(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 September 2014 (18.09.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/144786 A1

(51) International Patent Classification:
A61K 31/711 (2006.01)

(21) International Application Number:
PCT/US2014/029341

(22) International Filing Date:
14 March 2014 (14.03.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/801,792 15 March 2013 (15.03.2013) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

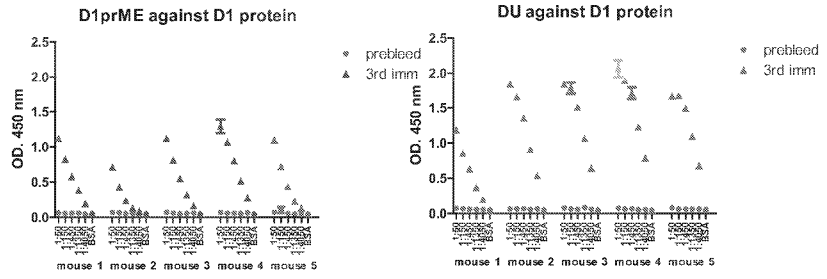
Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: NOVEL VACCINES AGAINST MULTIPLE SUBTYPES OF DENGUE VIRUS



Comparison of binding antibodies against D1-DIII protein in mice immunized with DU or D1prME construct

FIGURE 1

(57) **Abstract**: An aspect of the present invention is related to nucleic acid constructs capable of expressing a polypeptide, such as a consensus dengue prME that elicits an immune response in a mammal against more than one subtype of dengue virus, and methods of use thereof. Additionally, there are DNA plasmid vaccines capable of generating in a mammal an immune response against a plurality of dengue virus subtypes, comprising a DNA plasmid and a pharmaceutically acceptable excipient, and methods of use thereof. The DNA plasmid is capable of expressing a consensus dengue antigen in a cell of the mammal in a quantity effective to elicit an immune response in the mammal that is cross reactive against all 4 dengue subtypes.

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NOVEL VACCINES AGAINST MULTIPLE SUBTYPES OF DENGUE VIRUS

FIELD OF THE INVENTION

The present invention relates to improved dengue vaccines, improved methods for inducing immune responses, and for prophylactically and/or therapeutically immunizing individuals against dengue virus.

BACKGROUND

Dengue virus (DENV) is an emerging mosquito-borne pathogen that causes dengue fever (DF) and severe life threatening illness, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). DENV is a small, enveloped, positive-stranded RNA virus that belongs to the Flavivirus genus of the Flaviviridae family. Four distinct subtypes or serotypes of dengue viruses (DV-1 to DV-4) are transmitted to humans through the bites of mosquito species *Aedes aegypti* and *Aedes albopictus*. It has been estimated that 50–100 million cases of DF and 250,000–500000 cases of DHF occur every year. Dengue constitutes a significant international public health concern, as two-fifths of the world's population live in dengue endemic regions, and an estimated 50–100 million cases of dengue infection occur annually. Furthermore 2.5 billion people are at risk for infection in subtropical and tropical regions of the world in the absence of effective intervention.

More than 100 tropical countries have endemic dengue virus infections, and DHF has been documented in >60 of these countries. Surveillance for DF/DHF is poor in most countries, and in the past has focused primarily on DHF; the number of DF cases that occur each year can therefore only be estimated. In 1998, however, major epidemics occurred throughout Asia and the Americas, with >1.2 million cases of DF/DHF reported to the World Health Organization (WHO). Global reports of DHF have increased on average by five-fold in the past 20 years. At the beginning of the 21st century it is estimated that between 50 and 100 million cases of DF and several hundred thousand cases of DHF occur each year, depending on the epidemic activity. The case fatality rate (CFR) varies among countries, but can be as high as 10–15% in some and <1% in others.

There are four dengue virus subtypes: dengue-1 (DV-1), dengue-2 (DV-2), dengue-3 (DV-3), and dengue-4 (DV-4). Each one of these subtypes form an antigenically distinct subgroup within the flavivirus family. They are enveloped, RNA viruses that encode ten proteins: three structural proteins and seven non-structural proteins. The structural proteins are capsid (C), envelope (E), and pre-membrane precursor (preM). The intracellular life cycle of DV begins with receptor-mediated endocytosis of the virus in to cells followed by fusion of the viral envelope protein with the late endosomal membrane, which results in the release of the viral genome into the cytoplasm for replication.

Infection by DV may either be asymptomatic or characterized by fever, chills, frontal headache, myalgia, arthralgia, and rash. Subsequent infection with different serotypes may result in more severe manifestations of the disease involving plasma leakage or hemorrhage (dengue hemorrhagic fever) and shock (dengue shock syndrome). Although extensive studies have been carried out over the years to understand the pathogenicity of DENV infection, little progress has been made in the development of specific anti-DV compounds. Currently there are no specific anti viral agents or vaccines against Dengue infections approved in the U.S.

The envelope (E) glycosylated protein, being the major structural protein present on the surface of the mature dengue virions, is a type I integral membrane protein. It has been demonstrated that the E protein of the mature Dengue forms homodimers in the anti-parallel manner (head to tail orientation). Each monomer is folded into three distinct domains, namely domain I (DI, the central N-terminal domain), domain II (DII, the dimerization domain), and domain III (PRM/E, immunoglobulin (Ig) like C terminal domain). The PRM/E domain of E protein consists of 100 amino acids (residues 303-395) of the C-terminus. This domain has been suggested to be the receptor recognition and binding domain. Ig-like fold present in the PRM/E protein is commonly associated with structures that have an adhesion function. This domain extends perpendicularly to the surface of the virus, with a tip that projects further from the virion surface than any other part of the E protein. In addition, studies have demonstrated that both recombinant PRM/E proteins and antibodies generated against PRM/E of E protein of flavivirus can inhibit entry of the flavivirus into target cells. Further, flavivirus with mutation in PRM/E of the E protein shows either attenuated virulence or the ability to escape immune neutralization.

Development of a safe and effective vaccine against dengue virus infection remains a principal public health goal. Given that the primary correlate of immunity to dengue virus is thought to be the presence of neutralizing antibodies, a prerequisite for comparing and optimizing vaccine candidates is the ability to precisely measure the neutralizing antibody responses evoked by vaccines. A combination of live attenuated virus-containing vaccines from all four serotypes has been shown to result in several complications (Guy B, Almond JW, Comp Immunol Microbiol Infect Dis. 2008 Mar, 31(2-3):239-52). Further, there are few reports on an adenovirus-based delivery of dengue antigens. Nevertheless, the one well recognized problem with adenovirus systems is a majority of the human population is known to have antibodies against one of the adenoviruses, and such pre-existing antibodies can cause these adeno-based vaccines to be ineffective.

Therefore, there remains a need to develop a vaccine that provides broad immunity against multiple and preferably all four serotypes of dengue virus, or universal immunity, and preferably a vaccine which is economical and effective across all serotypes. Further, there remains a need for an effective method of administering vaccines, such as DNA vaccines or DNA plasmid vaccines, to a mammal in order to provide immunization against dengue virus, either prophylatically or therapeutically.

SUMMARY OF THE INVENTION

One aspect of the present invention provides nucleic acid constructs capable of expressing a polypeptide that elicits an immune response in a mammal against more than one subtype of dengue virus. The nucleic acid constructs are comprised of an encoding nucleotide sequence and a promoter operably linked to the encoding nucleotide sequence. The encoding nucleotide sequence expresses the polypeptide, wherein the polypeptide includes domain III of envelope protein (PRM/E domain or PRM/E) from at least two different dengue virus subtypes. The promoter regulates expression of the polypeptide in the mammal.

Another aspect of the present invention provides DNA plasmid vaccines that are capable of generating in a mammal an immune response against a plurality of dengue virus subtypes. The DNA plasmid vaccines are comprised of a DNA plasmid capable of expressing a consensus dengue antigen in the mammal and a pharmaceutically acceptable excipient. The

DNA plasmid is comprised of a promoter operably linked to a coding sequence that encodes the consensus dengue PRM/E antigen. The consensus dengue antigen is comprised of consensus PRM/E domains of dengue virus-subtype 1, dengue virus-subtype 2, dengue virus-subtype 3, or dengue virus-subtype 4.

Another aspect of the present invention provides methods of eliciting an immune response against a plurality of subtypes of a virus in a mammal, comprising delivering a DNA plasmid vaccine to tissue of the mammal, the DNA plasmid vaccine comprising a DNA plasmid capable of expressing a plurality of consensus antigens derived from the subtypes of the virus in a cell of the mammal to elicit an immune response in the mammal, the plurality of consensus antigens comprising an antigenic domain from at least two different subtypes of the virus, and electroporating cells of the tissue with a pulse of energy at a constant current effective to permit entry of the DNA plasmids into the cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 displays binding titers against all dengue PRM/E domains from subtype 1 for sera from D1prME vaccinated mice versus sera from DU vaccinated mice.

Figure 2 displays binding titers against all dengue PRM/E domains from subtype 2 for sera from D2prME vaccinated mice versus sera from DU vaccinated mice.

Figure 3 displays binding titers against all dengue PRM/E domains from subtype 3 for sera from D3prME vaccinated mice versus sera from DU vaccinated mice.

Figure 4 displays binding titers against all dengue PRM/E domains from subtype 4 for sera from D4prME vaccinated mice versus sera from DU vaccinated mice.

Figure 5 displays stained gels showing the binding antibodies generated against prME proteins types D1, D2, D3, and D4 for mice immunized with a PRM/E domain from serotype 1, 2, 3, or 4.

Figure 6 displays graphs showing neutralizing antibodies against each one of dengue PRM/E protein types (1 through 4) for control guinea pig sera.

Figure 7 displays graphs showing neutralizing antibodies against each one of dengue PRM/E protein types (1 through 4) for DU-DIII guinea pig sera.

Figure 8 displays graphs showing neutralizing antibodies against each one of dengue PRM/E protein types (1 through 4) for D1-D4 prME guinea pig sera (all four vaccines combined in one mixture and administered together).

Figure 9 displays graphs showing neutralizing antibodies against each one of dengue PRM/E protein types (1 through 4) for D1-D4 prME guinea pig sera (each vaccine administered separately).

Figure 10 displays graphs showing neutralizing antibodies against each one of dengue PRM/E protein types (1 through 4) for D1 prME guinea pig sera.

Figure 11 displays graphs showing neutralizing antibodies against each one of dengue PRM/E protein types (1 through 4) for D2 prME guinea pig sera.

Figure 12 displays graphs showing neutralizing antibodies against each one of dengue PRM/E protein types (1 through 4) for D3 prME guinea pig sera.

Figure 13 displays graphs showing neutralizing antibodies against each one of dengue PRM/E protein types (1 through 4) for D4 prME guinea pig sera.

Figure 14 displays a graph showing neutralizing antibodies against Dengue 1 virus for sera from animals vaccinated with all four D1-D4 prME.

Figure 15 displays a graph showing neutralizing antibodies against Dengue 2 virus for sera from animals vaccinated with all four D1-D4 prME.

Figure 16 displays a graph showing neutralizing antibodies against Dengue 3 virus for sera from animals vaccinated with all four D1-D4 prME.

Figure 17 displays a graph showing neutralizing antibodies against Dengue 4 virus for sera from animals vaccinated with all four D1-D4 prME.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The following abbreviated, or shortened, definitions are given to help the understanding of the preferred embodiments of the present invention. The abbreviated definitions given here are by no means exhaustive nor are they contradictory to the definitions as understood in the field or dictionary meaning. The abbreviated definitions are given here to supplement or more clearly define the definitions known in the art.

Definitions

Sequence homology for nucleotides and amino acids as used herein may be determined using FASTA, BLAST and Gapped BLAST (Altschul et al., Nuc. Acids Res., 1997, 25, 3389, which is incorporated herein by reference in its entirety) and PAUP* 4.0b10 software (D. L. Swofford, Sinauer Associates, Massachusetts). Briefly, the BLAST algorithm, which stands for Basic Local Alignment Search Tool is suitable for determining sequence similarity (Altschul et al., J. Mol. Biol., 1990, 215, 403-410, which is incorporated herein by reference in its entirety). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to another if the smallest sum probability in comparison of the test nucleic acid to the other nucleic acid is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. "Percentage of similarity" can be calculated using PAUP* 4.0b10 software (D. L. Swofford, Sinauer Associates, Massachusetts). The average similarity of the consensus sequence is calculated compared to all sequences in the phylogenic tree.

As used herein, the term “nucleic acid construct” refers to the DNA or RNA molecules that comprise a nucleotide sequence that encodes protein. The coding sequence, or “encoding nucleic acid sequence,” can include initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the individual to whom the nucleic acid molecule is administered.

As used herein, the term "expressible form" refers to nucleic acid constructs that contain the necessary regulatory elements operably linked to a coding sequence that encodes a protein such that when present in the cell of the individual, the coding sequence will be expressed.

The term “constant current” is used herein to define a current that is received or experienced by a tissue, or cells defining said tissue, over the duration of an electrical pulse delivered to same tissue. The electrical pulse is delivered from the electroporation devices described herein. This current remains at a constant amperage in said tissue over the life of an electrical pulse because the electroporation device provided herein has a feedback element, preferably having instantaneous feedback. The feedback element can measure the resistance of the tissue (or cells) throughout the duration of the pulse and cause the electroporation device to alter its electrical energy output (e.g., increase voltage) so current in same tissue remains constant throughout the electrical pulse (on the order of microseconds), and from pulse to pulse. In some embodiments, the feedback element comprises a controller.

The term “feedback” or “current feedback” is used interchangeably and means the active response of the provided electroporation devices, which comprises measuring the current in tissue between electrodes and altering the energy output delivered by the EP device accordingly in order to maintain the current at a constant level. This constant level is preset by a user prior to initiation of a pulse sequence or electrical treatment. Preferably, the feedback is accomplished by the electroporation component, e.g., controller, of the electroporation device, as the electrical circuit therein is able to continuously monitor the current in tissue between electrodes and compare that monitored current (or current within tissue) to a preset current and continuously make energy-output adjustments to maintain the monitored current at preset levels. In some embodiments, the feedback loop is instantaneous as it is an analog closed-loop feedback.

The terms "electroporation," "electro-permeabilization," or "electro-kinetic enhancement" ("EP"), as used interchangeably herein, refer to the use of a transmembrane electric field pulse to induce microscopic pathways (pores) in a bio-membrane; their presence allows biomolecules such as plasmids, oligonucleotides, siRNA, drugs, ions, and/or water to pass from one side of the cellular membrane to the other.

The term "decentralized current" is used herein to define the pattern of electrical currents delivered from the various needle electrode arrays of the electroporation devices described herein, wherein the patterns minimize, or preferably eliminate, the occurrence of electroporation related heat stress on any area of tissue being electroporated.

The term "feedback mechanism" as used herein refers to a process performed by either software or hardware (or firmware), which process receives and compares the impedance of the desired tissue (before, during, and/or after the delivery of pulse of energy) with a present value, preferably current, and adjusts the pulse of energy delivered to achieve the preset value. The term "impedance" is used herein when discussing the feedback mechanism and can be converted to a current value according to Ohm's law, thus enabling comparisons with the preset current. In a preferred embodiment, the "feedback mechanism" is performed by an analog closed loop circuit.

The term "immune response" is used herein to mean the activation of a host's immune system, e.g., that of a mammal, in response to the introduction of a dengue antigen, e.g., universal dengue antigen, via the provided DNA plasmid vaccines. The immune response can be in the form of a cellular or humoral response, or both.

The term "consensus" or "consensus sequence" is used herein to mean a synthetic nucleic acid sequence, or corresponding polypeptide sequence, constructed based on analysis of an alignment of multiple strains of a specific dengue subtype, which yields the consensus dengue sequences of subtype-1, subtype-2, subtype-3, subtype-4, and the universal dengue described below. The consensus universal dengue can be used to induce broad immunity against multiple subtypes or serotypes of dengue virus.

The term "adjuvant" is used herein to mean any molecule added to the DNA plasmid vaccines described herein to enhance antigenicity of the dengue antigen encoded by the DNA plasmids and encoding nucleic acid sequences described hereinafter.

The term "subtype" or "serotype" is used herein interchangeably and in reference to a virus, for example dengue virus, and means genetic variants of that virus antigen such that one subtype is recognized by an immune system apart from a different subtype. For example, dengue virus subtype 1 is immunologically distinguishable from dengue virus subtype 2.

One aspect of the present invention provides nucleic acid constructs capable of expressing a polypeptide that elicits an immune response in a mammal against more than one subtype of dengue virus. The nucleic acid constructs are comprised of an encoding nucleotide sequence and a promoter operably linked to the encoding nucleotide sequence. The encoding nucleotide sequence expresses the polypeptide, wherein the polypeptide includes a PRM/E domain from at least two different dengue virus subtypes. The promoter regulates expression of the polypeptide in the mammal.

In some embodiments the nucleic acid construct can further include an IgE leader sequence operatively linked to an N-terminal end of the coding sequence and operably linked to the promoter. Preferably, the IgE leader has the sequence of SEQ ID NO: 11. The nucleic acid construct can also comprise a polyadenylation sequence attached to the C-terminal end of the coding sequence. Preferably, the nucleic acid construct is codon optimized.

In some embodiments, the encoding nucleotide sequence encodes a polypeptide that includes PRM/E domain from Dengue virus-subtype 1, Dengue virus-subtype 2, Dengue virus-subtype 3, and Dengue virus-subtype 4. In preferred embodiments, the encoding nucleotide sequence is selected from the group consisting of:

SEQ ID NO:	Description
1	Den1-prME DNA sequence
2	Den1-prME protein sequence
3	Den2-prME DNA sequence
4	Den2-prME Protein sequence
5	Den3-prME DNA sequence

6	Den3-prME protein sequence
7	Den4-prME DNA sequence
8	Den4-prME protein sequence

Another aspect of the present invention provides DNA plasmid vaccines that are capable of generating in a mammal an immune response against a plurality of dengue virus subtypes. The DNA plasmid vaccines are comprised of a DNA plasmid capable of expressing a consensus dengue antigen in the mammal and a pharmaceutically acceptable excipient. The DNA plasmid is comprised of a promoter operably linked to a coding sequence that encodes the consensus dengue antigen. The consensus dengue antigen is comprised of consensus PRM/E domains of dengue virus-subtype 1, dengue virus-subtype 2, dengue virus-subtype 3, or dengue virus-subtype 4. Preferably, the DNA plasmid comprises a consensus dengue antigen that encodes a consensus dengue antigen selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, and SEQ ID NO: 8.

In some embodiments, the DNA plasmid further comprises an IgE leader sequence attached to an N-terminal end of the coding sequence and operably linked to the promoter. Preferably, the IgE leader has the sequence Met Arg Trp Thr Trp Ile Leu Phe Leu Val Ala Ala Ala Thr Arg Val His Ser.

The DNA plasmid can further include a polyadenylation sequence attached to the C-terminal end of the coding sequence. Preferably, the DNA plasmid is codon optimized.

In some embodiments, the pharmaceutically acceptable excipient is an adjuvant. Preferably, the adjuvant is selected from the group consisting of: IL-12 and IL-15. In some embodiments, the pharmaceutically acceptable excipient is a transfection facilitating agent. Preferably, the transfection facilitating agent is a polyanion, polycation, or lipid, and more preferably poly-L-glutamate. Preferably, the poly-L-glutamate is at a concentration less than 6 mg/mL. Preferably, the DNA plasmid vaccine has a concentration of total DNA plasmid of 1 mg/mL or greater.

In some embodiments, the DNA plasmid comprises a plurality of unique DNA plasmids, wherein each of the plurality of unique DNA plasmids encodes a polypeptide comprising a

prME dengue virus-subtype 1, dengue virus-subtype 2, dengue virus-subtype 3, or dengue virus-subtype 4.

The DNA plasmid vaccines can include a DNA plasmid comprising encoding nucleotide sequences: SEQ ID NO: 1, nucleotide sequence encoding SEQ ID NO: 2, SEQ ID NO: 3,nucleotide sequence encoding SEQ ID NO: 4, SEQ ID NO: 5,nucleotide sequence encoding SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8.

In some embodiments, the DNA plasmid vaccines comprises at least two different DNA plasmids that express a dengue virus prME. In some embodiments, the DNA plasmid vaccines can include four consensus dengue virus prME (subtypes 1-4).

In some embodiments, the mammal in which the DNA plasmid vaccines generate an immune response is a primate. Preferably, the mammal is a primate. The immune response can be either a humoral response or cellular response, and preferably both.

Another aspect of the present invention provides methods of eliciting an immune response against a plurality of dengue virus subtypes in a mammal, comprising delivering a DNA plasmid vaccine to tissue of the mammal and electroporating cells of the tissue with a pulse of energy at a constant current effective to permit entry of the DNA plasmids into the cells.

In some embodiments, the methods of eliciting an immune response includes a delivering step that comprises injecting the DNA plasmid vaccine into intradermic, subcutaneous or muscle tissue.

In some embodiments, the methods of eliciting an immune response can further comprise presetting a current that is desired to be delivered to the tissue; and electroporating cells of the tissue with a pulse of energy at a constant current that equals the preset current.

In some embodiments, the methods of eliciting an immune response further comprise measuring the impedance in the electroporated cells; adjusting energy level of the pulse of energy relative to the measured impedance to maintain a constant current in the

electroporated cells. The measuring and adjusting steps preferably occur within a lifetime of the pulse of energy.

In some embodiments, the electroporating step comprises delivering the pulse of energy to a plurality of electrodes according to a pulse sequence pattern that delivers the pulse of energy in a decentralized pattern.

In some embodiments of the present invention, the DNA plasmid vaccines can further include an adjuvant. In some embodiments, the adjuvant is selected from the group consisting of: alpha-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNFα, TNFβ, GM-CSF, epidermal growth factor (EGF), cutaneous T cell-attracting chemokine (CTACK), epithelial thymus-expressed chemokine (TECK), mucosae-associated epithelial chemokine (MEC), IL-12, IL-15, MHC, CD80, CD86 including IL-15 having the signal sequence deleted and optionally including the signal peptide from IgE. Other genes which may be useful adjuvants include those encoding: MCP-1, MIP-l-alpha, MIP-1p, IL-8, RANTES, L-selectin, P-selectin, E-selectin, CD34, GlyCAM-1, MadCAM-1, LFA-1, VLA-1, Mac-1, pl50.95, PECAM, ICAM-1, ICAM-2, ICAM-3, CD2, LFA-3, M-CSF, G-CSF, IL-4, mutant forms of IL-18, CD40, CD40L, vascular growth factor, fibroblast growth factor, IL-7, nerve growth factor, vascular endothelial growth factor, Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6, Caspase ICE, Fos, c-jun, Sp-1, Ap-1, Ap-2, p38, p65Rel, MyD88, IRAK, TRAF6, IkB, Inactive NIK, SAP K, SAP-1, JNK, interferon response genes, NFkB, Bax, TRAIL, TRAILrec, TRAILrecDRC5, TRAIL-R3, TRAIL-R4, RANK, RANK LIGAND, Ox40, Ox40 LIGAND, NKG2D, MICA, MICB, NKG2A, NKG2B, NKG2C, NKG2E, NKG2F, TAP1, TAP2 and functional fragments thereof. In some preferred embodiments, the adjuvant is selected from IL-12, IL-15, CTACK, TECK, or MEC.

In some embodiments, the pharmaceutically acceptable excipient is a transfection facilitating agent, which can include the following: surface active agents, such as immune-stimulating complexes (ISCOMS), Freunds incomplete adjuvant, LPS analog including monophosphoryl lipid A, muramyl peptides, quinone analogs, vesicles such as squalene and squalene, hyaluronic acid, lipids, liposomes, calcium ions, viral proteins, polyanions, polycations, or nanoparticles, or other known transfection facilitating agents. Preferably, the transfection

facilitating agent is a polyanion, polycation, including poly-L-glutamate (LGS), or lipid. Preferably, the transfection facilitating agent is poly-L-glutamate, and more preferably, the poly-L-glutamate is present in the DNA plasmid vaccine at a concentration less than 6 mg/mL. In some embodiments, the concentration of poly-L-glutamate in the DNA plasmid vaccine is less than 4 mg/mL, less than 2 mg/mL, less than 1 mg/mL, less than 0.750 mg/mL, less than 0.500 mg/mL, less than 0.250 mg/mL, less than 0.100 mg/mL, less than 0.050 mg/mL, or less than 0.010 mg/mL.

In some embodiments, the DNA plasmid vaccine can be delivered to a mammal to elicit an immune response; preferably the mammal is a primate, including human and nonhuman primate, a cow, pig, chicken, dog, or ferret. More preferably, the mammal is a human primate.

One aspect of the present invention relates to methods of eliciting an immune response against a plurality of subtypes of a virus in a mammal. The methods include delivering a DNA plasmid vaccine to tissue of the mammal, and electroporating cells of the tissue with a pulse of energy at a constant current effective to permit entry of the DNA plasmids into the cells. The DNA plasmid vaccine comprises a DNA plasmid capable of expressing a plurality of consensus antigens derived from the subtypes of the virus in a cell of the mammal to elicit an immune response in the mammal, the plurality of consensus antigens comprising an antigenic domain from at least two different subtypes of the virus.

One aspect of the present invention relates to methods of eliciting an immune response against a plurality of dengue virus subtypes in a mammal. The methods include delivering a DNA plasmid vaccine to tissue of the mammal, the DNA plasmid vaccine comprising a DNA plasmid capable of expressing a consensus dengue antigen in a cell of the mammal to elicit an immune response in the mammal, the consensus dengue antigen comprising consensus sequences coding for prME protein from at least two dengue subtypes, and preferably all four dengue subtypes. The dengue subtypes include subtype-1, subtype-2, subtype-3, and subtype-4. The methods of eliciting an immune response including electroporating cells of the tissue with a pulse of energy at a constant current effective to permit entry of the DNA plasmids in the cells.

In some embodiments, the methods of the present invention include the delivering step, which comprises injecting the DNA plasmid vaccine into intradermic, subcutaneous or muscle tissue. Preferably, these methods include using an in vivo electroporation device to preset a current that is desired to be delivered to the tissue; and electroporating cells of the tissue with a pulse of energy at a constant current that equals the preset current. In some embodiments, the electroporating step further comprises: measuring the impedance in the electroporated cells; adjusting energy level of the pulse of energy relative to the measured impedance to maintain a constant current in the electroporated cells; wherein the measuring and adjusting steps occur within a lifetime of the pulse of energy.

In some embodiments, the electroporating step comprises delivering the pulse of energy to a plurality of electrodes according to a pulse sequence pattern that delivers the pulse of energy in a decentralized pattern.

The present invention also comprises DNA fragments that encode a polypeptide capable of eliciting an immune response in a mammal substantially similar to that of the non-fragment for at least one dengue virus subtype. The DNA fragments are fragments selected from at least one of the various encoding nucleotide sequences of the present invention, including SEQ ID NO: 1, nucleotide sequence encoding SEQ ID NO: 2, SEQ ID NO: 3,nucleotide sequence encoding SEQ ID NO: 4, SEQ ID NO: 5,nucleotide sequence encoding SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, and can be any of the following described DNA fragments, as it applies to the specific encoding nucleic acid sequence provided herein. In some embodiments, DNA fragments can comprise 30 or more, 45 or more, 60 or more, 75 or more, 90 or more, 120 or more, 150 or more, 180 or more, 210 or more, 240 or more, 270 or more, 300 or more, 320 or more, 340 or more, or 360 or more nucleotides. In some embodiments, DNA fragments can comprise coding sequences for the immunoglobulin E (IgE) leader sequences. In some embodiments, DNA fragments can comprise fewer than 60, fewer than 75, fewer than 90, fewer than 120, fewer than 150, fewer than 180, fewer than 210, fewer than 240, fewer than 270, fewer than 300, fewer than 320, fewer than 340, or fewer than 360 nucleotides.

The present invention includes polypeptides encoded by the encoding nucleotide sequences and can include polypeptides having amino acid sequences of SEQ ID NOS: 2, 4, 6, and 8.

The present invention also comprises polypeptide fragments that are capable of eliciting an immune response in a mammal substantially similar to that of the non-fragment for at least one dengue subtype. The polypeptide fragments are selected from at least one of the various polypeptide sequences of the present invention, including SEQ ID NOS: 2, 4, 6, and 8, and can be any of the following described polypeptide fragments, as it applies to the specific polypeptide sequence provided herein. In some embodiments, polypeptide fragments can comprise 15 or more, 30 or more, 45 or more, 60 or more, 75 or more, 90 or more, 100 or more, 110 or more, or 120 or more amino acids. In some embodiments, polypeptide fragments can comprise fewer than 30, fewer than 45, fewer than 60, fewer than 75, fewer than 90, fewer than 100, fewer than 110, or fewer than 120 amino acids.

The determination of a functional fragment eliciting an immune response in a mammal substantially similar to that of the non-fragment for at least one dengue subtype can be readily determined by one of ordinary skill. The fragment can be analyzed to contain at least one, preferably more, antigenic epitopes as provided by a publicly available database, such as National Center for Biotechnology Information (NCBI). In addition, immune response studies can be routinely assessed using mice and antibody titers and ELISpots analysis, such as that shown in the Examples below.

Vaccines

In some embodiments, the invention provides improved vaccines by providing proteins and genetic constructs that encode proteins with epitopes that make them particularly effective as immunogens against which immune responses can be induced. Accordingly, vaccines can be provided to induce a therapeutic or prophylactic immune response.

According to some embodiments of the invention, a vaccine according to the invention is delivered to an individual to modulate the activity of the individual's immune system and thereby enhance the immune response. When a nucleic acid molecule that encodes the protein is taken up by cells of the individual the nucleotide sequence is expressed in the cells and the protein is thereby delivered to the individual. Aspects of the invention provide methods of delivering the coding sequences of the protein on nucleic acid molecule such as plasmid.

According to some aspects of the present invention, compositions and methods are provided which prophylactically and/or therapeutically immunize an individual.

When taken up by a cell, the DNA plasmids can remain in the cell as separate genetic material. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication. Genetic constructs include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for gene expression of the sequence that encodes the target protein or the immunomodulating protein. It is necessary that these elements be operable linked to the sequence that encodes the desired proteins and that the regulatory elements are operably in the individual to whom they are administered.

Initiation codons and stop codon are generally considered to be part of a nucleotide sequence that encodes the desired protein. However, it is necessary that these elements are functional in the mammals to whom the nucleic acid construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from simian virus 40 (SV40), mouse mammary tumor virus (MMTV) promoter, human immunodeficiency virus (HIV) such as the bovine immunodeficiency virus (BIV) long terminal repeat (LTR) promoter, Moloney virus, avian leukosis virus (ALV), cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr virus (EBV), Rous sarcoma virus (RSV) as well as promoters from human genes such as human actin, human myosin, human hemoglobin, human muscle creatine and human metalothionein; in other embodiments, promoters can be tissue specific promoters, such as muscle or skin specific promoters, natural or synthetic. Examples of such promoters are described in U.S. Patent Application

Publication No. US20040175727, which is incorporated hereby in its entirety.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to SV40 polyadenylation signals, LTR polyadenylation signals, bovine growth hormone (bGH) polyadenylation signals, human growth hormone (hGH) polyadenylation signals, and human β-globin polyadenylation signals. In particular, the SV40 polyadenylation signal that is in pCEP4 plasmid (Invitrogen, San Diego, CA), referred to as the SV40 polyadenylation signal, can be used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human actin, human myosin, human hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pVAX1, pCEP4 and pREP4 from Invitrogen (San Diego, CA) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the cells the construct is administered into. Moreover, codons that encode said protein may be selected which are most efficiently transcribed in the host cell. One having ordinary skill in the art can produce DNA constructs that are functional in the cells.

In some embodiments, nucleic acid constructs may be provided in which the coding sequences for the proteins described herein are linked to IgE signal peptide. In some embodiments, proteins described herein are linked to IgE signal peptide.

In some embodiments for which protein is used, for example, one having ordinary skill in the art can, using well known techniques, produce and isolate proteins of the invention using well known techniques. In some embodiments for which protein is used, for example, one having ordinary skill in the art can, using well known techniques, inserts DNA molecules that encode a protein of the invention into a commercially available expression vector for use in well-known expression systems. For example, the commercially available plasmid pSE420 (Invitrogen, San Diego, CA) may be used for production of protein in *Escherichia coli* (*E. coli*). The commercially available plasmid pYES2 (Invitrogen, San Diego, CA) may, for example, be used for production in Saccharomyces cerevisiae strains of yeast. The commercially available MAXBAC™ complete baculovirus expression system (Invitrogen, San Diego, CA) may, for example, be used for production in insect cells. The commercially available plasmid pcDNA or pcDNA3 (Invitrogen, San Diego, CA) may, for example, be used for production in mammalian cells such as Chinese hamster ovary (CHO) cells. One having ordinary skill in the art can use these commercial expression vectors and systems or others to produce protein by routine techniques and readily available starting materials. (See e.g., Sambrook et al., Molecular Cloning a Laboratory Manual, Second Ed. Cold Spring Harbor Press (1989)). Thus, the desired proteins can be prepared in both prokaryotic and eukaryotic systems, resulting in a spectrum of processed forms of the protein.

One having ordinary skill in the art may use other commercially available expression vectors and systems or produce vectors using well known methods and readily available starting materials. Expression systems containing the requisite control sequences, such as promoters and polyadenylation signals, and preferably enhancers are readily available and known in the art for a variety of hosts. See e.g., Sambrook et al., Molecular Cloning a Laboratory Manual, Second Ed. Cold Spring Harbor Press (1989). Genetic constructs include the protein coding sequence operably linked to a promoter that is functional in the cell line, or cells of targeted tissue, into which the constructs are transfected. Examples of constitutive promoters include promoters from cytomegalovirus (CMV) or SV40. Examples of inducible promoters include mouse mammary leukemia virus or metallothionein promoters. Those having ordinary skill in the art can readily produce genetic constructs useful for transfecting cells with DNA that encodes protein of the invention from readily available starting materials. The expression vector including the DNA that encodes the protein is used to transform the compatible host which is then cultured and maintained under conditions wherein expression of the foreign

DNA takes place.

The protein produced is recovered from the culture, either by lysing the cells or from the culture medium as appropriate and known to those in the art. One having ordinary skill in the art can, using well known techniques, isolate protein that is produced using such expression systems. The methods of purifying protein from natural sources using antibodies which specifically bind to a specific protein as described above may be equally applied to purifying protein produced by recombinant DNA methodology.

In addition to producing proteins by recombinant techniques, automated peptide synthesizers may also be employed to produce isolated, essentially pure protein. Such techniques are well known to those having ordinary skill in the art and are useful if derivatives which have substitutions not provided for in DNA-encoded protein production.

The nucleic acid molecules may be delivered using any of several well-known technologies including DNA injection (also referred to as DNA vaccination) with and without in vivo electroporation, liposome mediated, nanoparticle facilitated, recombinant vectors such as recombinant adenovirus, recombinant adenovirus associated virus and recombinant vaccinia. Preferably, the nucleic acid molecules such as the DNA plasmids described herein are delivered via DNA injection and along with in vivo electroporation.

Routes of administration include, but are not limited to, intramuscular, intranasally, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraoccularly and oral as well as topically, transdermally, by inhalation or suppository or to mucosal tissue such as by lavage to vaginal, rectal, urethral, buccal and sublingual tissue. Preferred routes of administration include intramuscular, intraperitoneal, intradermal and subcutaneous injection. Genetic constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, "microprojectile bombardment gone guns", or other physical methods such as electroporation (“EP”), “hydrodynamic method”, or ultrasound.

Examples of electroporation devices and electroporation methods preferred for facilitating delivery of the DNA vaccines of the present invention, include those described in U.S. Patent No. 7,245,963 by Draghia-Akli, et al., U.S. Patent Application Publication No. 2005/0052630

submitted by Smith, et al., the contents of which are hereby incorporated by reference in their entirety. Also preferred, are electroporation devices and electroporation methods for facilitating delivery of the DNA vaccines provided in co-pending and co-owned U.S. Patent Application Serial No. 11/874072, filed October 17, 2007, which claims the benefit under 35 USC 119(e) to U.S. Provisional Applications Serial No. 60/852,149, filed October 17, 2006, and U.S. Provisional Applications Serial No. 60/978,982, filed October 10, 2007, all of which are hereby incorporated in their entirety.

U.S. Patent No. 7,245,963 by Draghia-Akli, et al. describes modular electrode systems and their use for facilitating the introduction of a biomolecule into cells of a selected tissue in a body or plant. The modular electrode systems comprise a plurality of needle electrodes; a hypodermic needle; an electrical connector that provides a conductive link from a programmable constant-current pulse controller to the plurality of needle electrodes; and a power source. An operator can grasp the plurality of needle electrodes that are mounted on a support structure and firmly insert them into the selected tissue in a body or plant. The biomolecules are then delivered via the hypodermic needle into the selected tissue. The programmable constant-current pulse controller is activated and constant-current electrical pulse is applied to the plurality of needle electrodes. The applied constant-current electrical pulse facilitates the introduction of the biomolecule into the cell between the plurality of electrodes. The entire content of U.S. Patent No. 7,245,963 is hereby incorporated by reference.

U.S. Patent Application Publication No. 2005/0052630 submitted by Smith, et al. describes an electroporation device which may be used to effectively facilitate the introduction of a biomolecule into cells of a selected tissue in a body or plant. The electroporation device comprises an electro-kinetic device ("EKD device") whose operation is specified by software or firmware. The EKD device produces a series of programmable constant-current pulse patterns between electrodes in an array based on user control and input of the pulse parameters, and allows the storage and acquisition of current waveform data. The electroporation device also comprises a replaceable electrode disk having an array of needle electrodes, a central injection channel for an injection needle, and a removable guide disk. The entire content of U.S. Patent Application Publication No. 2005/0052630 is hereby

incorporated by reference.

The electrode arrays and methods described in U.S. Patent No. 7,245,963 and U.S. Patent Application Publication No. 2005/0052630 are adapted for deep penetration into not only tissues such as muscle, but also other tissues or organs. Because of the configuration of the electrode array, the injection needle (to deliver the biomolecule of choice) is also inserted completely into the target organ, and the injection is administered perpendicular to the target issue, in the area that is pre-delineated by the electrodes. The electrodes described in U.S. Patent No. 7,245,963 and U.S. Patent Application Publication No. 2005/005263 are preferably 20 mm long and 21 gauge.

The following is an example of methods of the present invention, and is discussed in more detail in the patent references discussed above: electroporation devices can be configured to deliver to a desired tissue of a mammal a pulse of energy producing a constant current similar to a preset current input by a user. The electroporation device comprises an electroporation component and an electrode assembly or handle assembly. The electroporation component can include and incorporate one or more of the various elements of the electroporation devices, including: controller, current waveform generator, impedance tester, waveform logger, input element, status reporting element, communication port, memory component, power source, and power switch. The electroporation component can function as one element of the electroporation devices, and the other elements are separate elements (or components) in communication with the electroporation component. In some embodiments, the electroporation component can function as more than one element of the electroporation devices, which can be in communication with still other elements of the electroporation devices separate from the electroporation component. The present invention is not limited by the elements of the electroporation devices existing as parts of one electromechanical or mechanical device, as the elements can function as one device or as separate elements in communication with one another. The electroporation component is capable of delivering the pulse of energy that produces the constant current in the desired tissue, and includes a feedback mechanism. The electrode assembly includes an electrode array having a plurality of electrodes in a spatial arrangement, wherein the electrode assembly receives the pulse of energy from the electroporation component and delivers same to the desired tissue through the electrodes. At least one of the plurality of electrodes is neutral during delivery of the

pulse of energy and measures impedance in the desired tissue and communicates the impedance to the electroporation component. The feedback mechanism can receive the measured impedance and can adjust the pulse of energy delivered by the electroporation component to maintain the constant current.

In some embodiments, the plurality of electrodes can deliver the pulse of energy in a decentralized pattern. In some embodiments, the plurality of electrodes can deliver the pulse of energy in the decentralized pattern through the control of the electrodes under a programmed sequence, and the programmed sequence is input by a user to the electroporation component. In some embodiments, the programmed sequence comprises a plurality of pulses delivered in sequence, wherein each pulse of the plurality of pulses is delivered by at least two active electrodes with one neutral electrode that measures impedance, and wherein a subsequent pulse of the plurality of pulses is delivered by a different one of at least two active electrodes with one neutral electrode that measures impedance.

In some embodiments, the feedback mechanism is performed by either hardware or software. Preferably, the feedback mechanism is performed by an analog closed-loop circuit. Preferably, this feedback occurs every 50 μs, 20 μs, 10 μs or 1 μs, but is preferably a real-time feedback or instantaneous (i.e., substantially instantaneous as determined by available techniques for determining response time). In some embodiments, the neutral electrode measures the impedance in the desired tissue and communicates the impedance to the feedback mechanism, and the feedback mechanism responds to the impedance and adjusts the pulse of energy to maintain the constant current at a value similar to the preset current. In some embodiments, the feedback mechanism maintains the constant current continuously and instantaneously during the delivery of the pulse of energy.

A pharmaceutically acceptable excipient can include such functional molecules as vehicles, adjuvants, carriers or diluents, which are known and readily available to the public. Preferably, the pharmaceutically acceptable excipient is an adjuvant or transfection facilitating agent. In some embodiments, the nucleic acid molecule, or DNA plasmid, is delivered to the cells in conjunction with administration of a polynucleotide function enhancer or a genetic vaccine facilitator agent (or transfection facilitating agent). Polynucleotide function enhancers are described in U.S. Patent No. 5,593,972, U.S. Patent

No. 5,962,428, and International Patent Application No. PCT/US94/00899 filed January 26, 1994, which are each incorporated herein by reference. Genetic vaccine facilitator agents are described in U.S. Patent Application Serial No. 021,579 filed April 1, 1994, which is incorporated herein by reference. The transfection facilitating agent can be administered in conjunction with nucleic acid molecules as a mixture with the nucleic acid molecule or administered separately simultaneously, before or after administration of nucleic acid molecules. Examples of transfection facilitating agents includes surface active agents such as immune-stimulating complexes (ISCOMS), Freunds incomplete adjuvant, LPS analog including monophosphoryl lipid A, muramyl peptides, quinone analogs and vesicles such as squalene and squalene, and hyaluronic acid may also be used administered in conjunction with the genetic construct. In some embodiments, the DNA plasmid vaccines may also include a transfection facilitating agent such as lipids, liposomes, including lecithin liposomes or other liposomes known in the art, as a DNA-liposome mixture (see for example W09324640), calcium ions, viral proteins, polyanions, polycations, or nanoparticles, or other known transfection facilitating agents. Preferably, the transfection facilitating agent is a polyanion, polycation, including poly-L-glutamate (LGS), or lipid.

In some preferred embodiments, the DNA plasmids are delivered with an adjuvant that are genes for proteins which further enhance the immune response against such target proteins. Examples of such genes are those which encode other cytokines and lymphokines such as alpha-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNFα, TNFβ, GM-CSF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18, MHC, CD80,CD86 and IL-15 including IL-15 having the signal sequence deleted and optionally including the signal peptide from IgE. Other genes which may be useful include those encoding: MCP-1, MIP-lα, MIP-1p, IL-8, RANTES, L-selectin, P-selectin, E-selectin, CD34, GlyCAM-1, MadCAM-1, LFA-1, VLA-1, Mac-1, pl50.95, PECAM, ICAM-1, ICAM-2, ICAM-3, CD2, LFA-3, M-CSF, G-CSF, IL-4, mutant forms of IL-18, CD40, CD40L, vascular growth factor, fibroblast growth factor, IL-7, nerve growth factor, vascular endothelial growth factor, Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6, Caspase ICE, Fos, c-jun, Sp-1, Ap-1, Ap-2, p38, p65Rel, MyD88, IRAK, TRAF6, IkB, Inactive NIK, SAP K, SAP-1, JNK, interferon response genes, NFkB, Bax, TRAIL, TRAILrec, TRAILrecDRC5, TRAIL-R3, TRAIL-R4, RANK, RANK LIGAND, Ox40, Ox40 LIGAND, NKG2D, MICA,

MICB, NKG2A, NKG2B, NKG2C, NKG2E, NKG2F, TAP1, TAP2 and functional fragments thereof.

The DNA plasmid vaccines according to the present invention comprise DNA quantities of from about 1 nanogram to 10 milligrams; about 1 microgram to about 10 milligrams; or preferably about 0.1 microgram to about 10 milligrams; or more preferably about 100 microgram to about 1 milligram. In some preferred embodiments, DNA plasmid vaccines according to the present invention comprise about 5 nanograms to about 1000 micrograms of DNA. In some preferred embodiments, the DNA plasmid vaccines contain about 10 nanograms to about 800 micrograms of DNA. In some preferred embodiments, the DNA plasmid vaccines contain about 0.1 to about 500 micrograms of DNA. In some preferred embodiments, the DNA plasmid vaccines contain about 1 to about 350 micrograms of DNA. In some preferred embodiments, the DNA plasmid vaccines contain about 25 to about 250 micrograms of DNA. In some preferred embodiments, the DNA plasmid vaccines contain about 100 microgram to about 1 milligram DNA.

The DNA plasmid vaccines according to the present invention are formulated according to the mode of administration to be used. In cases where DNA plasmid vaccines are injectable compositions, they are sterile, and/or pyrogen free and/or particulate free. An isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation. In some embodiments, a stabilizing agent that allows the formulation to be stable at room or ambient temperature for extended periods of time, such as LGS or other polycations or polyanions is added to the formulation.

In some embodiments, methods of eliciting an immune response in mammals against a consensus dengue antigen include methods of inducing mucosal immune responses. Such methods include administering to the mammal one or more of CTACK protein, TECK protein, MEC protein and functional fragments thereof or expressible coding sequences thereof in combination with a DNA plasmid including a consensus dengue antigen, described above. The one or more of CTACK protein, TECK protein, MEC protein and functional

fragments thereof may be administered prior to, simultaneously with or after administration of the DNA plasmid dengue vaccines provided herein. In some embodiments, an isolated nucleic acid molecule that encodes one or more proteins of selected from the group consisting of: CTACK, TECK, MEC and functional fragments thereof is administered to the mammal.

The present invention is further illustrated in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Preferably the DNA formulations for use with a muscle or skin EP device described herein have high DNA concentrations, preferably concentrations that include microgram to tens of milligram quantities, and preferably milligram quantities, of DNA in small volumes that are optimal for delivery to the skin, preferably small injection volume, ideally 25-200 microliters (μL). In some embodiments, the DNA formulations have high DNA concentrations, such as 1 mg/mL or greater (mg DNA/volume of formulation). More preferably, the DNA formulation has a DNA concentration that provides for gram quantities of DNA in 200 μL of formula, and more preferably gram quantities of DNA in 100 μL of formula.

The DNA plasmids for use with the EP devices of the present invention can be formulated or manufactured using a combination of known devices and techniques, but preferably they are manufactured using an optimized plasmid manufacturing technique that is described in U.S. Patent Application No. 12/126611 which published as U.S. Patent Application Publication No. 20090004716, which published January 1, 2009. In some examples, the DNA plasmids used in these studies can be formulated at concentrations greater than or equal to 10 mg/mL. The manufacturing techniques also include or incorporate various devices and protocols that are commonly known to those of ordinary skill in the art, in addition to those described in U.S. Patent Application Publication No. 20090004716 and those described in U.S. Patent No.

7,238,522, which issued on July 3, 2007. The high concentrations of plasmids used with the skin EP devices and delivery techniques described herein allow for administration of plasmids into the ID/SC space in a reasonably low volume and aids in enhancing expression and immunization effects. The publications, U.S. Patent Application Publication No. 20090004716 and U.S. Patent No. 7,238,522, are hereby incorporated in their entirety.

EXAMPLES

Example 1

prM prevents premature fusion of the E protein during virus maturation by forming a non-infectious immature virus particles, prM-E heterodimeric complex. The immature particles transit through a low pH environment of the Golgi compartment, and at this stage, a reversible conformational change occurs in E protein prior to processing of prM. After cleavage of prM to M by cellular serine protease in the trans-Golgi network, the result is an irreversible conformational change in E which maintains the integrity of the neutralizing epitopes.

D1prME, D2prME, D3prME, and D4prME were constructs encoding an antigen comprising the consensus sequence of both the pRM (pre-membrane precursor) protein and the E (envelope) protein for serotype D1, D2, D3, or D4, respectively.

DU was a construct encoding an antigen comprising the consensus sequence of the DIII domain of the E (envelope) protein for serotype D1, the consensus sequence of the DIII domain of the E (envelope) protein for serotype D2, the consensus sequence of the DIII domain of the E (envelope) protein for serotype D3, and the consensus sequence of the DIII domain of the E (envelope) protein for serotype D4, with each separated by a linker. A construct combining all four DIII domains (universal or universal DIII) separated by proteolytic cleavage sequences was constructed and referred to as DU (SEQ ID NO: 9). The consensus DIII domain DU that has the sequence SEQ ID NO: 10 consists of linker sequences connecting each of the four subtypes of DIII (DV-1-DIII, DV-2-DIII, DV-3-DIII, and DV-4-DIII). The linker has the sequence RGRKRRS, which is a known cleavage site and allowed cleavage of DU into separate DIII sequences of a specific subtype. However, the

linker sequence can be any other linker sequence available in the art that has similar characteristics, and it is within the ordinary skill in the art to replace such linker for RGRKRRS currently utilized.

Mice were immunized with 100 μg of D1prME, D2prME, D3prME, D4prME, or DU. Mice received 3 immunizations with 3P Cellectra (ID), each 3 weeks apart, and bled every 3 weeks at the same injection site. Each group included 5 animals (n=5).

Serial dilutions (1:50, 1:150, 1:450, 1:1350, and 1:4050) were made of serum from each mouse. The serum was contacted with DIII (domain III of the envelope protein E) from each of the four serotypes D1, D2, D3, or D4. Binding of the antibodies in the serum to the DIII protein was determined by measuring the absorbance at 450 nm. BSA was used as a control.

A summary of the experimental design is below.

Table 1. Experimental Design

GROUP	NO. OF ANIMALS	PLASMID	NO. OF INJECTION SITES	PLASMID DOSE PER INJECTION
1	5	D1prME	1	100 ug
2	5	D2prME	1	100 ug
3	5	D3prME	1	100 ug
4	5	D4prME	1	100 ug
5	5	DU	1	100 ug
6	5	D1-D4 prME Separate	4	100 ug each; 400 ug in total
7	5	D1-D4 prME Mixed together	1	100 ug each; 400 ug in total
8	3	control	NONE	NONE

The results are shown in Figures 1-5. As shown in Figure 1, all mice immunized with D1prME generated antibodies that bound to DIII from D1, and all mice immunized with DU also generated antibodies that bound to DIII from D1. As shown in Figure 2, all mice immunized with D2prME generated antibodies that bound to DIII from D2, and all mice immunized with DU also generated antibodies that bound to DIII from D2. As shown in Figure 3, mice 3, 4, and 5 immunized with D3prME generated antibodies that bound to DIII from D3, but not as well as the binding shown in Figures 1 and 2. Mice 1 and 2 immunized with D3prME did not generate antibodies that bound well to DIII from D3. All mice immunized with DU generated antibodies that bound to DIII from D3. As shown in Figure 4, mice 4 and 5 immunized with D1prME generated antibodies that bound to DIII from D4, although the antibodies from mice 1, 2, and 3 did not. All mice immunized with DU generated antibodies that bound to DIII from D4.

Serum from mice immunized with D1prME, D2prME, D3prME, or D4prME was analyzed with Western blot analysis for protein E and protein prM. As shown in Figure 5, protein E and prM were separated and present in serum from all mice.

Example 2: Dengue Li-Cor Assay

Guinea pigs were immunized with 100 μg of D1prME, D2prME, D3prME, D4prME, or DU. In some groups, as detailed below, each guinea pig was immunized with all four D1prME, D2prME, D3prME, or D4prME, in either a single or separate immunization sites. In the single administration site, all four were mixed together in a single administration. For the separate immunization sites, each was administered separately. As in Example 2, D1prME, D2prME, D3prME, and D4prME were constructs encoding an antigen comprising both the pRM (pre-membrane precursor) protein and the E (envelope) protein from serotype D1, D2, D3, or D4, respectively. DU was a construct encoding an antigen comprising the consensus sequence of the DIII domain of the E (envelope) protein for serotype D1, the consensus sequence of the DIII domain of the E (envelope) protein for serotype D2, the consensus sequence of the DIII domain of the E (envelope) protein for serotype D3, and the consensus sequence of the DIII domain of the E (envelope) protein for serotype D4, with each separated by a linker, as detailed in Example 1.

Serial dilutions (1:50, 1:150, 1:450, 1:1350, and 1:4050) were made of serum from each guinea pig. Binding of the antibodies in the serum to the Dengue virus was determined by measuring the absorbance at 450 nm.

2-fold serial dilutions of serum were made and placed in a 96-well plate, with 50 μL per well. 50 pfu (50 μL per well) of Dengue virus was added to each well. The plates were incubated at 37°C for 1 hour to allow for neutralization of the virus with antibodies from the serum. The entire mixture (100 μL) was added to VERO cells (seed 1.5 x 10^4 / well). The plates were incubated for 4 days at 37°C. Cells were fixed using 3.7% formaldehyde for 30 minutes. Cells were washed and permeabilized using 0.1% TritonX-100/PBS. A cell-based ELISA was performed, using Mouse 4G2 mAb, Biotinylated anti-mouse IgG, and IRDye 800CW Streptavidin + 5 mM DRAQ5 cocktail solution. The plates were washed, dried, and scanned using a Li-Cor Aerius system. 800 nm/700 nm ratios were calculated.

Results are shown in Figures 6-13. As shown in Figure 6, the sera from two control guinea pigs (no immunization) did not bind DIII from D1, D2, D3, or D4 serotypes. As shown in Figure 7, guinea pigs were immunized with DU, and only guinea pig 4 generated antibodies that bound to DIII protein from D1, D2, D3, and D4 serotypes. As shown in Figure 8, five guinea pigs were immunized with a mixture of 100 μg of D1prME, 100 μg of D2prME, 100 μg of D3prME, and 100 μg of D4prME, for a total of 400 μg all in one immunization site per guinea pig. All guinea pigs generated antibodies that bound DIII protein from D1, D2, and D3. However, only two guinea pigs generated antibodies that bound DIII protein from D4, and the binding was attenuated relative to the other serotypes. As shown in Figure 9, five guinea pigs were immunized with 100 μg of D1prME, 100 μg of D2prME, 100 μg of D3prME, and 100 μg of D4prME at four separate sites, for a total of 400 μg per guinea pig. All guinea pigs generated antibodies that bound DIII protein from D1, D2, and D3. However, only three or four guinea pigs generated antibodies that bound DIII protein from D4, and the binding was attenuated relative to the other serotypes. As shown in Figure 10, five guinea pigs were immunized with 100 μg of D1prME each. All guinea pigs generated antibodies that bound DIII protein from D1. However, only a few guinea pigs generated antibodies that bound DIII protein from D2, D3, or D4, and the binding was attenuated relative to D1. As shown in Figure 11, five guinea pigs were immunized with 100 μg of D2prME each. All guinea pigs generated antibodies that bound DIII protein from D2. However, only one guinea

pig generated antibodies that bound DIII protein from D1, and the binding was attenuated relative to D1. No guinea pigs generated antibodies that bound DIII protein from D3, or D4. As shown in Figure 12, five guinea pigs were immunized with 100 μg of D3prME each. All guinea pigs generated antibodies that bound DIII protein from D1 and D3. However, only one guinea pig generated antibodies that bound DIII protein from D2. No guinea pigs generated antibodies that bound DIII protein from D4. As shown in Figure 13, five guinea pigs were immunized with 100 μg of D4prME each. Four guinea pigs generated antibodies that bound DIII protein from D4. Guinea pig 1 generated antibodies that bound DIII protein from D1 and D2. Only a few guinea pigs generated antibodies that bound (minimally) to DIII protein from D2 and D3.

A comparison of Figures 8 and 9 with Figure 7 indicates immunization with a combination of D1prME, D2prME, D3prME, and D4prME, whether separate or in a mixture, resulted in better immunogenicity to DIII than did immunization with DU.

Example 3: Dengue $PRNT_{50}$ and FRNT Assays

Guinea pigs were immunized with 100 μg of DU, D1prME, D2prME, D3prME, or D4prME. In some groups, as detailed below, each guinea pig was immunized with all four D1prME, D2prME, D3prME, or D4prME, in either a single or separate immunization sites. As in Examples 2 and 3, D1prME, D2prME, D3prME, and D4prME were constructs encoding an antigen comprising both the pRM (pre-membrane precursor) protein and the E (envelope) protein from serotype D1, D2, D3, or D4, respectively. DU was a construct encoding an antigen comprising the consensus sequence of the DIII domain of the E (envelope) protein for serotype D1, the consensus sequence of the DIII domain of the E (envelope) protein for serotype D2, the consensus sequence of the DIII domain of the E (envelope) protein for serotype D3, and the consensus sequence of the DIII domain of the E (envelope) protein for serotype D4, with each separated by a linker, as detailed in Example 2.

For the Dengue PRNT50 Assay, VERO cells were plated in a 6 well format ($7.5x10^5$ to $1.0x10^6$ cells/well) on Day 1. On Day 2, 50 pfu of Dengue virus/well was incubated with serum from guinea pigs for 1 hour at 37°C. The Dengue virus used was one of the strains Hawaii, NGC, H87, or H241. The mixture was added to the cell monolayer in the plate and

incubated for 1 hour at 37°C. Cells were overlayed with 1% methyl-cellulose in 2% medium 199. Cells were incubated for 5 days post-infection at 37°C. On Day 7, cells were fixed and stained with a Crystal violet/20% methanol mixture (incubation for 2 hours). The plates were washed with dH_2O and dried. Plaques were counted. Neutralization titers were defined as the reciprocal of the highest serum dilution that reduced plaque count by >50%, compared to controls without sera.

For the Focus Diagnostics FRNT Assay, 24-well plates were used with a 4-fold dilution of serum. Plates were incubated for 4 days. Immunofocus was used to determine and count the development of plaques.

Results are shown in Table 2 below and in Figures 14-17. As shown in Figure 14, sera from guinea pigs immunized with all of D1prME, D2prME, D3prME, or D4prME constructs, as well as guinea pigs immunized with just D1prME, generated antibodies that bound proteins from the Hawaii Dengue virus. Immunization with DU did not. As shown in Figure 15, sera from guinea pigs immunized with all of D1prME, D2prME, D3prME, or D4prME constructs, as well as guinea pigs immunized with just D2prME, generated antibodies that bound proteins from the NGC Dengue virus. Immunization with DU did not. As shown in Figure 16, sera from guinea pigs immunized with all of D1prME, D2prME, D3prME, or D4prME constructs, as well as guinea pigs immunized with just D3prME, generated antibodies that bound proteins from the H87 Dengue virus. The observed titer after immunization with DU was minimal. As shown in Figure 17, sera from guinea pigs immunized with all of D1prME, D2prME, D3prME, or D4prME constructs, as well as guinea pigs immunized with just D4prME, generated antibodies that bound proteins from the H241 Dengue virus. Immunization with DU did not.

Table 2. Ph1 Clinical Study Virus Neutralizing Antibody Responses determined by LiCor, FRNT and PRNT assays.

Protocol	Treatment Group	Time Point	Blinding ID	Merck Li-Cor DEN1 Titers	HBI PRNT DEN1 Titers	Focus FRNT DEN1 Titers
HBV-001		week 10	69	320	44	152
HBV-001		week 10	42	80	30	32
HBV-001	10µg DEN1-80E	week 10	84	5	5	5
HBV-001	1.25 mg Alhyd.	week 10	7	320	91	137
HBV-001		week 10	18	40	22	28
HBV-001		week 10	49	5	5	5
			GMT	46	21	28
HBV-001		week 10	77	640	502	370
HBV-001		week 10	28	160	55	25
HBV-001	50µg DEN1-80E	week 10	95	80	5	5
HBV-001	1.25 mg Alhyd.	week 10	66	80	37	20
HBV-001		week 10	53	320	62	82
HBV-001		week 10	47	80	14	29
			GMT	160	41	34
HBV-001		week 10	25	5	5	5
HBV-001	Placebo	week 10	86	5	5	5
HBV-001		week 10	20	5	5	5
HBV-001		week 10	12	5	5	5
			GMT	5	5	5

What is claimed:

1. A nucleic acid construct capable of expressing a polypeptide that elicits an immune response in a mammal against more than one subtype of Dengue virus, comprising:

an encoding nucleotide sequence that expresses the polypeptide, wherein the polypeptide includes a PRM/E domain from at least two different Dengue virus subtypes,

a promoter that regulates expression of the polypeptide in the mammal and is operably linked to the encoding nucleotide sequence.

2. The nucleic acid construct of claim 1, further comprising an IgE leader sequence operatively linked to an N-terminal end of the coding sequence and operably linked to the promoter.

3. The nucleic acid construct of any one of claims 1-2, further comprising a polyadenylation sequence attached to the C-terminal end of the coding sequence.

4. The nucleic acid construct of any one of claims 1-3, wherein the nucleic acid construct is codon optimized.

5. The nucleic acid construct of any one of claims 1-4, wherein the encoding nucleotide sequence encodes a polypeptide that includes a DIII domain from dengue virus-subtype 1, dengue virus-subtype 2, dengue virus-subtype 3, or dengue virus-subtype 4, or a combination thereof.

6. The nucleic acid construct of any one of claims 1-5, wherein the encoding nucleotide sequence is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

7. A DNA plasmid vaccine capable of generating in a mammal an immune response against a plurality of dengue virus subtypes, comprising:

a DNA plasmid capable of expressing a consensus dengue antigen in a cell of the mammal in a quantity effective to elicit an immune response in the mammal, the consensus

dengue antigen comprising consensus DIII domain of dengue virus-subtype 1, dengue virus-subtype 2, dengue virus-subtype 3, or dengue virus-subtype 4, or a combination thereof, and a pharmaceutically acceptable excipient;

the DNA plasmid comprising a promoter operably linked to a coding sequence that encodes the consensus dengue antigen.

8. The DNA plasmid vaccine of claim 7, wherein the DNA plasmid further comprises an IgE leader sequence attached to an N-terminal end of the coding sequence and operably linked to the promoter.

9. The DNA plasmid vaccine of any one of claims 7-8, wherein the DNA plasmid further comprises a polyadenylation sequence attached to the C-terminal end of the coding sequence.

10. The DNA plasmid vaccine of any one of claims 7-9, wherein the DNA plasmid is codon optimized.

11. The DNA plasmid vaccine of any one of claims 7-10 wherein the pharmaceutically acceptable excipient is an adjuvant.

12. The DNA plasmid vaccine of claim 11, wherein the adjuvant is selected from the group consisting of: IL-12 and IL-15.

13. The DNA plasmid vaccine of any one of claims 7-10, wherein the pharmaceutically acceptable excipient is a transfection facilitating agent.

14. The DNA plasmid vaccine of claim 13, wherein the transfection facilitating agent is a polyanion, polycation, or lipid.

15. The DNA plasmid vaccine of claim 13, wherein the transfection facilitating agent is poly-L-glutamate at a concentration less than 6 mg/mL.

16. The DNA plasmid vaccine of any one of claims 7-15, wherein the DNA plasmid vaccine has a concentration of total DNA plasmid of 1 mg/mL or greater.

17. The DNA plasmid vaccine of any one of claims 7-16, wherein the DNA plasmid comprises a plurality of unique DNA plasmids, wherein each of the plurality of unique DNA plasmids encodes a polypeptide comprising a PRM/E domain from dengue virus-subtype 1, dengue virus-subtype 2, dengue virus-subtype 3, or dengue virus-subtype 4, or a combination thereof.

18. The DNA plasmid vaccine of any one of claims 7-17, wherein the encoding nucleotide sequence comprises SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5.

19. The DNA plasmid vaccine of any one of claims 7-18, comprising at least two different DNA plasmids that express a Dengue virus PRM/E domain, the plasmids selected from the group consisting of:

a DNA plasmid comprising a sequence that encodes a consensus dengue virus-subtype 1 PRM/E domain,

a DNA plasmid comprising a sequence that encodes a consensus dengue virus-subtype 2 PRM/E domain,

a DNA plasmid comprising a sequence that encodes a consensus dengue virus-subtype 3 PRM/E domain, and

a DNA plasmid comprising a sequence that encodes a consensus dengue virus-subtype 4 PRM/E domain.

20. The DNA plasmid vaccine of any one of claims 7-19, comprising:

the DNA plasmid comprising a sequence that encodes a consensus dengue virus - subtype 1 PRM/E domain,

the DNA plasmid comprising a sequence that encodes a consensus dengue virus-subtype 2 PRM/E domain,

the DNA plasmid comprising a sequence that encodes a consensus dengue virus-subtype 3 PRM/E domain, and

the DNA plasmid comprising a sequence that encodes a consensus dengue virus-subtype 4 PRM/E domain.

21. The DNA plasmid vaccine of any one of claims 7-20, wherein the consensus dengue virus-subtype 1 PRM/E domain is SEQ ID NO: 1.

22. The DNA plasmid vaccine of any one of claims 7-21, wherein the consensus dengue virus-subtype 2 PRM/E domain is SEQ ID NO: 2.

23. The DNA plasmid vaccine of any one of claims 7-22, wherein the consensus dengue virus-subtype 3 PRM/E domain is SEQ ID NO: 3.

24. The DNA plasmid vaccine of any one of claims 7-23, wherein the consensus dengue virus-subtype 4 PRM/E domain is SEQ ID NO: 4.

25. The DNA plasmid vaccine of any one of claims 7-24, wherein the mammal is a nonhuman primate.

26. The DNA plasmid vaccine of any one of claims 7-25, wherein the immune response is a humoral response.

27. The DNA plasmid vaccine of any one of claims 7-25, wherein the immune response is a cellular response.

28. The DNA plasmid vaccine of any one of claims 7-25, wherein the immune response is a combined humoral response and cellular response.

29. A method of eliciting an immune response against a plurality of subtypes of a virus in a mammal, comprising,

delivering a DNA plasmid vaccine to tissue of the mammal, the DNA plasmid vaccine comprising a DNA plasmid capable of expressing a plurality of consensus antigens derived from the subtypes of the virus in a cell of the mammal to elicit an immune response in the mammal, the plurality of consensus antigens comprising an antigenic domain from at least two different subtypes of the virus, and

electroporating cells of the tissue with a pulse of energy at a constant current effective to permit entry of the DNA plasmids into the cells.

30. The method of claim 29, wherein the virus comprises west nile virus, human immunodeficiency virus, influenza virus, or dengue virus.

31. The method of any one of claims 29-30, wherein the delivering step comprises:

injecting the DNA plasmid vaccine into intradermic, subcutaneous or muscle tissue.

32. The method of any one of claims 29-31, further comprising:

presetting a current that is desired to be delivered to the tissue; and

electroporating cells of the tissue with a pulse of energy at a constant current that equals the preset current.

33. The method of any one of claims 29-32, wherein the electroporating step further comprises:

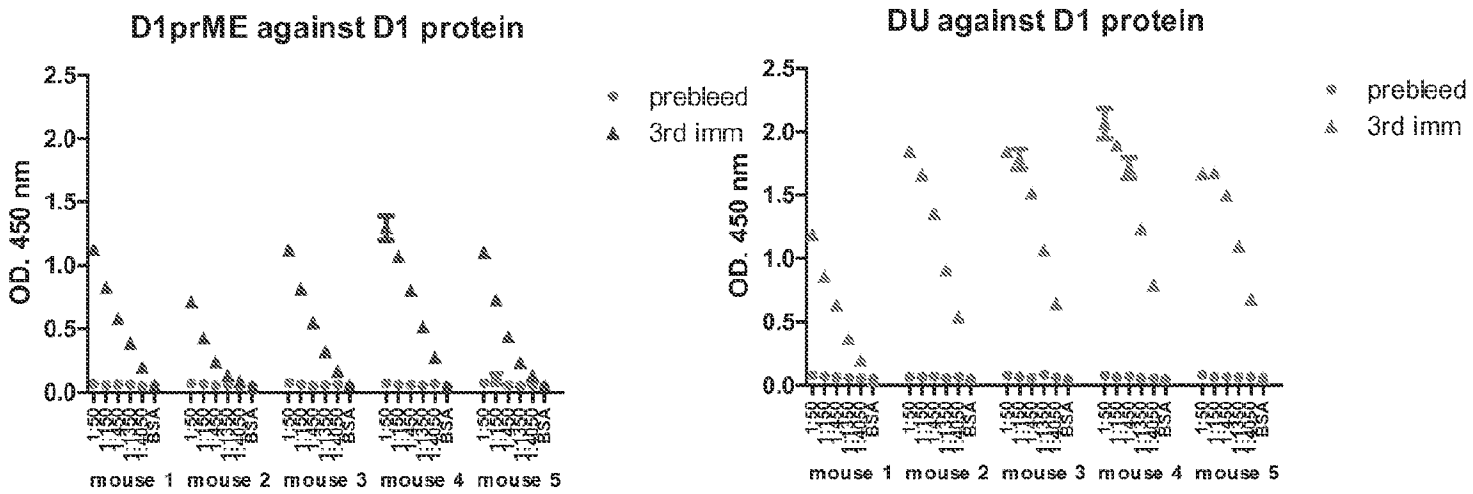
measuring the impedance in the electroporated cells;

adjusting energy level of the pulse of energy relative to the measured impedance to maintain a constant current in the electroporated cells;

wherein the measuring and adjusting steps occur within a lifetime of the pulse of energy.

34. The method of any one of claims 29-33, wherein the electroporating step comprises:

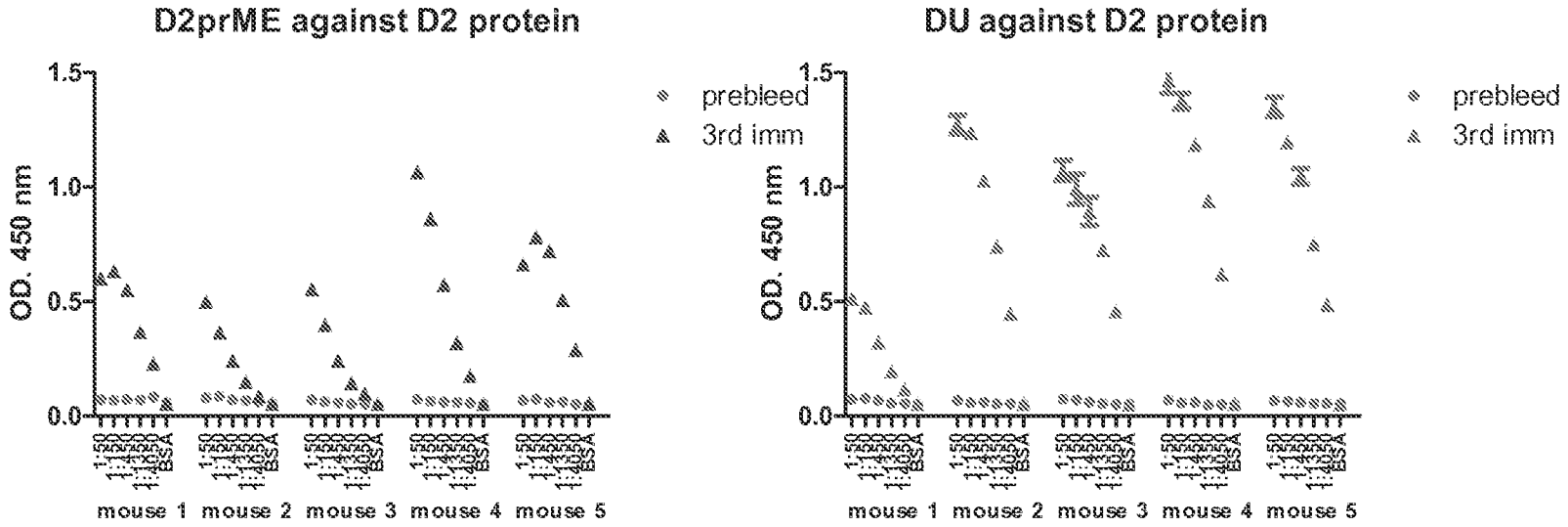
delivering the pulse of energy to a plurality of electrodes according to a pulse sequence pattern that delivers the pulse of energy in a decentralized pattern.



Comparison of binding antibodies against D1-DIII protein in mice immunized with DU or D1prME construct

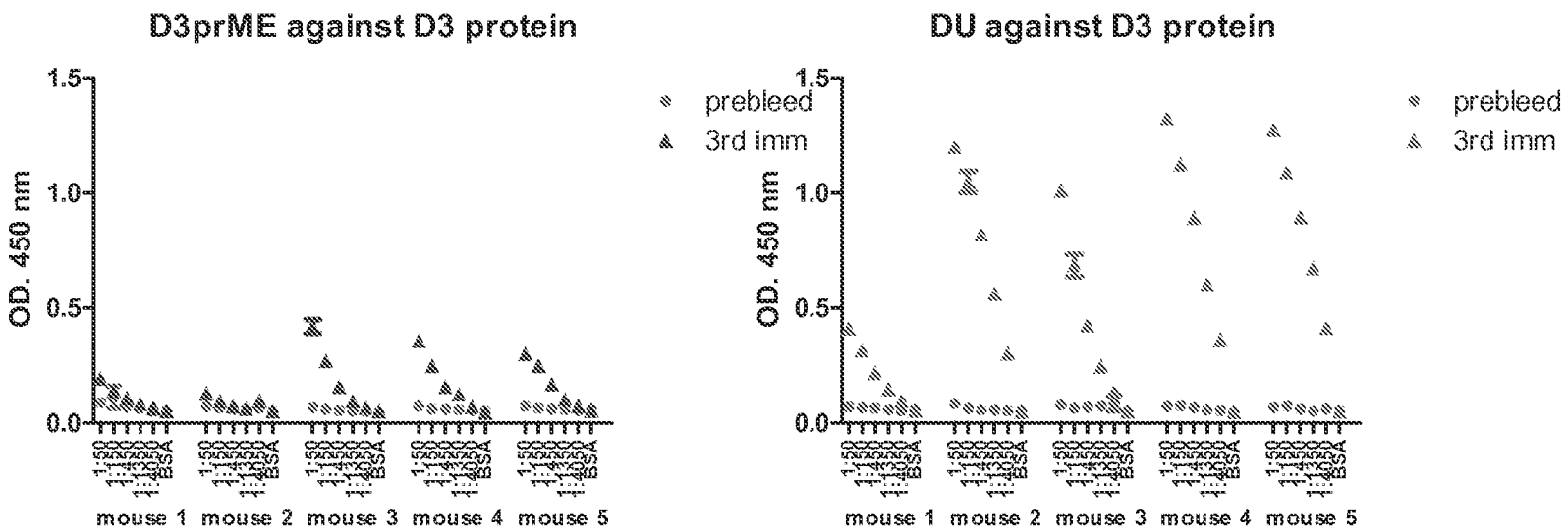
FIGURE 1

WO 2014/144786 PCT/US2014/029341



Comparison of binding antibodies against D2-DIII protein in mice immunized with DU or D2prME construct

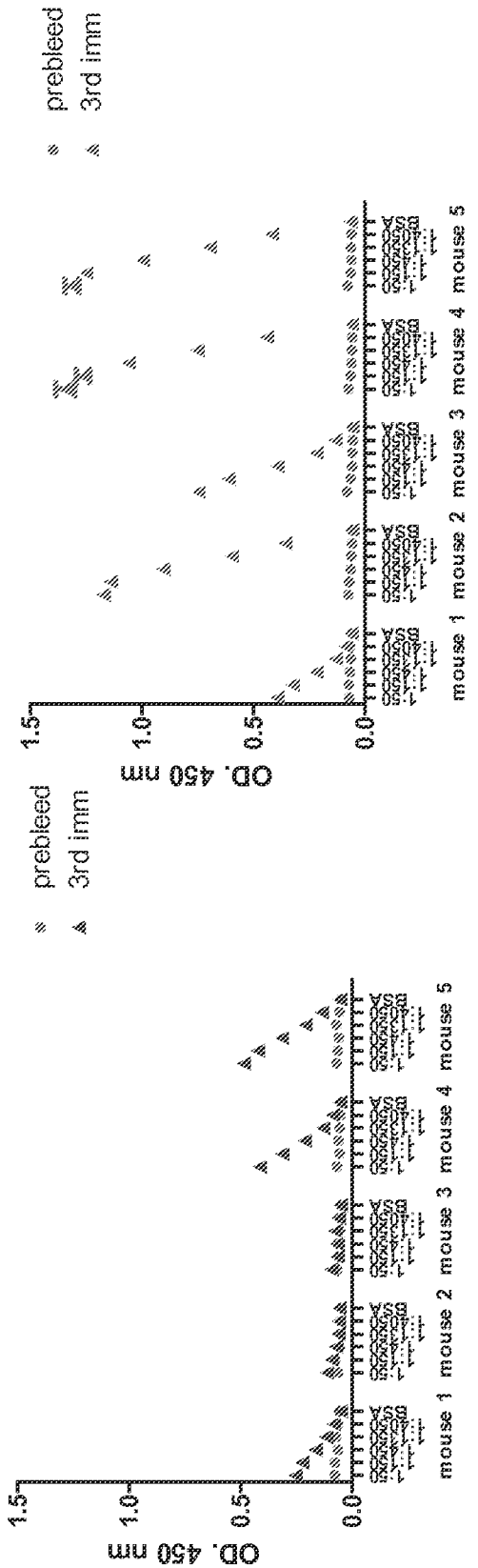
FIGURE 2



Comparison of binding antibodies against D3-DIII protein in mice immunized with DU or D3prME construct

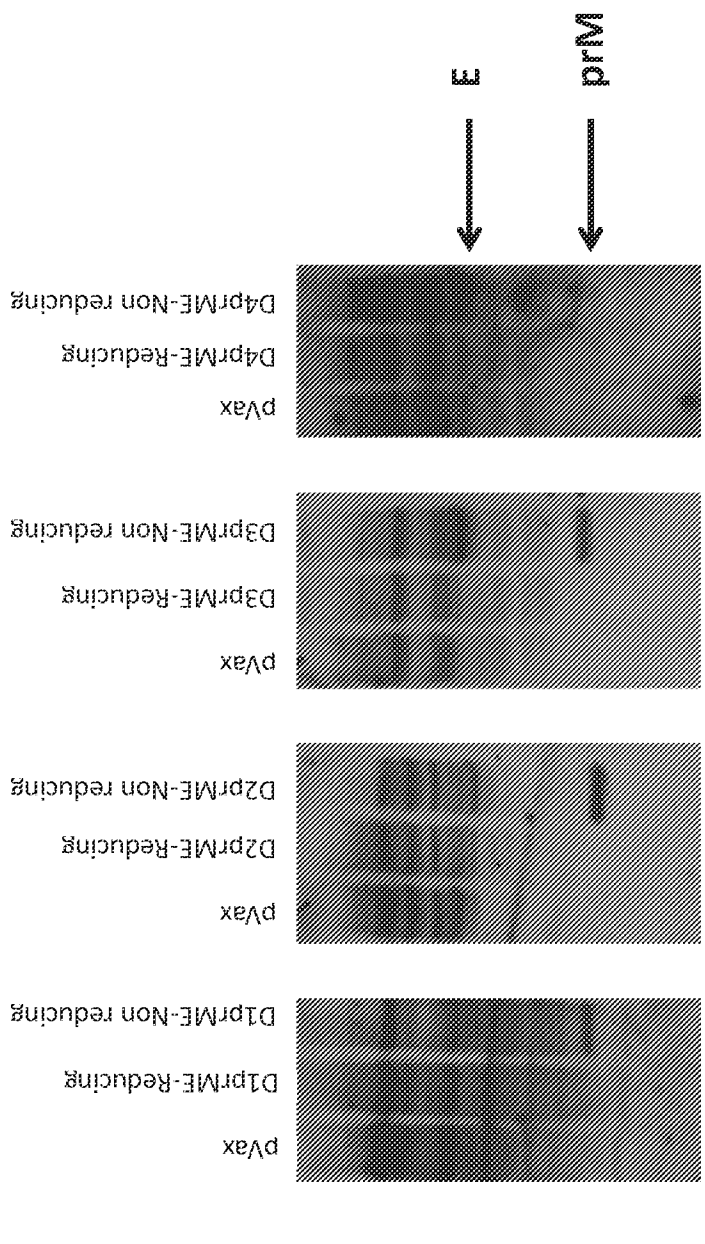
FIGURE 3

WO 2014/144786 PCT/US2014/029341



Comparison of binding antibodies against D4-DIII protein in mice immunized with DU or D4prME construct

FIGURE 4



Vaccination with Dengue prME constructs induced anti prM/E-specific antibodies in mice.

FIGURE 5

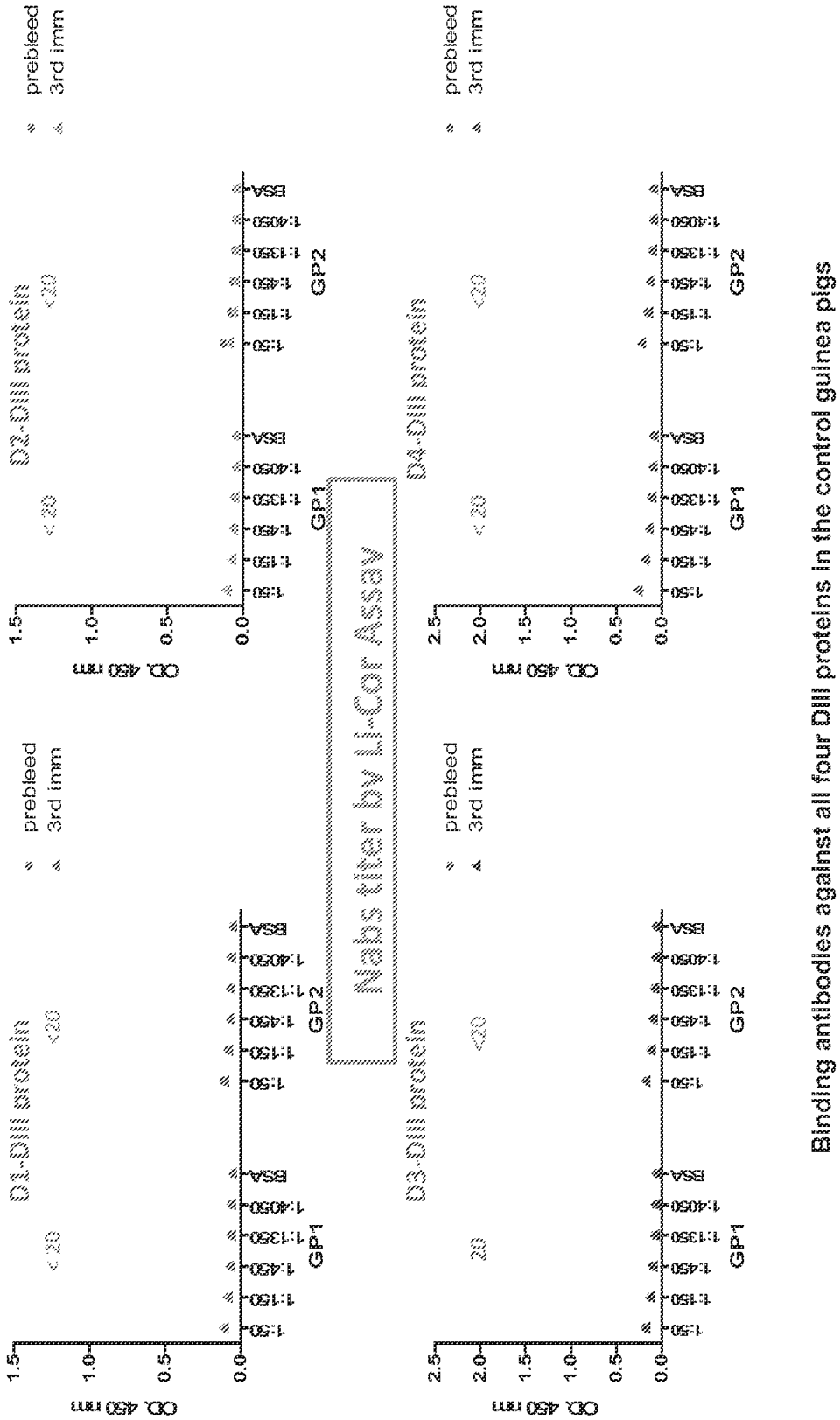


FIGURE 6

Binding antibodies against all four DIII proteins in the control guinea pigs

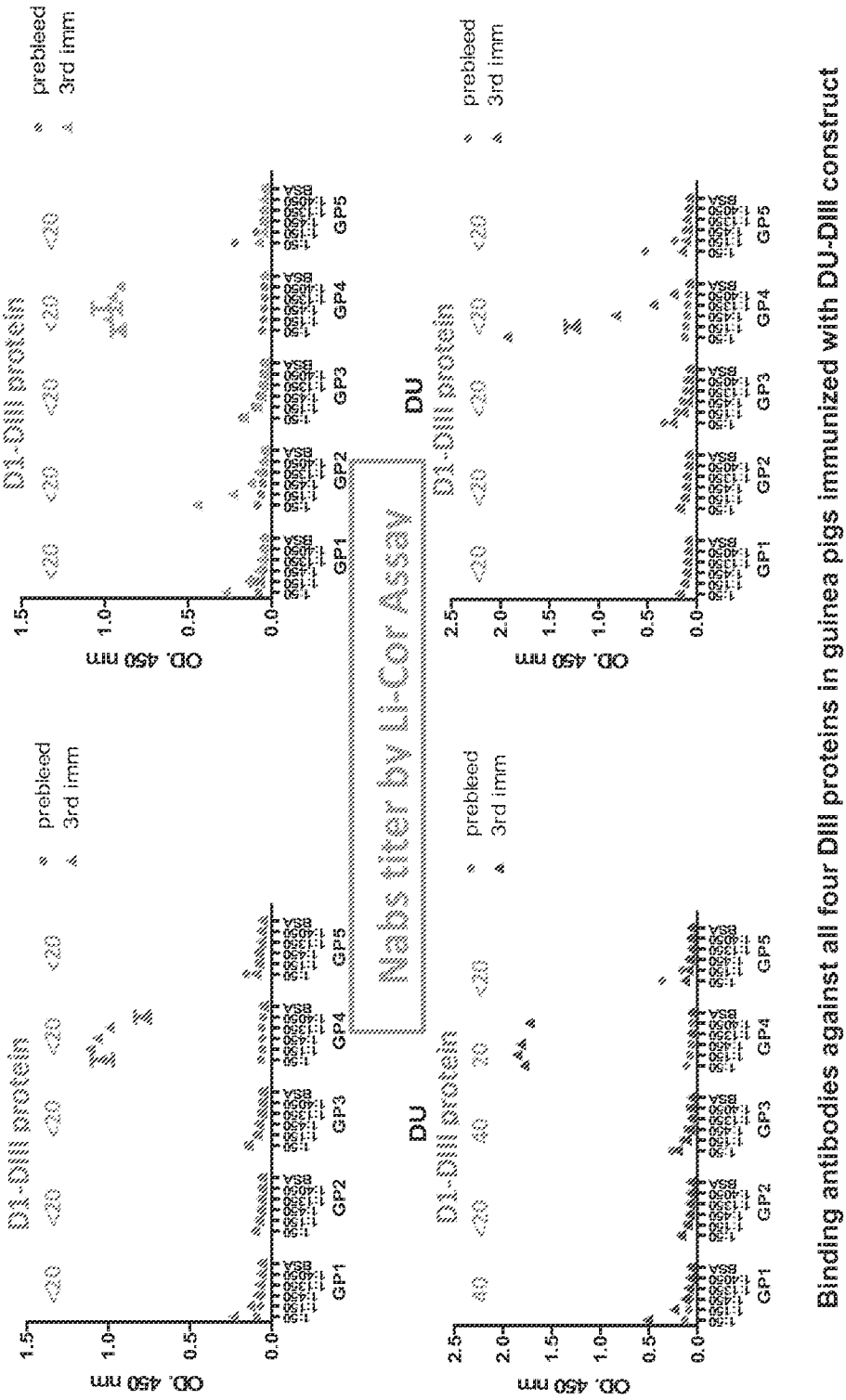


FIGURE 7

Binding antibodies against all four DIII proteins in guinea pigs immunized with DU-DIII construct

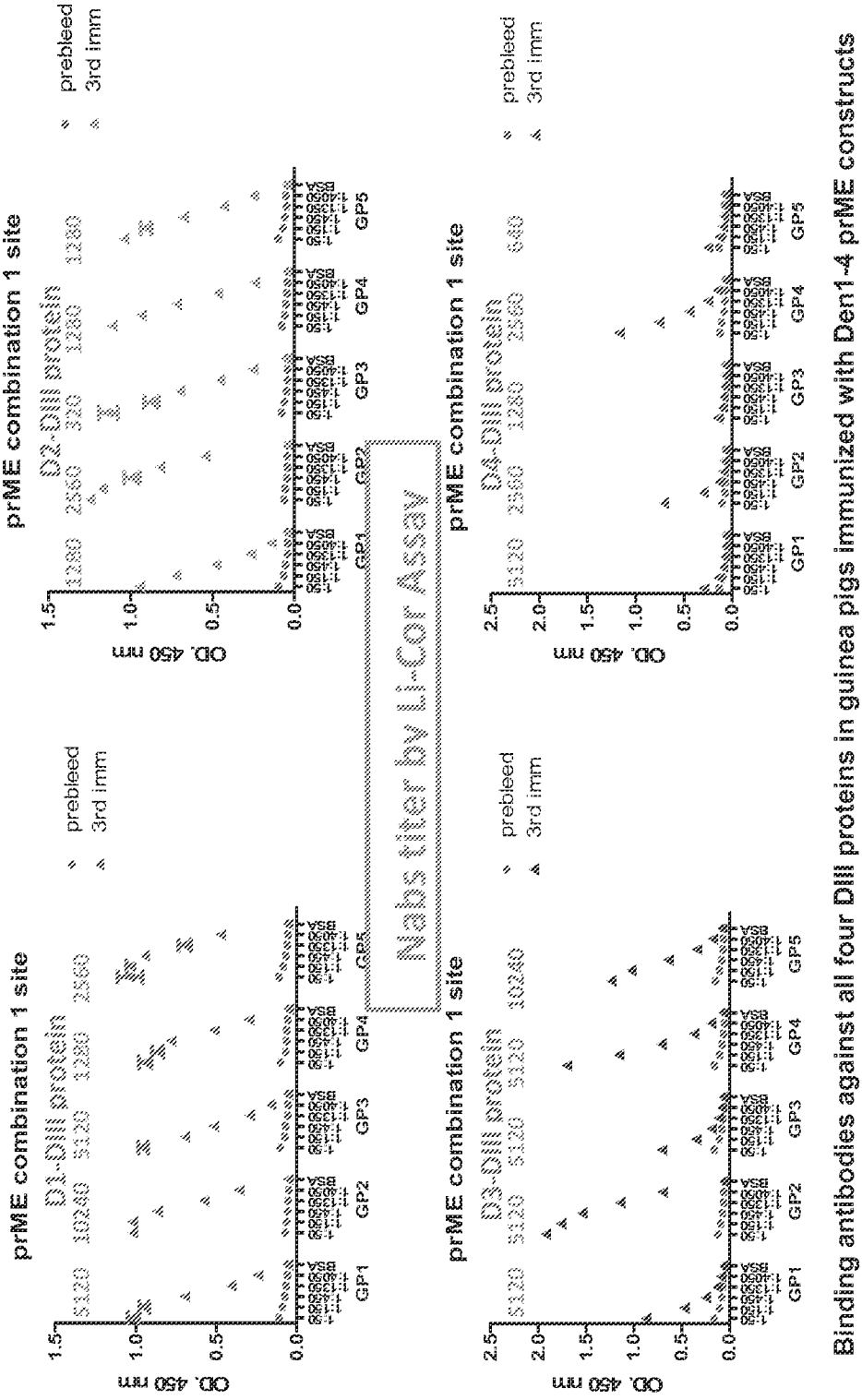


FIGURE 8

Binding antibodies against all four DIII proteins in guinea pigs immunized with Den1-4 prME constructs

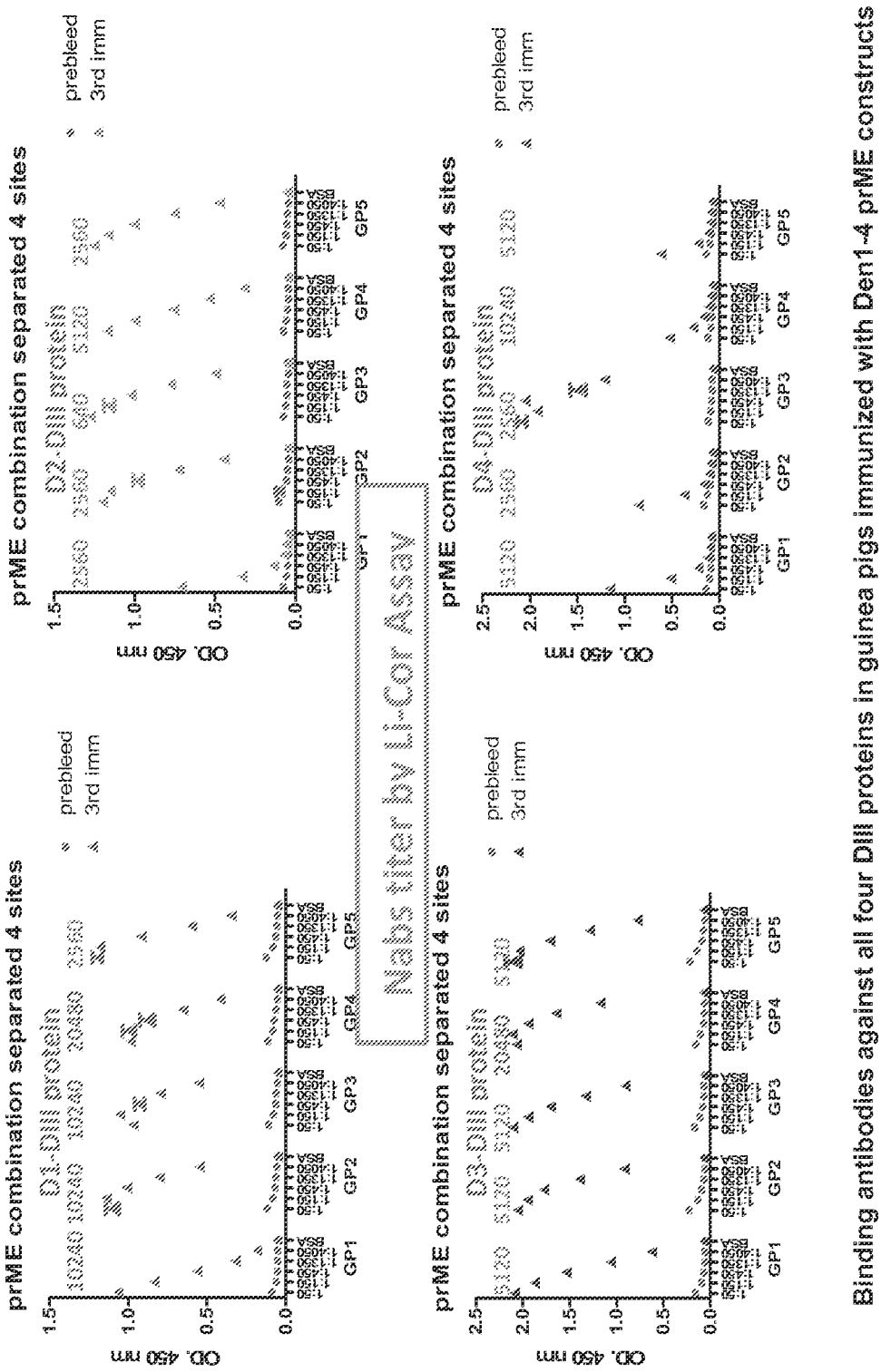


FIGURE 9

Binding antibodies against all four DIII proteins in guinea pigs immunized with Den1-4 prME constructs

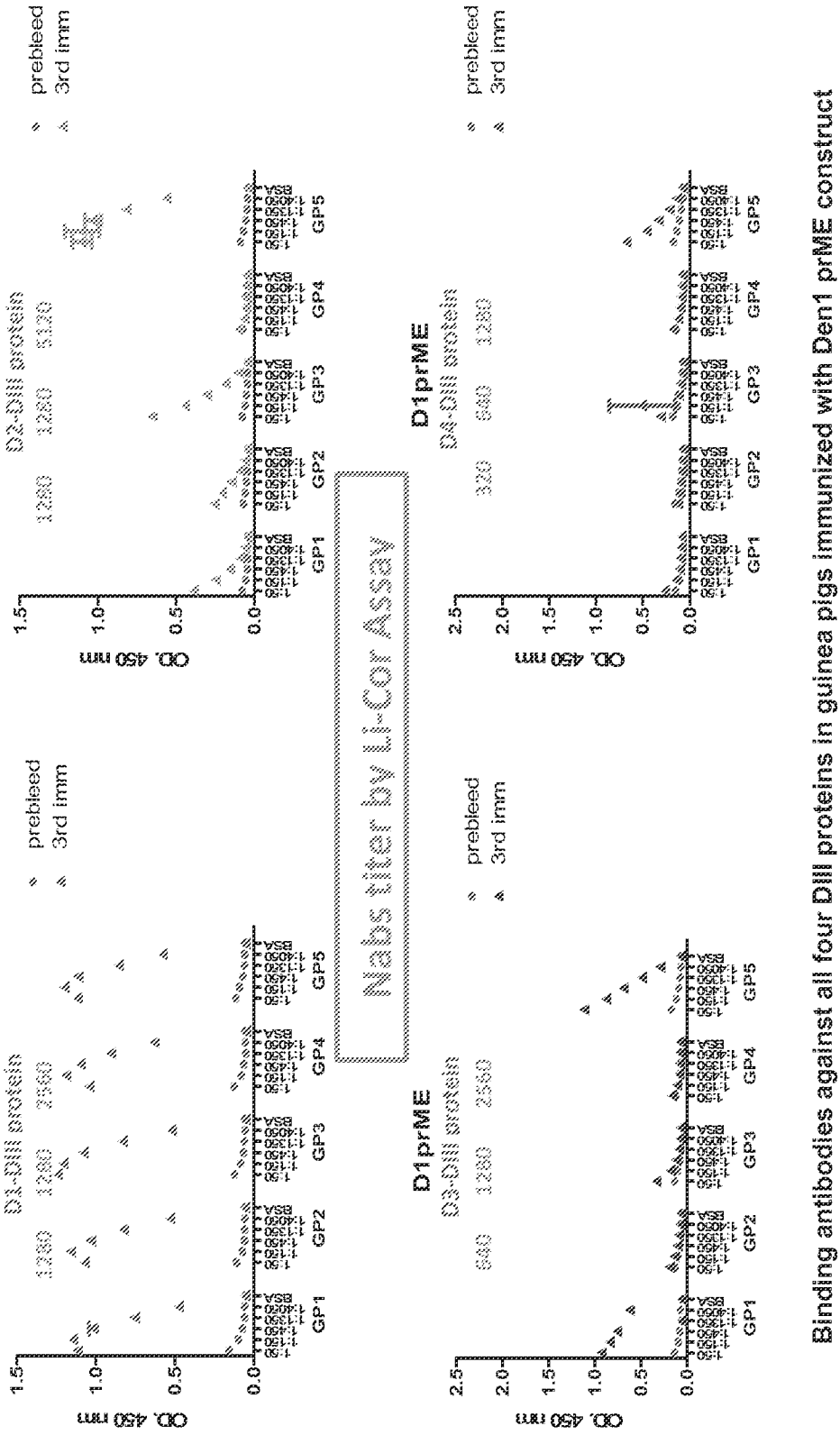


FIGURE 10

Binding antibodies against all four DIII proteins in guinea pigs immunized with Den1 prME construct

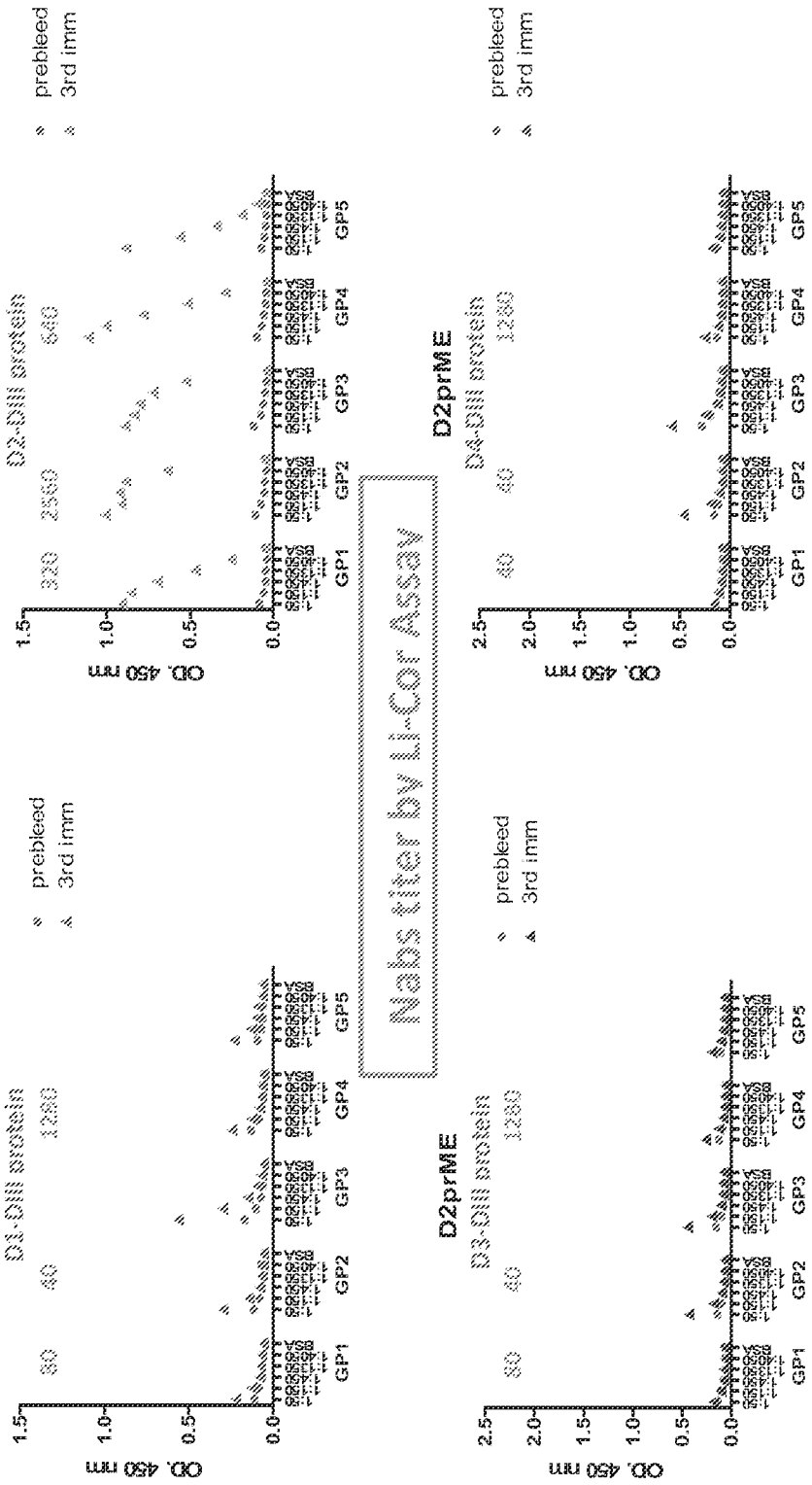


FIGURE 11

Binding antibodies against all four DIII proteins in guinea pigs immunized with Den2 prME construct

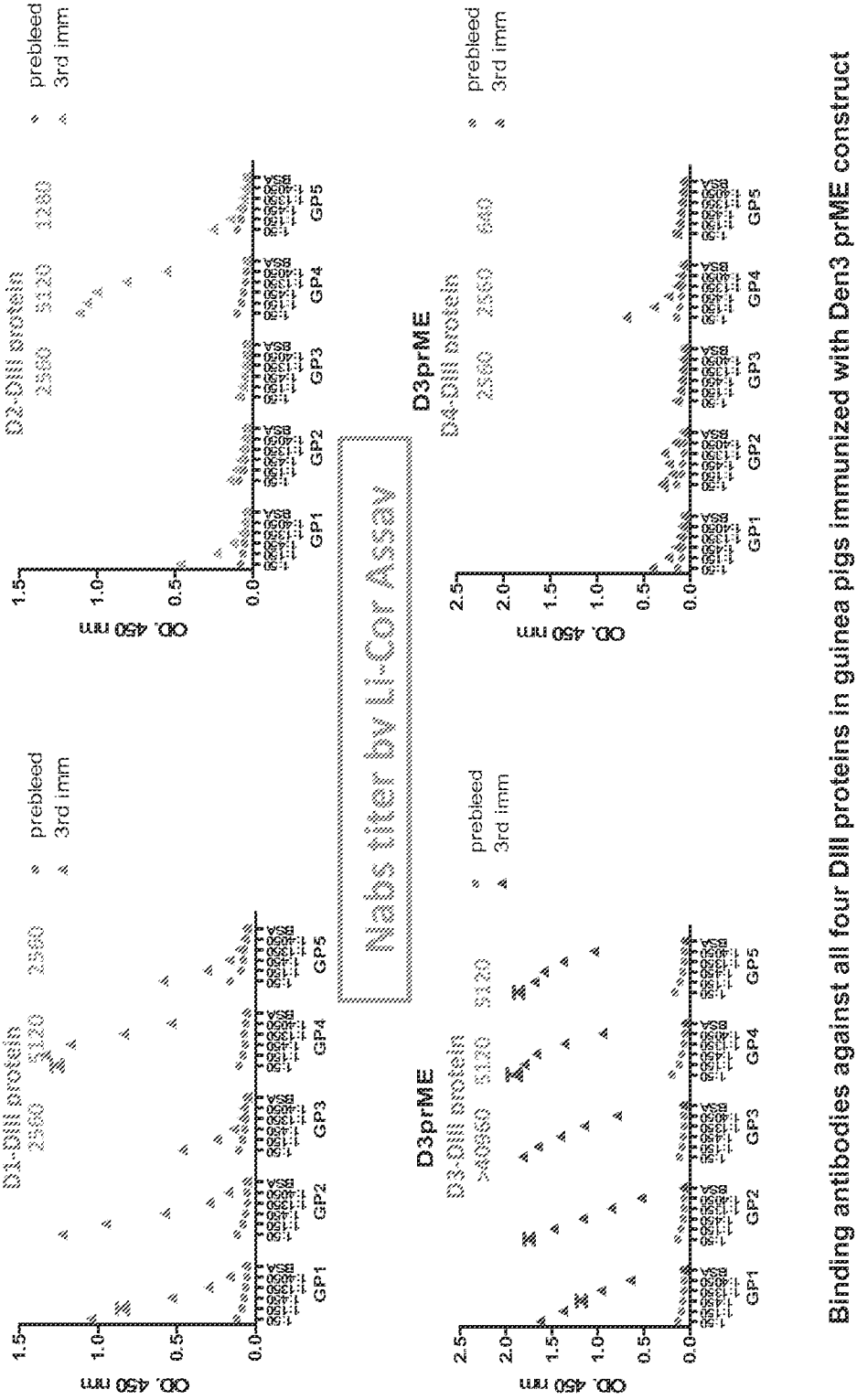


FIGURE 12

Binding antibodies against all four DIII proteins in guinea pigs immunized with Den3 prME construct

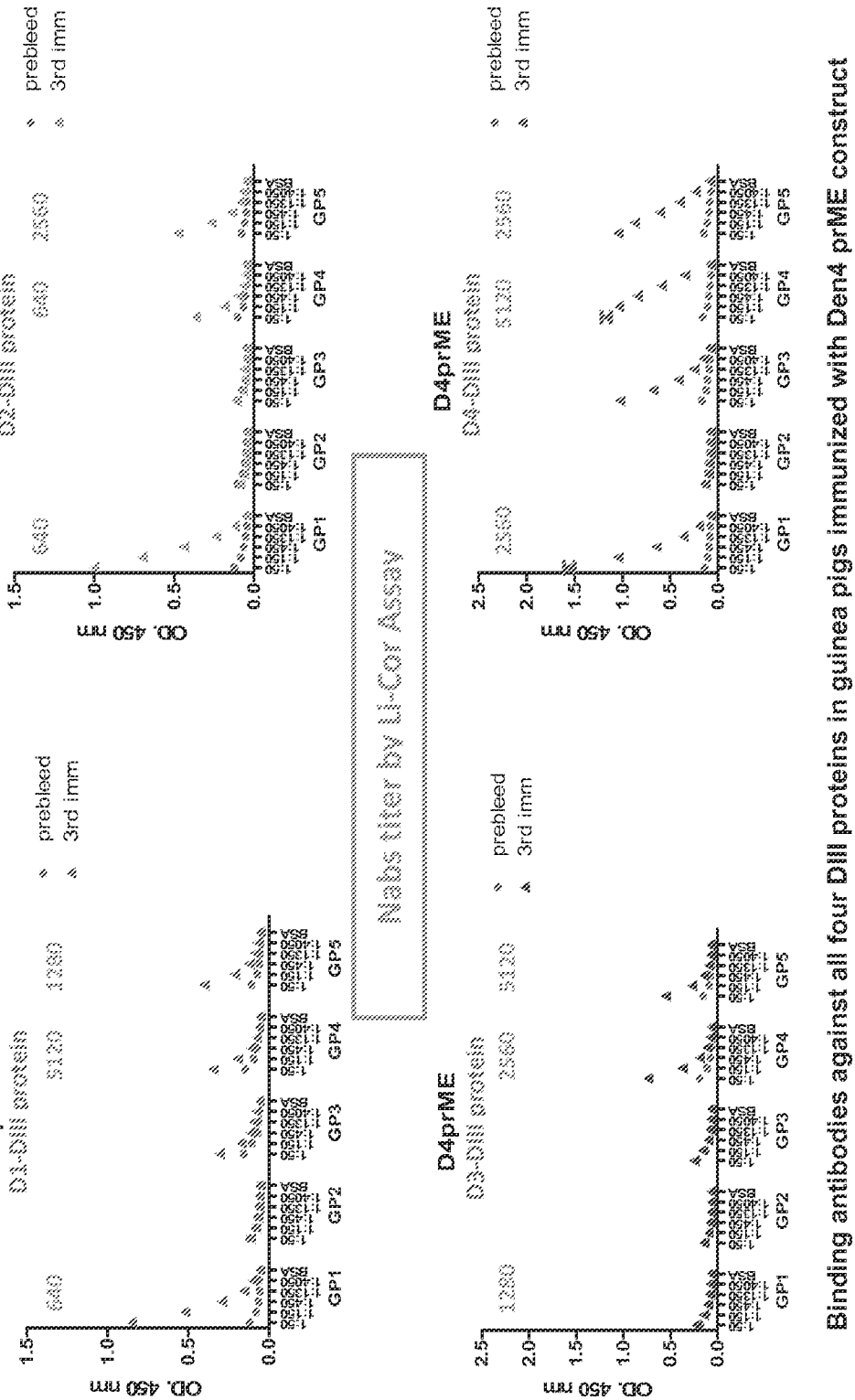


FIGURE 13

Binding antibodies against all four DIII proteins in guinea pigs immunized with Den4 prME construct

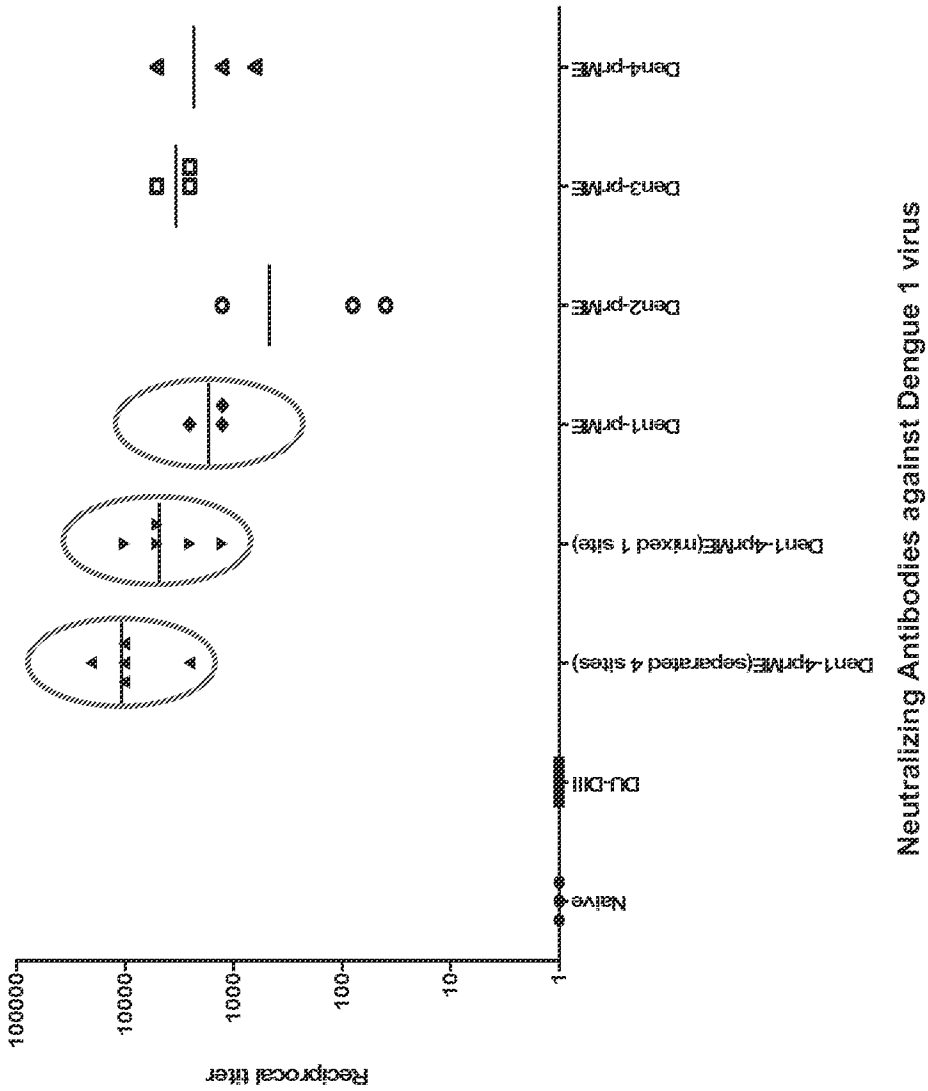
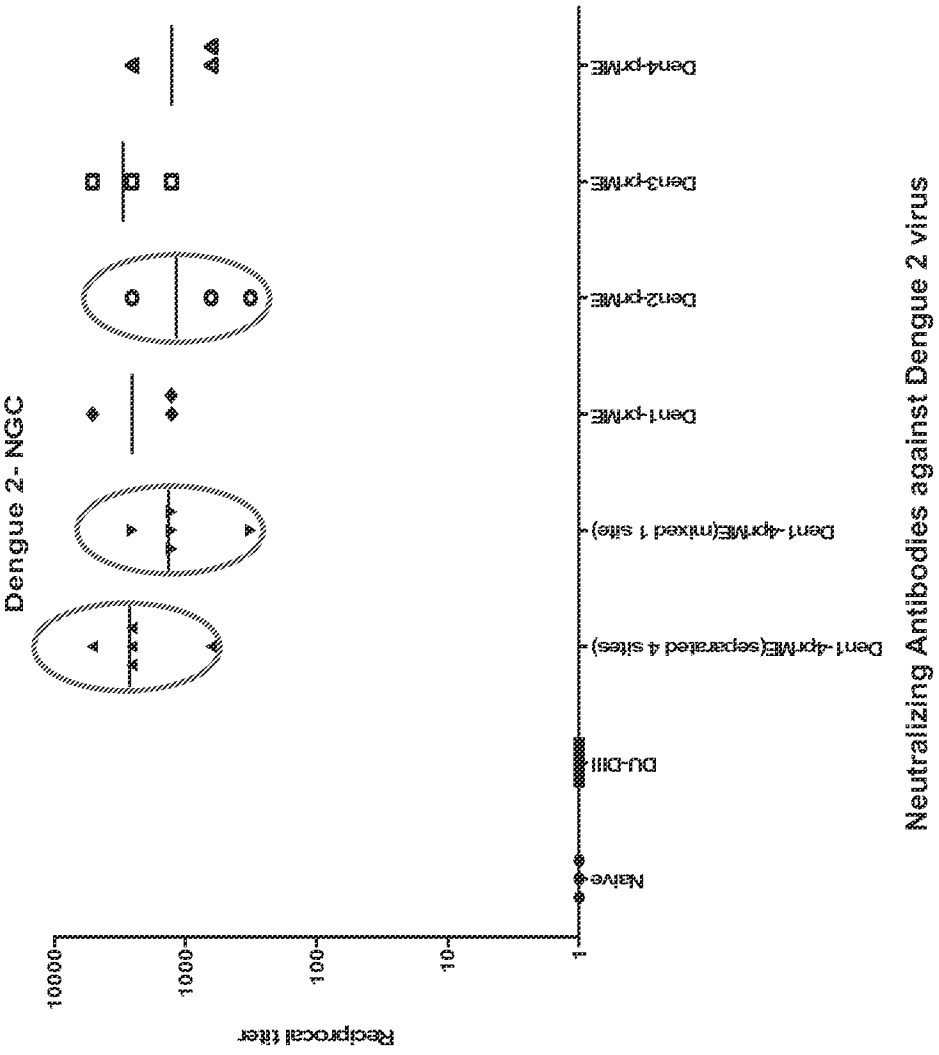


FIGURE 14

Neutralizing Antibodies against Dengue 1 virus



Neutralizing Antibodies against Dengue 2 virus

FIGURE 15

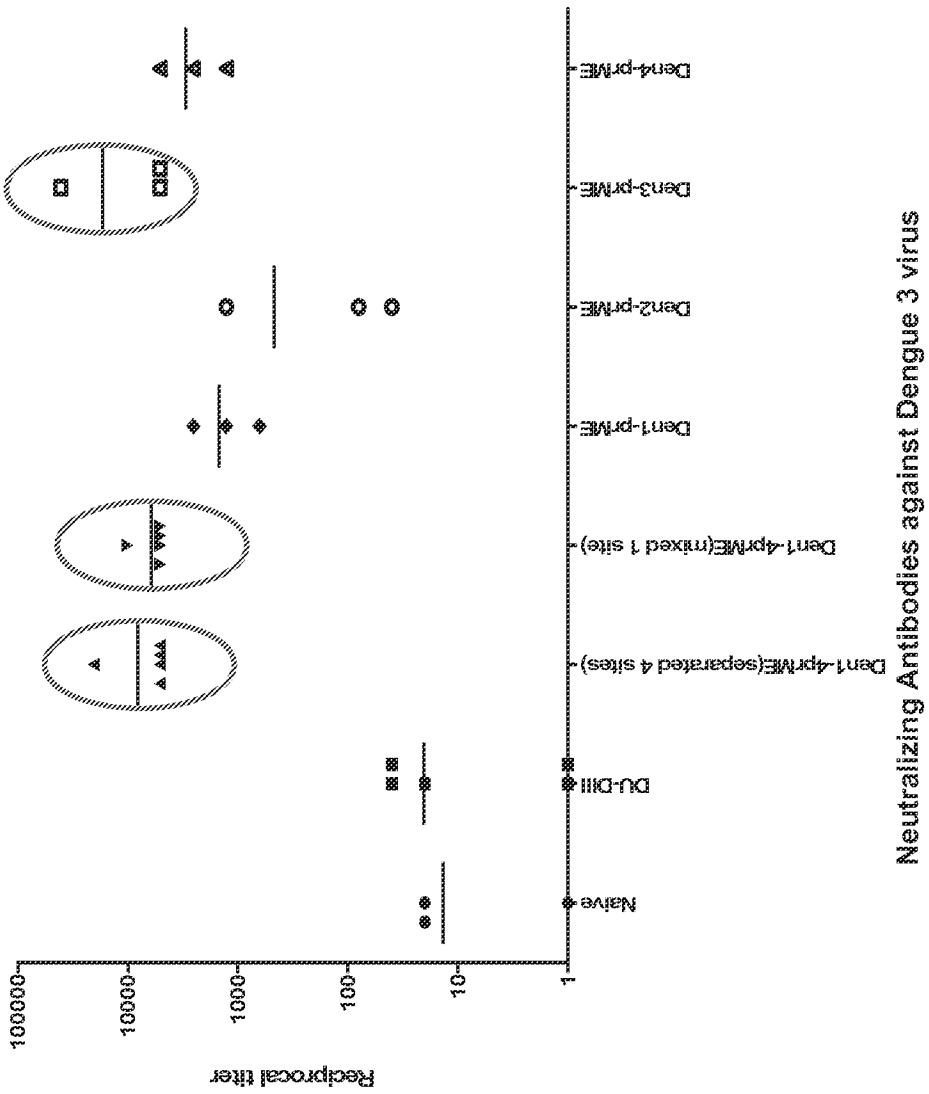
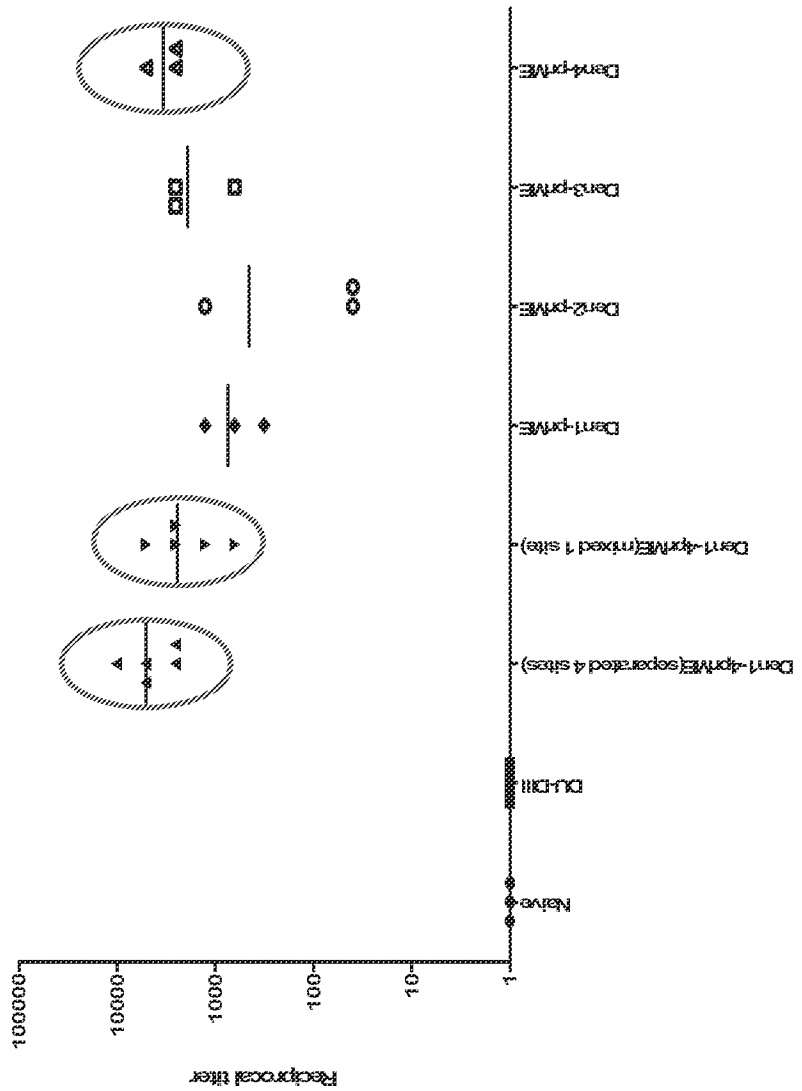

Dengue 3- H87
Reciprocal titer
100000
10000
1000
100
10
1
Naive
DU-DIII
Den1-4prME(separated 4 sites)
Den1-4prME(mixed 1 site)
Den1-prME
Den2-prME
Den3-prME
Den4-prME
Neutralizing Antibodies against Dengue 3 virus

FIGURE 16



Neutralizing Antibodies against Dengue 4 virus

FIGURE 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/029341

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/711 (2014.01)
USPC - 514/44R
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 31/7088, 31/711, 39/12 (2014.01)
USPC - 424/192.1; 514/44A, 44R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 31/7088, 31/711, 39/12 (2014.06)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0291144 A1 (RAMANATHAN et al) 18 November 2010 (18.11.2010) entire document	1-3, 7-9, 29-31
A	SCHMITZ et al, "Next generation dengue vaccines: A review of candidates in preclinical development," Vaccine, Vol. 29, Pgs. 7276-7284. 21 July 2011 (21.07.2011) entire document	1-3, 7-9, 29-31
A	AZEVEDO et al, "DNA Vaccines against Dengue Virus Type 2 Based on Truncate Envelope Protein or Its Domain III," Plos One, Vol. 6, No. 7, Pgs. 1-8. 11 July 2011 (11.07.2011) entire document	1-3, 7-9, 29-31

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 July 2014	07 AUG 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/029341

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☒ subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/029341

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-6, 10-28, 32-34
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.