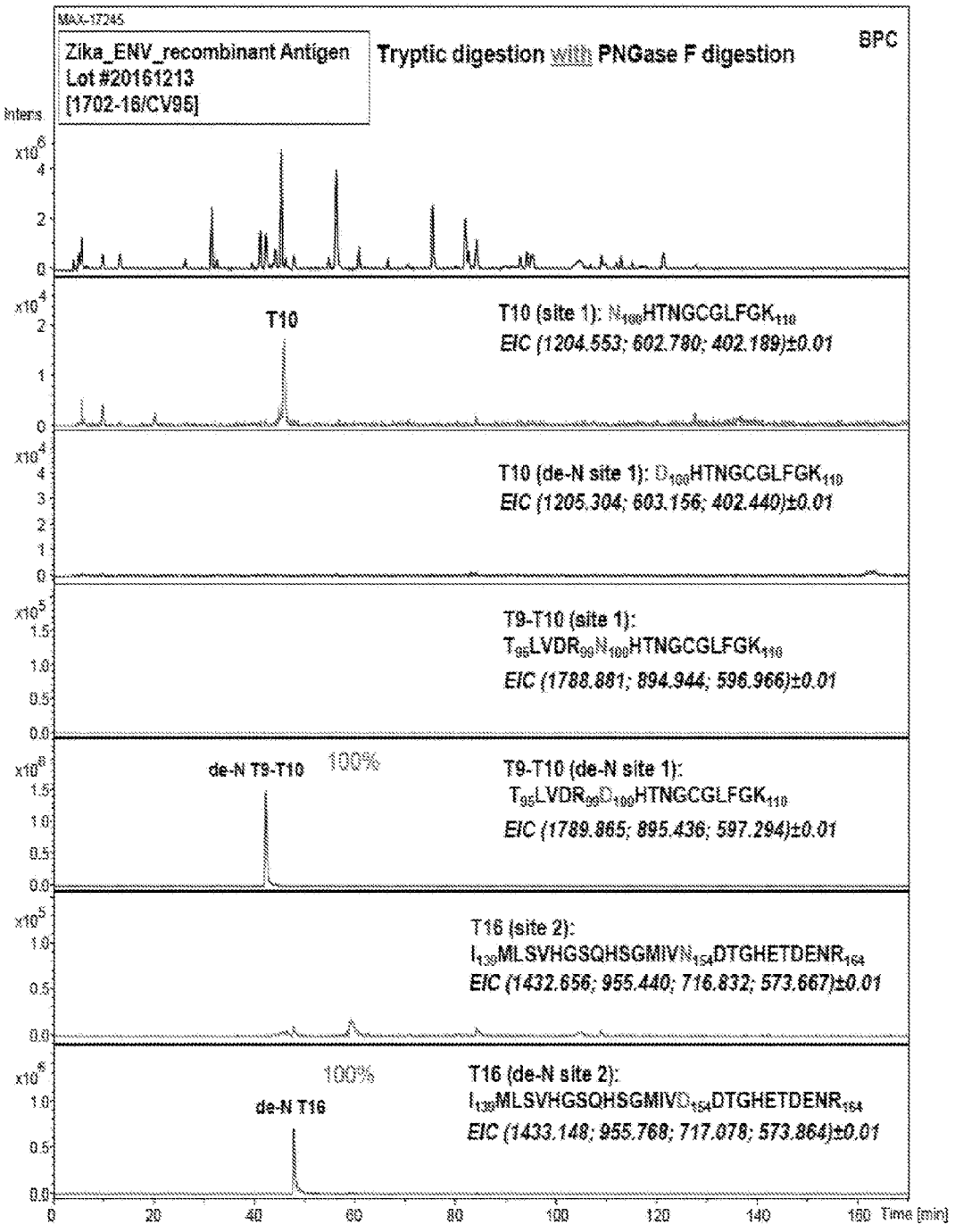

MAX-17245
Zika_ENV_recombinant Antigen
Lot #20161213
[1702-16/CV95]
Tryptic digestion with PNGase F digestion
BPC
Intens
T10
T10 (site 1): N100HTNGCGLFGK110
EIC (1204.553; 602.780; 402.189)±0.01
T10 (de-N site 1): D100HTNGCGLFGK110
EIC (1205.304; 603.156; 402.440)±0.01
T9-T10 (site 1):
T95LVDR99N100HTNGCGLFGK110
EIC (1788.881; 894.944; 596.966)±0.01
de-N T9-T10
100%
T9-T10 (de-N site 1):
T95LVDR99D100HTNGCGLFGK110
EIC (1789.865; 895.436; 597.294)±0.01
T16 (site 2):
I139MLSVHGSQHSGMIVN154DTGHETDENR164
EIC (1432.656; 955.440; 716.832; 573.667)±0.01
100%
de-N T16
T16 (de-N site 2):
I139MLSVHGSQHSGMIVD154DTGHETDENR164
EIC (1433.148; 955.768; 717.078; 573.864)±0.01
Time [min]

Fig. 3g

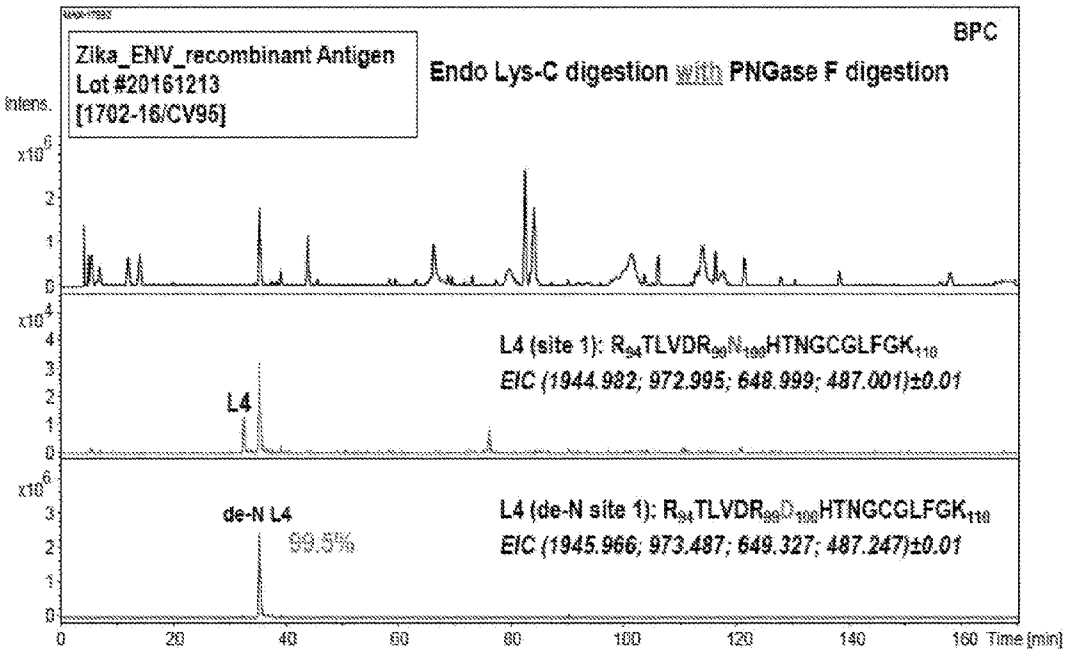

Zika_ENV_recombinant Antigen
Lot #20161213
[1702-16/CV95]
Endo Lys-C digestion with PNGase F digestion
BPC
Intens.
L4 (site 1): R94TLVDR99N154HTNGCGLFGK118
EIC (1944.982; 972.995; 648.999; 487.001)±0.01
L4
de-N L4
99.5%
L4 (de-N site 1): R94TLVDR99D154HTNGCGLFGK118
EIC (1945.966; 973.487; 649.327; 487.247)±0.01
Time [min]

Fig. 3h

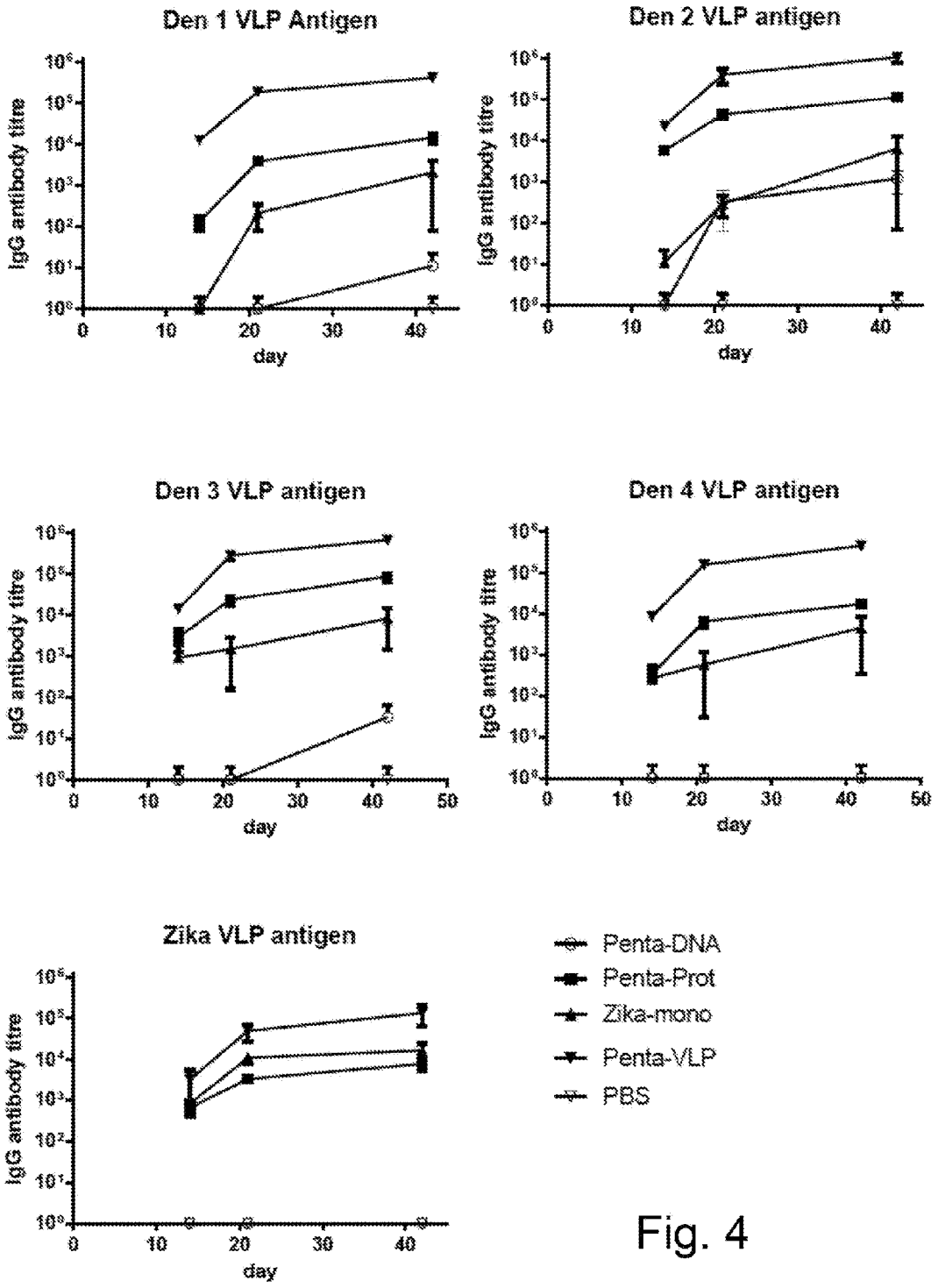

Den 1 VLP Antigen
Den 2 VLP antigen
Den 3 VLP antigen
Den 4 VLP antigen
Zika VLP antigen
IgG antibody titre
day
Penta-DNA
Penta-Prot
Zika-mono
Penta-VLP
PBS

Fig. 4

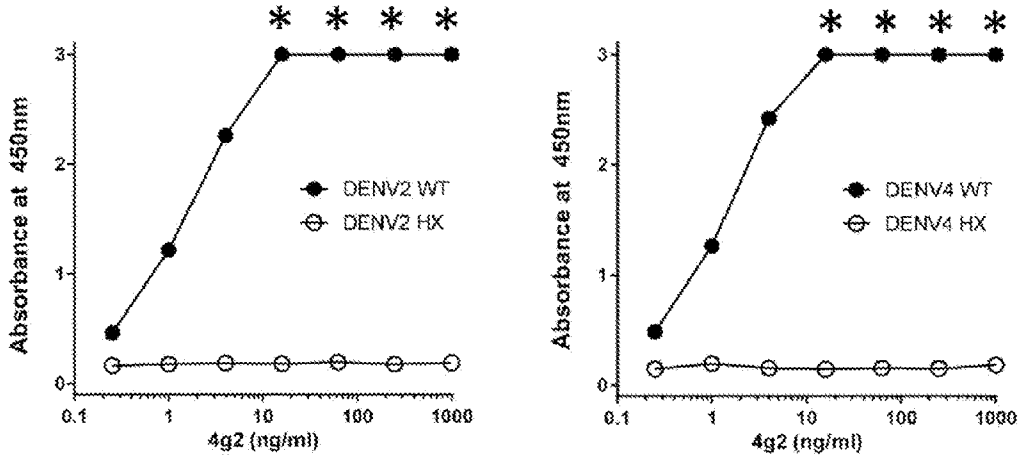

Absorbance at 450nm
DENV2 WT
DENV2 HX
4g2 (ng/ml)
DENV4 WT
DENV4 HX
4g2 (ng/ml)

Fig. 5a

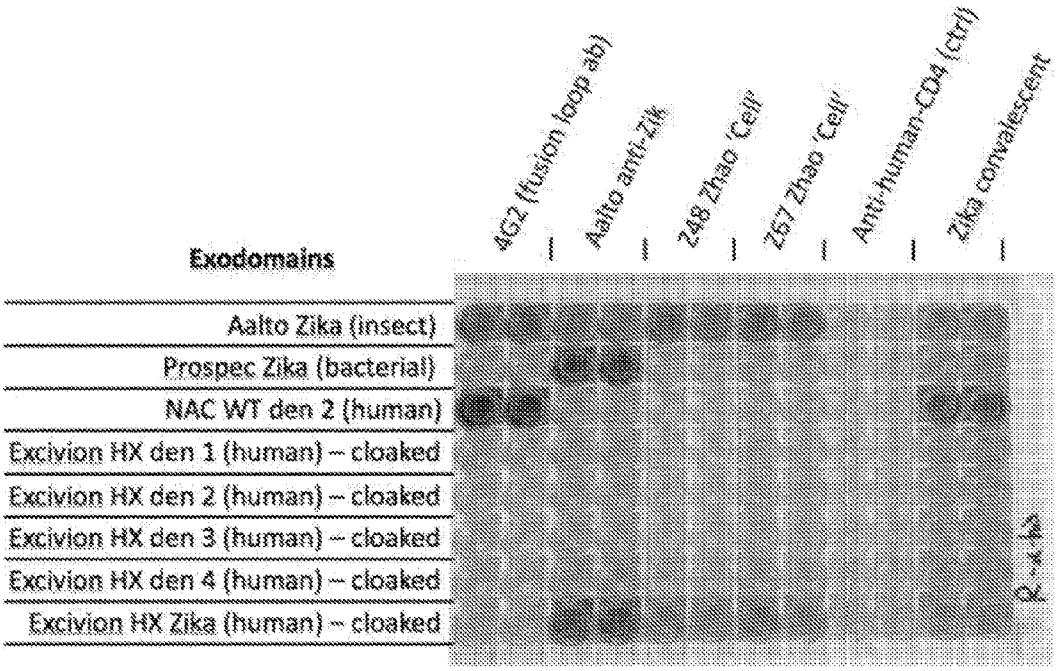

Exodomains
4G2 (fusion loop ab)
Aalto anti-Zik
248 Zhao 'Cell'
257 Zhao 'Cell'
Anti-human-CD4 (ctrl)
Zika convalescent
Aalto Zika (insect)
Prospec Zika (bacterial)
NAC WT den 2 (human)
Excivion HX den 1 (human) – cloaked
Excivion HX den 2 (human) – cloaked
Excivion HX den 3 (human) – cloaked
Excivion HX den 4 (human) – cloaked
Excivion HX Zika (human) – cloaked
2 × 4

Fig. 5b

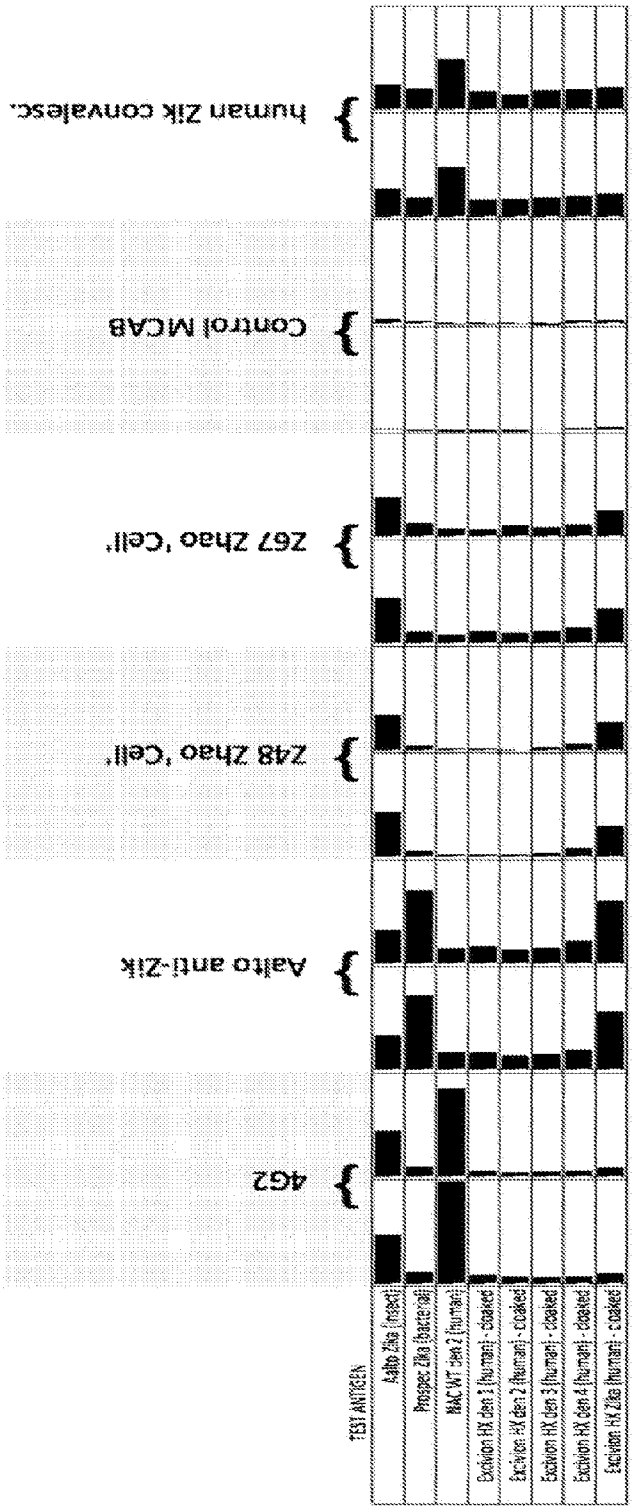

TEST ANTIGEN
Aalto Zika (insect)
Prospec Zika (bacterial)
MAC WT den 2 (human)
Exclusion HX den 1 (human) - cloaked
Exclusion HX den 2 (human) - cloaked
Exclusion HX den 3 (human) - cloaked
Exclusion HX den 4 (human) - cloaked
Exclusion HX Zika (human) - cloaked
4G2
Aalto anti-Zik
Z48 Zhao 'Cell'
Z67 Zhao 'Cell'
Control MCAB
human Zik convalesc.

Fig. 5c

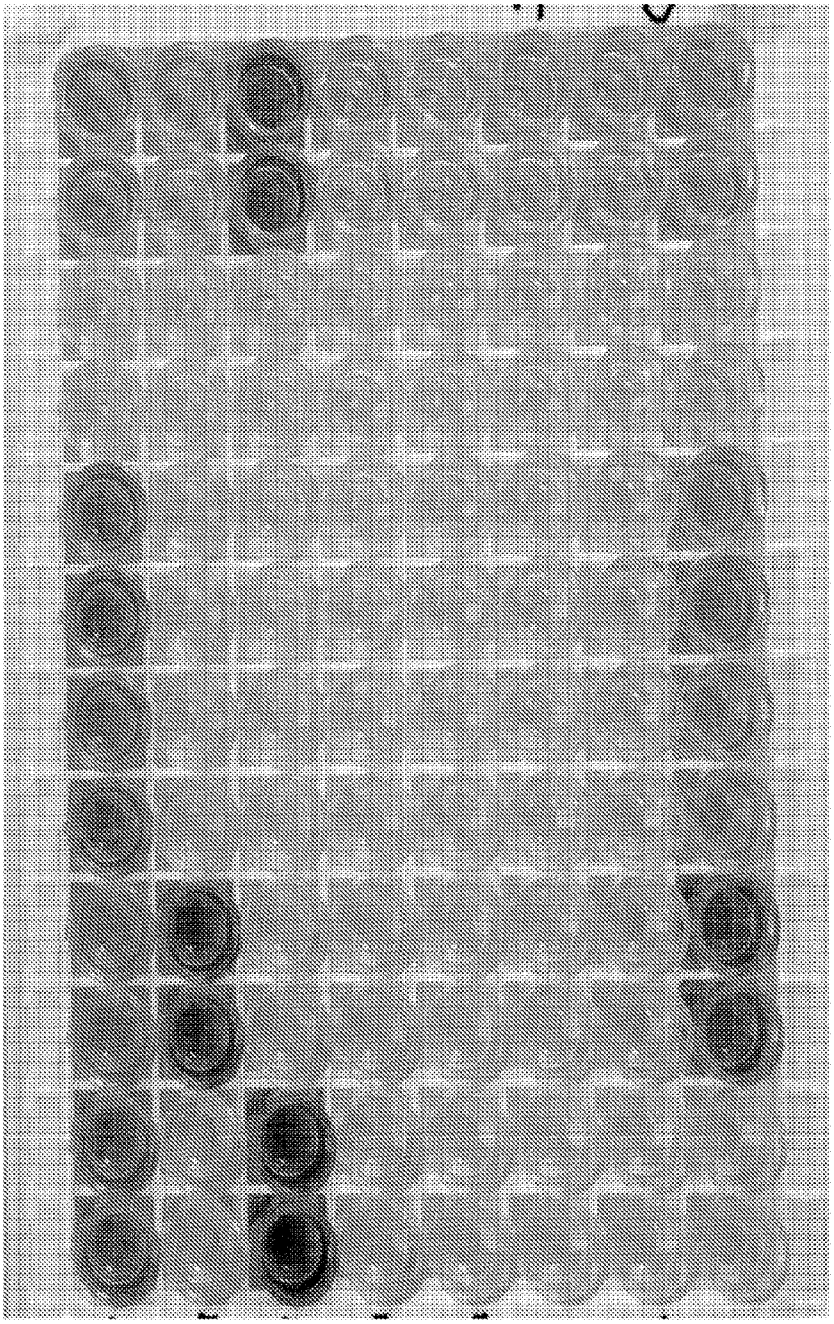


Fig. 5d

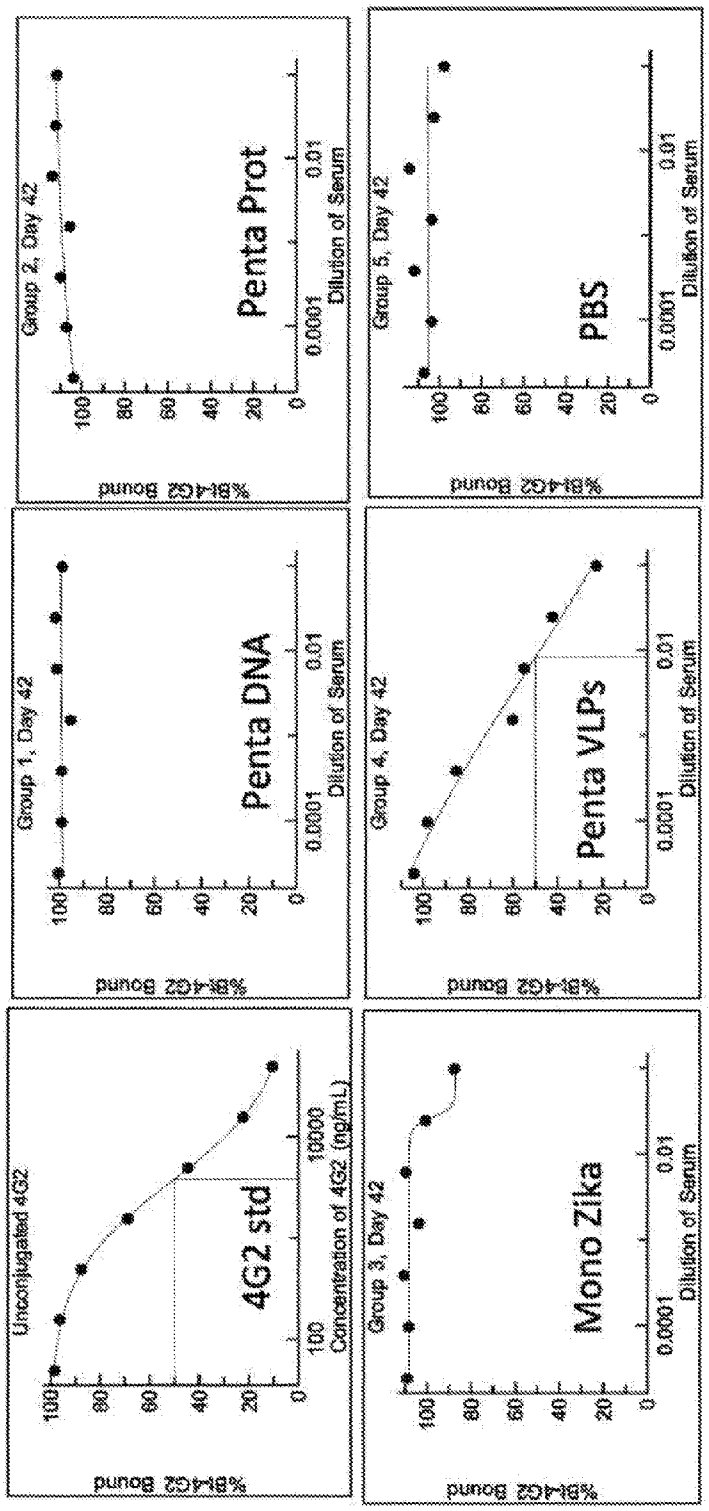

Unconjugated 4G2
4G2 std
Concentration of 4G2 (ng/mL)
%Bi-4G2 Bound
Group 1, Day 42
Penta DNA
Dilution of Serum
Group 2, Day 42
Penta Prot
Group 3, Day 42
Mono Zika
Group 4, Day 42
Penta VLPs
Group 5, Day 42
PBS

Fig. 6

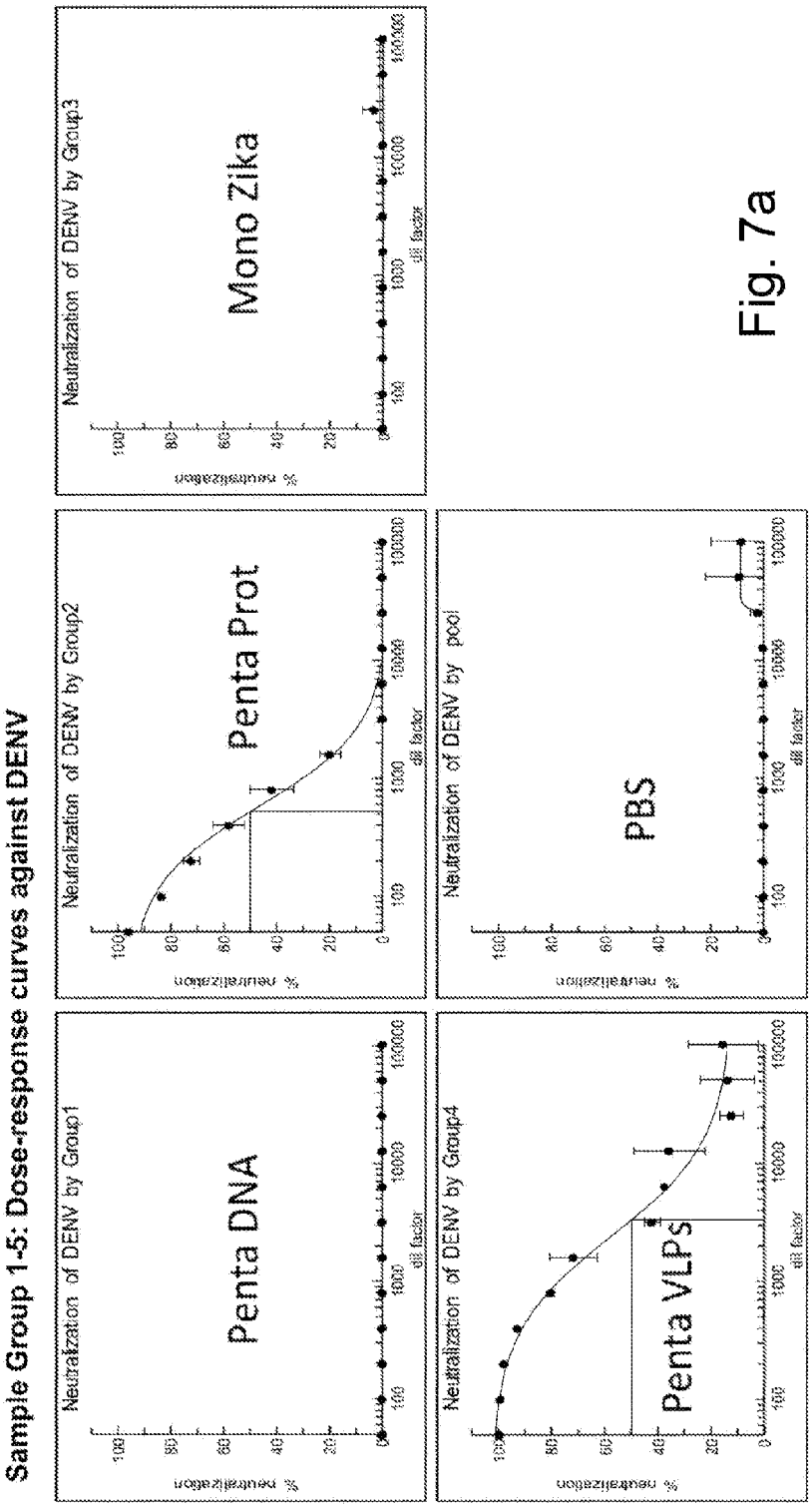

Sample Group 1-5: Dose-response curves against DENV
Neutralization of DENV by Group1
Penta DNA
Neutralization of DENV by Group2
Penta Prot
Neutralization of DENV by Group3
Mono Zika
Neutralization of DENV by Group4
Penta VLPs
Neutralization of DENV by pool
PBS
% neutralization
dil factor

Fig. 7a

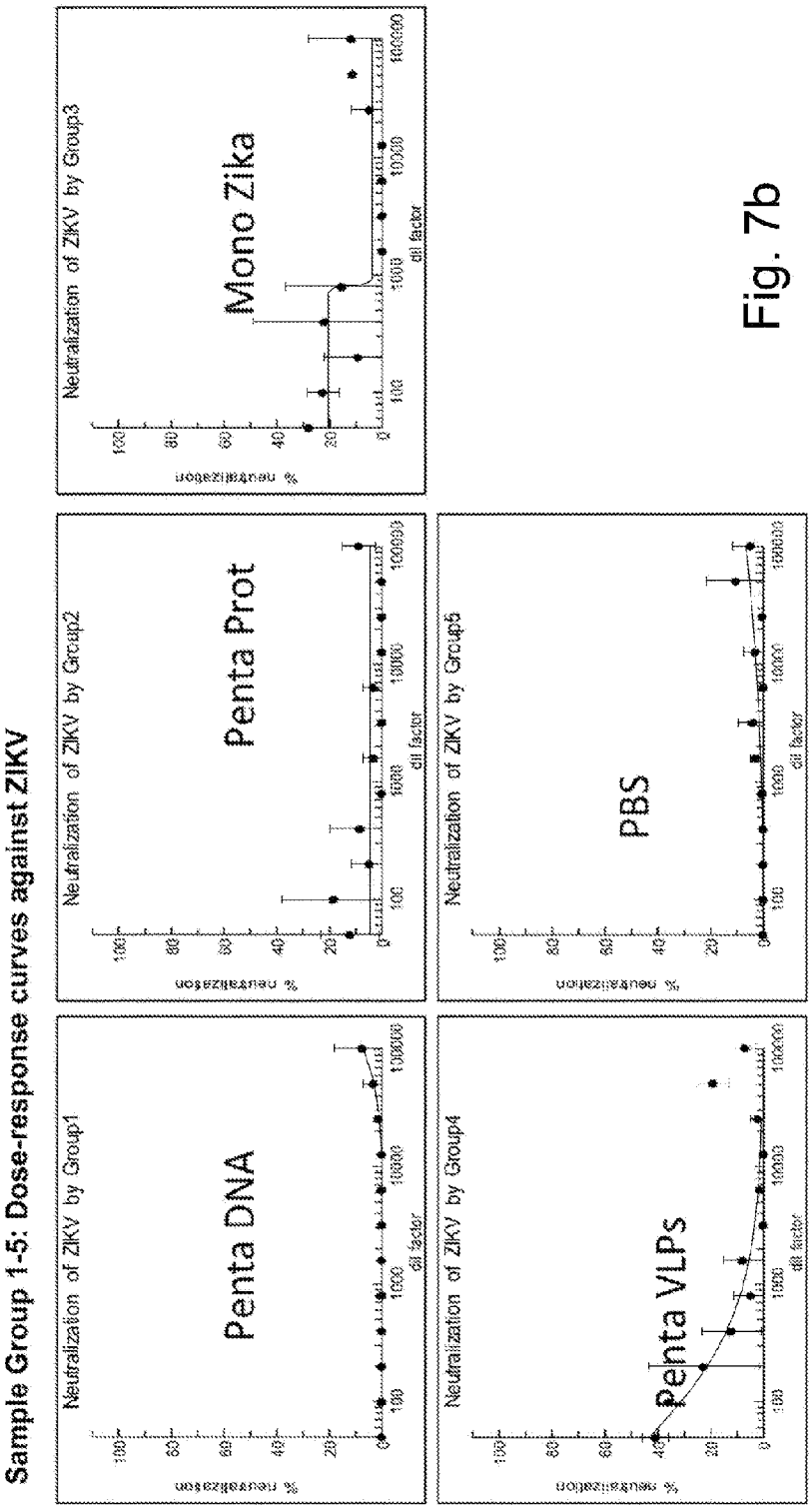

Sample Group 1-5: Dose-response curves against ZIKV
Neutralization of ZIKV by Group1
Penta DNA
Neutralization of ZIKV by Group2
Penta Prot
Neutralization of ZIKV by Group3
Mono Zika
Neutralization of ZIKV by Group4
Penta VLPs
Neutralization of ZIKV by Group5
PBS
% neutralization
dil factor

Fig. 7b

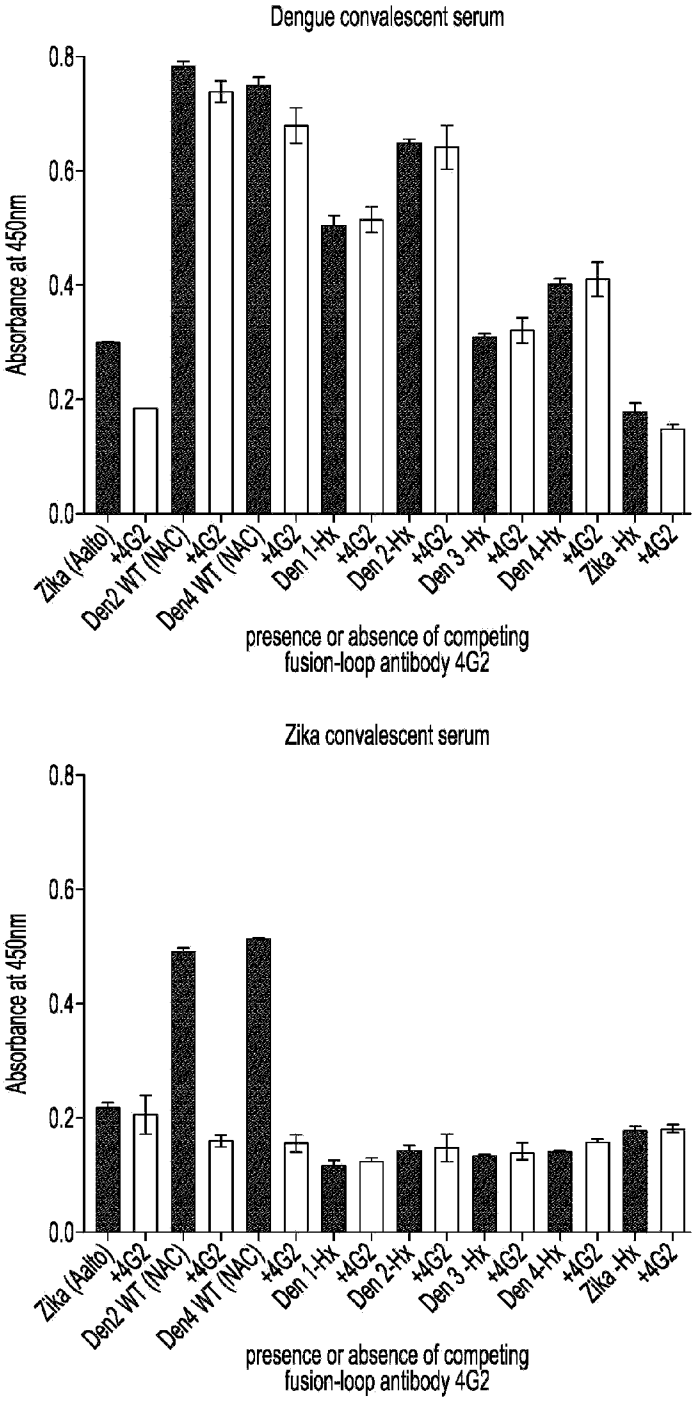

Dengue convalescent serum
Absorbance at 450nm
0.0
0.2
0.4
0.6
0.8
Zika (Aalto)
+4G2
Den2 WT (NAC)
+4G2
Den4 WT (NAC)
+4G2
Den 1-Hx
+4G2
Den 2-Hx
+4G2
Den 3 -Hx
+4G2
Den 4-Hx
+4G2
Zika -Hx
+4G2
presence or absence of competing
fusion-loop antibody 4G2
Zika convalescent serum
Absorbance at 450nm
0.0
0.2
0.4
0.6
0.8
Zika (Aalto)
+4G2
Den2 WT (NAC)
+4G2
Den4 WT (NAC)
+4G2
Den 1-Hx
+4G2
Den 2-Hx
+4G2
Den 3 -Hx
+4G2
Den 4-Hx
+4G2
Zika -Hx
+4G2
presence or absence of competing
fusion-loop antibody 4G2

Fig. 8