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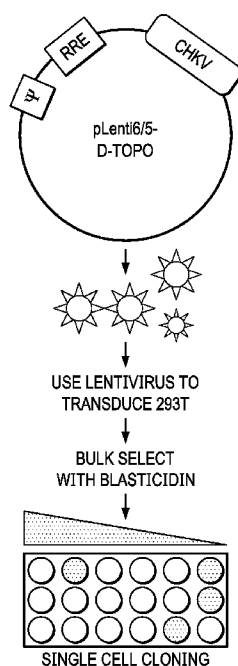


FIG. 4A

(57) **Abstract:** The present invention includes composition and methods for making multivalent vaccines for immunization against flavivirus and/or arboviruses including a multivalent Virus Like Particles (VLP) and mixtures thereof, the method comprising: method of making a flavivirus and/or arboviruses Virus Like Particles (VLP) comprising: inserting two or more nucleic acids that encode at least one flavivirus protein into a lentiviral backbone vector; generating a lentivirus by transfecting a first cell line with the lentiviral backbone vector and isolating the lentivirus therefrom; transducing a second cell line with the lentivirus; culturing the transduced cell line under conditions in which the multivalent flavivirus Virus Like Particles (VLP) are released from the cell line; and isolating the flavivirus Virus Like Particles (VLP) from a culture supernatant, wherein a cell line makes a virus-specific VLP, and the VLPs are purified and then mixed in different combinations to make the multivalent vaccine.

MULTIVALENT VIRUS LIKE PARTICLE VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 62/746,681, filed October 17, 2018, the entire contents of which are incorporated herein by reference.

5 STATEMENT OF FEDERALLY FUNDED RESEARCH

[0002] Not Applicable.

TECHNICAL FIELD OF THE INVENTION

[0003] The present invention relates in general to the field of vaccines, and more particularly, to novel multivalent vaccines for arboviruses.

10 INCORPORATION-BY-REFERENCE OF MATERIALS FILED ON COMPACT DISC

[0004] The present application includes a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 15, 2019, is named TECH2132WO_SeqList.txt and is 46, kilo bytes in size.

BACKGROUND OF THE INVENTION

15 [0005] Without limiting the scope of the invention, its background is described in connection with arboviruses, alphaviruses, and flaviviruses.

[0006] Arboviruses derive their name from the original manner in which they were identified and cataloged, namely, the commonality that they were transmitted via arthropod vectors. The name Arbovirus is an acronym for, ARthropod-BOrne virus. Arboviruses viruses have a typical
20 incubation period of 3-15 days, and the infection is typically resolved in 3-7 days. The most common symptoms of an Arbovirus infection is fever, headache, malaise and in certain cases encephalitis or hemorrhagic fever. Chikungunya virus is an alphavirus that is part of the larger group of Arboviruses.

[0007] Flavivirus is a genus of viruses in the family Flaviviridae, which derive their name from
25 the Latin work for yellow (*flavus*) and are characterized by the jaundice that accompanies infection with the first recognized members of this genus. The Flavivirus genus includes the

Zika virus, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue 1-4 Viruses, Tick Borne Encephalitis Virus, West Nile Virus, St. Louis Encephalitis Virus, and others. Flaviviruses are also Arboviruses.

[0008] It is known that Arboviruses have the tendency to co-circulate in similar geographical areas due to prevalence of conducive eco-epidemiological factors in tropical and sub-tropical countries. One of the key factors contributing to this prevalence is the occurrence of multiple vectors in an area capable of transmitting different diseases or the same vector capable of transmitting different pathogens. In this regard, countries with prevalent Zika virus (ZIKV) infection include Africa, Asia, Caribbean, Central, North, and South America and the Pacific Islands. Japanese Encephalitis Virus (JEV) infections are prevalent in almost all Asian countries. The Yellow Fever Virus (YFV) is found in tropical and subtropical areas of Africa and South America. Chikungunya virus (CHKV) cases and outbreaks have been identified in countries in Africa, Asia, Europe, Indian and Pacific Oceans, Mexico and North and South America. Thus there is a need for tailored multivalent arboviral vaccines depending on: (1) the geographical area that includes the components as per the prevalence of disease in a particular country/region, (2) members actively serving in the military and could be deployed to different geographical regions depending on need; and (3) pregnant women who are at risk or immunocompromised individuals that may be more prone to certain disease when compared to immunocompetent adults.

[0009] Thus, a need remains for the development of vaccines against members of the Flavivirus and/or Arbovirus families that provide robust immunity.

SUMMARY OF THE INVENTION

[0010] In one embodiment, the present invention includes a nucleic acid encoding two or more consensus, code optimized, or both consensus and codon optimized, multivalent flavivirus nucleic acid sequences that express one or more flavivirus and/or arbovirus proteins inserted into a lentiviral vector capable of forming Virus Like Particles (VLPs). In one aspect, a cell line makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine. In another aspect, the nucleic acids are selected from at least one of nucleic acid SEQ ID NOS:2, 6, or 8. In another aspect, the two or more nucleic acids are inserted into the lentiviral vector. In another aspect, the nucleic acid express two or more proteins selected from SEQ ID NO:1, 5, or 7. In another aspect, the nucleic

acid is transfected into a cell line. In another aspect, the nucleic acid is transfected into a human cell line. In another aspect, the nucleic acid is transfected into a 293T cell line. In another aspect, the nucleic acid is stably transduced in a cell line. In another aspect, the flavivirus proteins are selected from at least one of: Zika virus, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne Encephalitis Virus, West Nile Virus, St Louis Encephalitis Virus.

[0011] In another embodiment, the present invention includes a method of making a multivalent arbovirus Virus Like Particles (VLP) comprising: inserting two or more nucleic acids that encode at least one arbovirus protein into a lentiviral backbone vector; generating a lentivirus by transfecting a first cell line with the lentiviral backbone vector and isolating the lentivirus therefrom; transducing a second cell line with the lentivirus; culturing the transduced cell line under conditions in which the multivalent arbovirus Virus Like Particles (VLP) are released from the cell line; and isolating the multivalent arbovirus Virus Like Particles (VLP) from a culture supernatant. In one aspect, a cell line makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine. In another aspect, the nucleic acids are selected from at least one of nucleic acid SEQ ID NOS:2, 6, or 8. In another aspect, the nucleic acid is a consensus, a codon optimized, or both a consensus and codon optimized nucleic acid and expresses one or more proteins of SEQ ID NO:1, 5, or 7. In another aspect, the first, the second, or both the first and second cell line is a human cell line. In another aspect, the first, the second, or both the first and second cell line is a 293T cell line. In another aspect, the method further comprises generating two or more VLPs that express proteins from different arboviruses and/or flaviviruses, express proteins from different flavivirus strains, or express proteins from different flavivirus clades, wherein each of the VLPs is made in a different cell lines and a vaccine is prepared by mixing the different VLPs produced by different transduced cell lines. In another aspect, the mix of VLPs from different transduced cell lines into region-specific multivalent vaccines, wherein a ratio of different arbovirus VLPs is prepared based on the most prevalent arbovirus for such a region. In another aspect, the mix of VLPs is bivalent, trivalent, or tetravalent. In another aspect, the mix of VLPs from different transduced cell lines into region-specific multivalent vaccines is the following: immunization for a worldwide, a travel, or a military use: Zika, JEV, YFV and Chikungunya virus (CHIKV)(which is an arbovirus), or YFV and JEV; for an Asia or Australia JEV, CHIKV and

Zika; for a South America or Africa: YFV, CHIKV and Zika; or for a pacific region: JEV, CHIKV and Zika.

[0012] In another embodiment, the present invention includes a vaccine comprising an isolated and purified multivalent arbovirus Virus Like Particles (VLP) that comprises at least one structural protein from viruses selected from Japanese Encephalitis Virus (JEV), Chikungunya virus (CHIKV)(which is an arbovirus), Yellow Fever Virus (YFV), and Zika virus, wherein the vaccine is a combination of two or more different VLPs from two or more flaviviruses and/or arboviruses. In one aspect, a cell line transduced with a lentiviral vector that makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine. In another aspect, the vaccine is optimized for immunization worldwide, Asia and Australia, South America and Africa, or Pacific Region. In another aspect, the vaccine is bivalent, trivalent or tetravalent for a flavivirus selected from at least one of: Zika virus, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne Encephalitis Virus, West Nile Virus, St Louis Encephalitis Virus. In another aspect, the mix of VLPs from different transduced cell lines into region-specific multivalent vaccines is the following: immunization for a worldwide, a travel, or a military use: Zika, JEV, YFV and CHIKV, or YFV and JEV; for an Asia or Australia JEV, CHIKV and Zika; for a South America or Africa: YFV, CHIKV and Zika; or for a pacific region: JEV, CHIKV and Zika. In another aspect, the vaccine further comprises one or more vaccines selected from at least one of: influenza A vaccine, an influenza B vaccine, an influenza A (H1N1) vaccine, a hepatitis A vaccine, a hepatitis B vaccine, a haemophilus influenzae Type B (HiB) vaccine, a measles vaccine, a mumps vaccine, a rubella vaccine, a polio vaccine, a human papilloma virus (HPV) vaccine, a tetanus vaccine, a diptheria vaccine, a pertussis vaccine, a bubonic plague vaccine, a yellow fever vaccine, a cholera vaccine, a malaria vaccine, a smallpox vaccine, a pneumococcal vaccine, a rotavirus vaccine, a varicella vaccine or a meningococcus vaccine.

[0013] In another embodiment, the present invention includes a cell line transformed with a nucleic acid vector comprising a nucleic acid sequence that is a consensus, a codon optimized, or both a consensus and codon optimized nucleic acid that encodes one or more multivalent flavivirus and/or arbovirus proteins in a lentiviral vector. In one aspect, the one or more multivalent flavivirus and/or arbovirus proteins are expressed by a nucleic acid sequence codon optimized for expression in human cells. In another aspect, the one or more multivalent

flavivirus proteins are expressed by a nucleic acid sequence codon optimized for expression in human cells of SEQ ID NO:2, 6, or 8. In another aspect, the one or more multivalent flavivirus proteins have the amino acid sequence of SEQ ID NO:1, 5, or 7. In another aspect, the cell line is stably transfected with the nucleic acid vector.

5 [0014] In another embodiment, the present invention includes a purified multivalent flavivirus Virus Like Particles (MV-VLP) comprising the two or more proteins selected from different flaviviruses and the two or more proteins are from a portion of amino acid sequences SEQ ID NO:1, 5, or 7. In one aspect, a cell line transduced with a lentiviral vector that makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations
10 to make a multivalent vaccine. In another aspect, the MV-VLPs are produced in a stably transduced cell line. In another aspect, the MV-VLPs are produced in a stably transduced human cell line. In another aspect, the MV-VLPs are produced in a stably transduced 293T cell line. In another aspect, the MV-VLPs are isolated from at least one of: a culture supernatant, or the cell lines. In another aspect, the purified MV-VLP further comprises one or more vaccines selected
15 from at least one of: influenza A vaccine, an influenza B vaccine, an influenza A (H1N1) vaccine, a hepatitis A vaccine, a hepatitis B vaccine, a haemophilus influenzae Type B (HiB) vaccine, a measles vaccine, a mumps vaccine, a rubella vaccine, a polio vaccine, a human papilloma virus (HPV) vaccine, a tetanus vaccine, a diphtheria vaccine, a pertussis vaccine, a bubonic plague vaccine, a yellow fever vaccine, a cholera vaccine, a malaria vaccine, a
20 smallpox vaccine, a pneumococcal vaccine, a rotavirus vaccine, a varicella vaccine or a meningococcus vaccine.

[0015] In another embodiment, the present invention includes an immunogenic composition comprising at least one codon optimized multivalent Virus Like Particles (VLP) that comprises at least one structural protein from two or more viruses selected from Japanese Encephalitis
25 Virus (JEV), Chikungunya virus, Yellow Fever Virus (YFV), and Zika virus. In one aspect, a cell line transduced with a lentiviral vector that makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine. In another aspect, the immunogenic composition further comprises an adjuvant. In another aspect, the composition comprises at least two VLPs comprising different flavivirus E
30 proteins.

[0016] In another embodiment, the present invention includes a method of generating an immune response to one or more flaviviruses in a subject, the method comprising administering to the subject an effective amount of an immunogenic composition comprising at least one codon optimized multivalent flavivirus Virus Like Particles (VLP) that comprises at least one structural protein from two or more flaviviruses selected from Zika virus, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne Encephalitis Virus, West Nile Virus, or St Louis Encephalitis Virus. In one aspect, a cell line transduced with a lentiviral vector that makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine. In another aspect, the composition is administered mucosally, intradermally, subcutaneously, intramuscularly, or orally. In another aspect, the immune response vaccinates the subject against multiple serotypes or clades of one or more flaviviruses. In another aspect, the subject is a human. In another aspect, the method further comprises one or more vaccines selected from at least one of: influenza A vaccine, an influenza B vaccine, an influenza A (H1N1) vaccine, a hepatitis A vaccine, a hepatitis B vaccine, a haemophilus influenzae Type B (HiB) vaccine, a measles vaccine, a mumps vaccine, a rubella vaccine, a polio vaccine, a human papilloma virus (HPV) vaccine, a tetanus vaccine, a diphtheria vaccine, a pertussis vaccine, a bubonic plague vaccine, a yellow fever vaccine, a cholera vaccine, a malaria vaccine, a smallpox vaccine, a pneumococcal vaccine, a rotavirus vaccine, a varicella vaccine or a meningococcus vaccine. In another aspect, the composition is administered in a multiple dose schedule. In another aspect, the VLP further comprises antigens from Chikungunya virus.

[0017] In another embodiment, the present invention includes a nucleic acid vector comprising a lentiviral vector comprising one or more engineered restriction nuclease sites and a codon optimized Zika virus NC2B-3 protein downstream from an internal ribosomal entry site (IRES), wherein a flaviviral insert comprising one or more flavivirus protein sequences can be inserted into the vector at the restriction sites, wherein the vector produces a purified multivalent flavivirus Virus Like Particles (MV-VLP) when in a cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

5 [0019] FIGS. 1A to 1C show as follows. FIG. 1A shows a schematic of a Universal Lentiviral vector for generating cell lines secreting Flaviviral VLPs. Restriction sites introduced on either side of structural protein allow easy swapping of the structural proteins of any Flavivirus including but not limited to Zika, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne Encephalitis Virus, West Nile Virus, St Louis Encephalitis Virus etc., and
10 includes an downstream from an internal ribosomal entry site (IRES), and a codon optimized Zika NS2B3 Protein sequence with a Tag. FIG. 1B shows culture supernatants were harvested from 293T cell line stably expressing the JEV CprME (293T-JEV-Lenti) and analyzed for E protein expression by western blotting using specific antibody. FIG. 1C shows 293T-JEV-Lenti or control 293T cells were stained for E protein expression using the pan Flaviviral 4G2
15 monoclonal antibody followed by fluorescence microscopy.

[0020] FIGS. 2A and 2B show as follows. FIG. 2A shows a schematic of a lentiviral vector expressing Yellow Fever Virus (YFV) CprME along with the protease NS2B-3 for production of capsid containing virus like particles. FIG. 2B shows supernatants harvested from a 293T cell line stably expressing the YFV CprME proteins in the presence of the NSB2-3 protease that
20 is required for release of VLPs from cells. Expression of E protein was analyzed by western blotting using specific antibodies. Lanes 1 and 2 represent supernatants harvested at different days of culture and C represents Control supernatants from 293Y cells. M-molecular weight.

[0021] FIG. 3A is a schematic of a lentiviral vector expression that includes CHKV structural proteins for production of capsid containing virus like particles. FIG. 3B is a Western Blot
25 showing the culture supernatants harvested from 293T cells stably transfected and expressing the CHKV structural proteins (293T-CHKV-Lenti) and analyzed for CHKV E1-E2 protein expression.

[0022] FIGS. 4A and 4B show the establishment of a single cell clones expressing CHKV Structural proteins.

30 [0023] FIG. 5 is a flow cytometry analysis of single cell clones expressing the CHKV-E1/E2.

[0024] FIGS. 6A to 6F show the characterization of JEV, YFV and CHKV protein expression and VLP release.

[0025] FIGS. 7A to 7D show a strategy for JEV, YFV, Zika and CHIKV RVP generation and titration.

5 [0026] FIGS. 8A to 8D show the neutralization of RVP infection using disease specific sera.

[0027] FIGS. 9A to 9C show the generation and characterization of bicistronic lentiviral vectors expressing flaviviral structural proteins.

[0028] FIGS. 10A to 10C show the generation of stable cell lines secreting JEV, YFV and CHIKV VLPs.

10 [0029] FIGS. 11A and 11B show the characterization of stable single cell clones for Env and capsid protein expression.

[0030] FIGS. 12A and 12B show the VLP purification and schematic of mice immunization studies.

[0031] FIGS. 13A to 13D show the neutralization efficacy of VLP vaccine combinations.

15 [0032] FIGS. 14A to 14D show the quality of antibody response generated in VLP immunized mice.

[0033] FIGS. 15A to 15C show the adaptation of stable cell lines for growth in suspension culture.

DETAILED DESCRIPTION OF THE INVENTION

20 [0034] While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

25 [0035] To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may

be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not limit the invention, except as outlined in the claims.

[0036] The present invention includes a multivalent vaccine that forms Virus Like Particles (VLPs) in a cell line that stably produces multivalent arbovirus VLPs. Arbovirus virus genomes were cleaved to express the capsid (C), pre-membrane (PrM), envelope (E), and other non-structural proteins, none of which are capable of generating host infections, but are still able to elicit an immune response. These multivalent arbovirus VLPs were then coupled with the backbone of a West Nile virus (WNV) reporter gene, to generate reporter virus-like particles, which can be detected by luciferase assays, and when used as a vaccine, were able to trigger the production of a robust immune response in animals. The antibodies elicited were further shown to be neutralizing antibodies against multivalent arbovirus vaccine. The multivalent arbovirus VLP of the present invention includes combinations of, e.g., Japanese Encephalitis Virus, Chikungunya Virus (an arbovirus), Yellow Fever Virus, and/or Zika Virus in a single vaccine. The vaccine can be further customized for use in certain areas of the world where a subset of Arboviruses are more prevalent, thus providing customized immunity in those regions with the specific need for a specific need of immunity against certain Flaviviruses and/or Arboviruses.

[0037] As used throughout the present specification the following abbreviations are used: TF, transcription factor; ORF, open reading frame; kb, kilobase (pairs); UTR, untranslated region; kD, kilodalton; PCR, polymerase chain reaction; RT, reverse transcriptase.

[0038] The term “gene” is used to refer to a functional protein, polypeptide or peptide-encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences, or fragments or combinations thereof, as well as gene products, including those that may have been altered by the hand of man. Purified genes, nucleic acids, protein and the like are used to refer to these entities when identified and separated from at least one contaminating nucleic acid or protein with which it is ordinarily associated.

[0039] As used herein, the term “vector” is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The vector may be further defined as one designed to propagate multivalent arbovirus Virus Like Particle sequences, or as an expression vector that includes a promoter operatively linked to the multivalent flavivirus and/or arbovirus Virus Like Particle sequence, or one designed to cause such a promoter to be introduced. The

vector may exist in a state independent of the host cell chromosome, or may be integrated into the host cell chromosome.

[0040] The term “host cell” refers to cells that have been engineered to contain nucleic acid segment that encodes a multivalent arbovirus Virus Like Particle, or altered segments, whether
5 archeal, prokaryotic, or eukaryotic. Thus, engineered, or recombinant cells, are distinguishable from naturally occurring cells that do not contain recombinantly introduced genes through the hand of man.

[0041] As used herein, the terms “polynucleotide”, “nucleic acid sequence”, “nucleotide sequence”, or “nucleic acid fragment” are used interchangeably and is a polymer of RNA or
10 DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: “A” for adenylate or deoxyadenylate (for RNA or DNA, respectively), “C” for cytidylate or deoxycytidylate, “G” for guanylate or deoxyguanylate, “U” for uridylate, “T” for deoxythymidylate, “R” for purines (A or G), “Y” for
15 pyrimidines (C or T), “K” for G or T, “H” for A or C or T, “I” for inosine, and “N” for any nucleotide.

[0042] As used herein, the term “isolated” refers to materials, such as nucleic acid molecules and/or proteins that are substantially free or otherwise removed from components that normally accompany or interact with the materials in a naturally occurring environment.

[0043] The present invention can also be combined with other approved vaccines, including, but not limited to, one or more antigens from a pathogen selected from: *Haemophilus influenzae* b, *Neisseria meningitidis* type A, *Neisseria Meningitidis* type C, *Neisseria meningitidis* type W, *Neisseria meningitidis* type Y, *Neisseria meningitidis* type X, *Neisseria meningitidis* type B, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Salmonella typhi*, Hepatitis A, Hepatitis
25 B, RSV, Hepatitis C, diphtheria toxoid, tetanus toxoid, whole cell pertussis, acellular pertussis, *Staphylococcus aureus*, anthrax, *Vibrio cholera*, Zika, Ebola, Chikungunya, dengue, malaria, measles, mumps, rubella, BCG, Japanese encephalitis, Rotavirus, smallpox, *Shigella*, yellow fever, typhoid, CMV, Shingles, Varicella virus, HPV, HSV, and HIV. The multivalent arbovirus Virus Like Particles and variants of the present invention can be formulated into a
30 single vaccine with other vaccines, e.g., an influenza A vaccine, an influenza B vaccine, an

influenza A (H1N1) vaccine, a hepatitis A vaccine, a hepatitis B vaccine, a haemophilus influenzae Type B (HiB) vaccine, a measles vaccine, a mumps vaccine, a rubella vaccine, a polio vaccine, a human papilloma virus (HPV) vaccine, a tetanus vaccine, a diphtheria vaccine, a pertussis vaccine, a bubonic plague vaccine, a yellow fever vaccine, a cholera vaccine, a malaria vaccine, a smallpox vaccine, a pneumococcal vaccine, a rotavirus vaccine, a varicella vaccine or a meningococcus vaccine.

[0044] The multivalent arbovirus Virus Like Particles and variants of the present invention may contain alterations in the coding regions, non-coding regions, or both. Polynucleotide variants can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*), as is the case in certain embodiments of the present invention and which are known to those of skill in the art following, e.g., Sambrook and Russell, Molecular Cloning 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. 5 (2001) and by Ausubel et al., Current Protocols In Molecular Biology, John Wiley and Sons, Inc. (1998), and updates thereof.

[0045] Virus Like Particle (VLP) based vaccines fare superior to other vaccine platforms. For instance, Purified Inactivated Virus (PIV) vaccines bear the caveat of incomplete inactivation and handling large volumes of infectious virus, while live attenuated vaccines (LAVs) pose a safety challenge for administration in pregnant women and immunocompromised individuals. VLP based vaccines are safe to use in children and immunocompromised individuals due to their non-infectious nature, yet they are more immunogenic than purified viral proteins. As the structure of VLPs closely resemble the native virus particle, the antigens recognized by the immune system bear greater resemblance to a natural infection generating an effective immune response. Moreover, generation of stable cell lines producing VLPs provides a safe, viable and economical vaccine platform that can be scaled for worldwide use especially in developing and underdeveloped countries where the risk of arboviral infections remains high.

[0046] The nature of the Virus Like particle (VLP) vaccine of the present invention makes it ideal to develop as a mono, di, tri or tetravalent vaccine formulation depending on need. As each VLP vaccine is produced individually via a unique stable cell line, there is an opportunity to not only regulate the dose of each VLP in the vaccine but also tailor the combination vaccine as per need. For instance, the tetravalent formulation containing all 4 VLPs would be ideal for administration

in pregnant women and Military in the US and worldwide. A trivalent formulation containing Japanese Encephalitis Virus (JEV), Chikungunya virus, Yellow Fever Virus (YFV), and Zika virus would be ideal for the general population in Asia while a trivalent formulation containing JEV, YFV, CHIKV and Zika would be optimal for vaccination of the general population in South America (Table 2). Moreover, different monovalent and bivalent vaccine combinations depending on the geographical area are also recommended (Table 1). Tables 1 and 2 below list the vaccine combinations depending on the geographical region and the target population.

[0047] Table 1: Different vaccine combinations depending on the geographical region.

Vaccine	VLPs	Region
Monovalent	Zika	Worldwide
Monovalent	JEV	Asia, Australia
Monovalent	CHIKV	Worldwide
Monovalent	YFV	South America, Africa
Bivalent	JEV + YFV	Pacific region
Bivalent	Zika + JEV	Asia, Australia
Bivalent	Zika+ YFV	South America, Africa
Bivalent	Zika+ CHIKV	South America, Africa
Bivalent	JEV+ CHIKV	Asia, Australia
Bivalent	YFV+CHIKV	South America, Africa
Trivalent	Zika + YFV + CHIKV	South America, Africa
Trivalent	Zika + JEV+ CHIKV	Asia, Australia
Tetravalent	Zika + JEV + CHIKV + YFV	Worldwide

10 [0048] Table 2: Different vaccine combinations depending on the target population.

Target population	Vaccine version	VLPs included	Geographical region
Pregnant women	Tetravalent	Zika, JEV, YFV and CHIKV	US, Worldwide
US Military	Tetravalent	Zika, JEV, YFV and CHIKV	US, Worldwide
	Bivalent	YFV, JEV	US military, Pacom
General population Asia	Trivalent	JEV, CHIKV and Zika	Asia, Australia

Target population	Vaccine version	VLPs included	Geographical region
General population Africa	Trivalent	YFV, CHIKV and Zika	Africa
General population South America	Trivalent	YFV, CHIKV and Zika	South America
US Travelers	Tetravalent	Zika, JEV, YFV and CHIKV	Worldwide

[0049] A universal Lentiviral Vector for generating cell lines secreting Flaviviral VLPs. FIG. 1A show a Lentiviral vector for generating cell lines secreting Flaviviral VLPs that includes certain unique features. Restriction sites introduced on either side of structural protein allow easy swapping of the structural proteins of any Flavivirus including but not limited to Zika, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne Encephalitis Virus, West Nile Virus, St Louis Encephalitis Virus etc. Further, a codon optimized Zika NS2B3 sequence with an IRES sequence between the structural proteins and NS2B3.

[0050] Example I. Stable cell lines secreting Capsid containing Japanese Encephalitis Virus (JEV) Virus Like Particles (VLP) for Vaccine use.

[0051] Japanese Encephalitis (JE) is one the most important vector borne viral disease affecting Asian countries. It is estimated that 67900 cases of JE are seen every year in 24 JE endemic countries. Overall in these countries 2.6 billion people are at risk for JE. Although a vaccine for JE is available the cost and effectiveness of the vaccine makes it out of reach for most people. This is validated by the occurrence of approximately 55000 new cases of JE in areas with a vaccination program (WHO Bulletin). The current vaccine is also contraindicated in pregnant women.

[0052] The current approved vaccine for JEV is a purified inactivated virus vaccine (PIV). The immunogenicity, cost of production and safety of PIV vaccines is often limited. Virus like particles (VLP) on the other hand provide a cheaper, safer and more effective Vaccine platform for many viral diseases. The success of the VLP vaccine against Papilloma virus (HPV) exemplifies the success of this platform.

[0053] Currently there are no VLP vaccines against JEV approved for human use. Previous attempts to make JEV VLPs did not include the capsid protein, thereby limiting immunogenicity. The inventors have developed a capsid containing VLP platform for JEV related Zika virus and shown that this platform can provide an economical, safe and highly effective vaccine for use in humans. Our technology is built on three main innovations.

[0054] The present inventors have developed a vector system in which the structural proteins of JEV (CprME) are coexpressed with a Zika NS2B3 protein via a single vector by utilizing IRES sequence upstream for the NS2B3 coding region. The VLP platform produces Capsid containing VLPs that provide better immune response.

[0055] A consensus sequence of 158 published JEV sequences from year 2000 onwards was generated. The consensus sequence was then converted into the consensus sequence translation that is codon optimized for high expression of the proteins. The codon optimized vector is then used to produce a VLP that provide a vaccine that is most relevant to current outbreaks. Use a lentiviral system to generate stable cell lines that constitutively express JEV CprME VLP and secrete the same in the supernatant. Optimize production and purification is optimized for the VLPs from these stable cell lines.

[0056] Japanese Encephalitis Consensus Sequences used in the vaccine, amino acid sequence (SEQ ID NO:1):

[0057] MTKKPGGPGKNRAINMLKRGLPRVFPLVGVKRVVMSLLDGRGPVRFVLALITF
FKFTALAPTKALLGRWKAVEKSVAMKHLTSFKRELGTLDVAVNKRGRKQNKRRGGNEG
SIMWLASLAVVIACAGAMKLSNFQGKLLMTINNTDIADVIVIPTSKGENRCWVRAIDVG
YMCEDTITYECPKLTMGNDPEDVDCWCDNQEVYVQYGRCTRTRHSKRSRSSVSVQTH
GESSLVNKKEAWLDSTKATRYLMKTENWIIRNPGYAFLAAVLGWMLGSNNGQRVVFT
ILLLLVAPAYSFNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPTLDVRMINI
EASQLAEVRSYCYHASVTDISTVARCPTTGEAHNEKRADSSYVCKQGFTDRGWGNGC
GLFGKGSIDTCAKFSCTSKAIGRTIQPENIKYEVGIFVHGTTTSENHGNYS AQVGASQAA
KFTVTPNAPSITLKLGDYGEVTLDCPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALP
WTSPSSTAWRNRELLMEFEEAHATKQSVVALGSQEGGLHQALAGAI VVEYSSSVKLTS
GHLKCRLKMDKLALKGTTYGMCTEKFSFAKNPADTGHGTVVIELSYSGSDGPCKIPIVS
VASLNDMTPVGRLVTVPNFVATSSANSKVLVEMEPPFGDSYIVVGRGDKQINHHWHK

AGSTLGKAFSTTLKGAQRLAALGDTAWDFGSIGGVFNSIGKAVHQVFGGAFRTLFGGM
SWITQGLMGALLLWMGVNARDRSIALAFLATGGVLFVLATNVHAD

[0058] Japanese Encephalitis Codon optimized Sequences used in the vaccine, DNA sequence (SEQ ID NO:2)

5 [0059] ATGACCAAGAAGCCAGGCGGCCCTGGCAAGAACAGGGCCATCAATATGCTG
AAGAGGGGACTGCCCCGCGTGTTCCTCTGGTGGGCGTGAAGCGCGTGGTCATGAG
CCTGCTGGACGGCAGAGGCCCCGTGCGGTTTGTGCTGGCCCTGATCACATTCTTTAA
GTTACCGCCCTGGCACCAACAAAGGCCCTGCTGGGCCGGTGGGAAGGCAGTGGAG
AAGTCTGTGGCCATGAAGCACCTGACCAGCTTTAAGAGAGAGCTGGGCACACTGAT
10 CGATGCCGTGAACAAGAGGGGGCCGCAAGCAGAACAAGAGGGGGCGGCAATGAGGG
CTCTATCATGTGGCTGGCCAGCCTGGCAGTGGTCATCGCATGCGCAGGAGCCATGA
AGCTGTCTAACTTCCAGGGCAAGCTGCTGATGACAATCAACAATACCGACATCGCC
GATGTGATCGTGATCCCCACCTCCAAGGGCGAGAATAGGTGTTGGGTGCGCGCCAT
CGACGTGGGCTACATGTGCGAGGATACAATCACCTATGAGTGTCCCAAGCTGACCA
15 TGGGCAACGACCCTGAGGACGTGGATTGCTGGTGTGATAATCAGGAGGTGTACGTG
CAGTATGGCCGGTGCACACGGACCAGACACAGCAAGAGATCCCGGAGATCTGTGA
GCGTGCAGACCCACGGAGAGAGCTCCCTGGTGAACAAGAAGGAGGCCTGGCTGGA
CAGCACAAAGGCCACCAGGTACCTGATGAAGACAGAGAACTGGATCATCCGCAAT
CCCGGCTATGCCTTTCTGGCCGCCGTGCTGGGATGGATGCTGGGCTCCAACAATGG
20 CCAGAGGGTGGTGTTCACCATCCTGCTGCTGCTGGTGGCCCCTGCCTATTCTTTTAA
CTGCCTGGGCATGGGCAATAGGGATTTTCATCGAGGGAGCATCCGGAGCAACCTGGG
TGGACCTGGTGTGAGGAGGGCGATTCTTGTCTGACCATCATGGCCAACGACAAGCCA
AACTGGATGTGAGGATGATCAATATCGAGGCATCTCAGCTGGCAGAGGTGCGCAG
CTACTGCTATCACGCCAGCGTGACAGACATCTCCACCGTGGCAAGGTGTCCAACCA
25 CAGGAGAGGCCCACAACGAGAAGAGAGCCGACTCTAGCTACGTGTGCAAGCAGGG
CTTTACCGATCGGGGCTGGGGAAATGGATGTGGACTGTTTGGCAAGGGCAGCATCG
ATACCTGCGCCAAGTTCTCTTGTACAAGCAAGGCCATCGGCAGAACCATCCAGCCT
GAGAACATCAAGTACGAAGTGGGCATCTTTGTGCACGGCACCACAACCTCCGAGAA
CCACGGCAATTATTCGCCCCAAGTGGGAGCATCTCAGGCAGCAAAGTTCACAGTGA
30 CCCCTAACGCCCCATCTATCACCTGAAGCTGGGCGACTACGGCGAGGTGACACTG
GATTGCGAGCCACGGAGCGGCCTGAATACAGAGGCCTTTTATGTGATGACCGTGGG

CTCCAAGTCTTTTCTGGTGCACAGAGAGTGGTTCACGACCTGGCCCTGCCATGGAC
 CAGCCCCTCCTCTACAGCCTGGAGGAATCGCGAGCTGCTGATGGAGTTCGAGGAGG
 CACACGCAACCAAGCAGAGCGTGGTGGCCCTGGGCTCCCAGGAGGGAGGACTGCA
 CCAGGCCCTGGCAGGAGCCATCGTGGTGGAGTACAGCTCCTCTGTGAAGCTGACCA
 5 GCGGCCACCTGAAGTGCCGGCTGAAGATGGACAAGCTGGCCCTGAAGGGCACAAC
 CTATGGCATGTGCACAGAGAAGTTCTCCTTTGCCAAGAACCCTGCCGATACAGGCC
 ACGGCACCGTGGTCATCGAGCTGAGCTACTCCGGCTCTGACGGCCCTTGTAAGATC
 CCAATCGTGTCCGTGGCCTCTCTGAATGATATGACACCAGTGGGCAGACTGGTGAC
 CGTGAACCCCTTTGTGGCCACAAGCTCCGCCAATAGCAAGGTGCTGGTGGAGATGG
 10 AGCCCCCTTTTCGGCGACTCCTACATCGTGGTGGGCAGGGGCGATAAGCAGATCAAC
 CACCACTGGCACAAGGCAGGCTCCACCCTGGGCAAGGCCTTCTCTACAACCCTGAA
 GGGCGCCCAGCGCCTGGCCGCCCTGGGCGACACAGCCTGGGATTTTGGCAGCATCG
 GCGGCGTGTTCAATTCCATCGGCAAGGCAGTGCACCAGGTGTTTCGGAGGAGCCTTT
 CGGACCCTGTTTCGGAGGCATGAGCTGGATCACACAGGGACTGATGGGCGCCCTGCT
 15 GCTGTGGATGGGCGTGAACGCCCCGGGACAGATCCATCGCCCTGGCCTTTCTGGCAA
 CCGGAGGCGTGCTGGTGTTCCTGGCCACAAATGTGCACGCCGAT

[0060] FIGS. 1A to 1C shows as follows. FIG. 1A shows a schematic of a Universal Lentiviral vector for generating cell lines secreting Flaviviral VLPs. Restriction sites introduced on either side of structural protein allow easy swapping of the structural proteins of any Flavivirus including but not limited to Zika, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne Encephalitis Virus, West Nile Virus, St Louis Encephalitis Virus, etc. FIG. 1A shows that the lentiviral vector expresses, in this case, JEV CprME along with the Zika protease NS2B-3, which has been codon optimized, for production of capsid containing virus like particles. FIG. 1B shows culture supernatants were harvested from 293T cell line stably expressing the JEV CprME (293T-JEV-Lenti) and analyzed for E protein expression by western blotting using specific antibody. Lanes 1, 2, 3 represent supernatants harvested at different days and C represents Control supernatants from 293T cells. FIG. 1C shows 293T-JEV-Lenti or control 293T cells were stained for E protein expression using the pan Flaviviral 4G2 monoclonal antibody followed by fluorescence microscopy.

[0061] Zika NS2B3 Protein sequence with Hemagglutinin Tag. (SEQ ID NO:3)

[0062] MATTMSWPPSEVLTAVGLICALAGGFAKADIEMAGPMAAVGLLIVSYVVS
VDMYIERAGDITWEKDAEVTGNSPRLDVALDESGDFSLVEDDGPPMREILKVVLMTIC
GMNPIAIPFAAGAWYVYVKTGKRSGALWDVPAPKEVKKGETTDGVYRVMTRRLLGST
QVGVGVMQEGVFHTMWHVTKGSALRSGEGRDPYWGDVKQDLVSYCGPWKLDAA
5 WDGHSEVQLLAVPPGERARNIQTLPGIFKTKDGDIGAVALDYPAGTSGSPILDKCGRVI
GLYGNVVIKNGSYVSAITQGRREEETPVECFEPSMLKKKQLTVLDLHPGAGKTRRVL
PEIVREAIKTRLRTVILAPTRVVAEMEEALRGLPVRVYMTTAVNVTHSGTEIVDLMCHA
TFTSRLLQPIRVPNYNLYIMDEAHFTDPSSIAARGYISTRVEMGEAAIFMTATPPGTRD
AFPDSNSPIMDTEVEVPERAWSSGFDWVTDHSGKTVWFVPSVRNGNEIAACLTKAGKR
10 VIQLSRKTFETEFQKTKHQEWDVFTTDISEMGANFKADRVIDSRRLKPVILDGERVIL
AGPMPVTHASAAQRRGRIGRNPNKPGDEYLYGGGCAETDEDHAHWLEARMLLDNIYL
QDGLIASLYRPEADKVAIEGEFKLRTQKRTFVELMKRGDLPVWLAYQVASAGITYT
DRRWCFDGTNNTIMEDSVPAEVWTRHGEKRVLKPRWMDARVCSHAALKSFKEFA
AGKRYPYDVPDYA

- 15 [0063] DNA sequence comprising IRES sequence followed by codon optimized Zika NS2B3.
(SEQ ID NO:4)

[0064] CTCGAGGCCCTCTCCCTCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTG
GAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTG
GCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGT
20 CTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGT
TCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTCAGGCAGC
GGAACCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGAT
ACACCTGCAAAGGCGGCACAACCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGA
AAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGA
25 AGGTACCCCATTTGTATGGGATCTGATCTGGGGCCTCGGTACACATGCTTTACATGTG
TTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTTC
CTTTGAAAAACACGATGATAATATGGCCACAACCATGAGCTGGCCACCTTCTGAGG
TGCTGACAGCAGTGGGCCTGATCTGTGCACTGGCAGGAGGATTGCAAAGGCAGAC
ATCGAGATGGCAGGACCAATGGCAGCAGTGGGCCTGCTGATCGTGAGCTACGTGGT
30 GTCTGGCAAGAGCGTGGACATGTATATCGAGAGGGCCGGCGACATCACCTGGGAG
AAGGACGCCGAGGTGACAGGAACTCCCCTCGCCTGGACGTGGCCCTGGATGAGA

GCGGCGACTTCTCCCTGGTGGAGGACGATGGCCCACCCATGCGCGAGATCATCCTG
AAGGTGGTGCTGATGACCATCTGCGGCATGAATCCAATCGCAATCCCCTTTGCCGC
AGGAGCATGGTACGTGTATGTGAAGACAGGCAAGCGGTCTGGCGCCCTGTGGGATG
TGCCAGCACCAAAGGAGGTGAAGAAGGGCGAGACAACAGACGGCGTGTACAGAGT
5 GATGACCCGGAGACTGCTGGGCAGCACACAAGTGGGAGTGGGCGTGATGCAGGAG
GGCGTGTTTCACACCATGTGGCACGTGACAAAGGGCTCTGCCCTGAGGAGCGGAGA
GGGCCGCCTGGACCCATACTGGGGCGATGTGAAGCAGGACCTGGTGTCTATTGTG
GACCATGGAAGCTGGATGCAGCATGGGACGGACACTCTGAGGTGCAGCTGCTGGC
AGTGCCTCCAGGAGAGAGGGCCAGAAACATCCAGACCCTGCCCGGCATCTTCAAGA
10 CAAAGGACGGCGACATCGGAGCAGTGGCCCTGGATTACCCTGCAGGCACCTCTGGC
AGCCCAATCCTGGACAAGTGCGGCAGAGTGATCGGCCTGTACGGCAACGGCGTGGT
CATCAAGAATGGCTCCTACGTGAGCGCCATCACCCAGGGCAGGCGCGAGGAGGAG
ACACCTGTGGAGTGTTTTGAGCCCTCCATGCTGAAGAAGAAGCAGCTGACCGTGCT
GGATCTGCACCCAGGAGCAGGCAAGACAAGGAGAGTGCTGCCTGAGATCGTGAGG
15 GAGGCCATCAAGACCAGGCTGAGGACAGTGATCCTGGCACCAACCAGGGTGGTGG
CAGCAGAGATGGAGGAGGCCCTGCGGGGCCTGCCTGTGAGATACATGACCACAGC
CGTGAACGTGACCCACAGCGGCACAGAGATCGTGACCTGATGTGCCACGCCACCT
TCACATCCAGGCTGCTGCAGCCTATCCGCGTGCCAAACTACAATCTGTATATCATGG
ATGAGGCCCACTTTACCGACCCAAGCTCCATCGCAGCAAGGGGATATATCTCCACA
20 AGAGTGGAGATGGGCGAGGCTGCCGCCATCTTCATGACCGCAACACCACCTGGAAC
CAGGGATGCCTTTCCCGACTCCAATTCTCCTATCATGGACACAGAGGTGGAGGTGC
CAGAGAGGGCATGGTCTAGCGGCTTCGATTGGGTGACCGACCACTCCGGCAAGACA
GTGTGGTTTGTGCCCTCTGTGAGAAACGGCAATGAGATCGCCGCCTGTCTGACCAA
GGCCGGCAAGAGAGTGATCCAGCTGAGCCGCAAGACCTTCGAGACAGAGTTTCAG
25 AAGACAAAGCACCAGGAGTGGGATTTTCGTGGTGACCACAGACATCAGCGAGATGG
GCGCCAACTTTAAGGCCGATAGAGTGATCGACTCCAGGCGCTGCCTGAAGCCTGTG
ATCCTGGATGGCGAGAGAGTGATCCTGGCAGGACCTATGCCAGTGACCCACGCATC
CGCCGCACAGAGGAGAGGCCGGATCGGCAGAAACCCCAATAAGCCTGGCGACGAG
TACCTGTATGGCGGCGGCTGTGCCGAGACAGACGAGGATCACGCACACTGGCTGGA
30 GGCAAGGATGCTGCTGGATAATATCTACCTGCAGGACGGCCTGATCGCCAGCCTGT
ATAGACCTGAGGCCGATAAGGTGGCCGCCATCGAGGGCGAGTTCAAGCTGCGGAC

CGAGCAGAGAAAGACATTTGTGGAGCTGATGAAGAGGGGCGACCTGCCAGTGTGG
 CTGGCATAACCAGGTGGCATCTGCCGGCATCACCTATACAGATAGGCGCTGGTGCTT
 CGACGGCACCACAAACAATACCATCATGGAGGACAGCGTGCCCTGCCGAAGTGTGG
 ACAAGGCACGGCGAGAAGCGCGTGCTGAAGCCACGGTGGATGGATGCACGGGTGT
 5 GCTCTGACCACGCCGCCCTGAAGAGCTTCAAGGAGTTTGCCGCCGGCAAGAGATAT
 CCCTACGATGTCCCTGATTACGCATAACCGCGGGTTTAAAC

[0065] Example II. Stable cell lines secreting Capsid coating Yellow Fever Virus Like particles for Vaccine use.

[0066] Yellow fever is an acute viral hemorrhagic disease that causes fever and Jaundice. Forty
 10 countries in Africa and Americas are endemic for this disease. The estimated burden of yellow
 fever during 2013 was 84,000–170,000 severe cases and 29,000–60,000 deaths. Although a
 vaccine for YFV is currently available, this vaccine is a Live Attenuated Virus (LAV) vaccine
 which has its disadvantages due to its replication competent nature. There have been rare reports
 of serious side-effects from the yellow fever vaccine. This is described by severe ‘adverse
 15 events following immunization’ (AEFI), when the vaccine provokes an attack on the liver,
 kidneys or the nervous system. The risk of AEFI is higher for people over 60 years of age and in
 immunocompromised individuals like: (1) Infants aged less than 9 months; (2) Pregnant
 women; (3) People with severe allergies to egg protein; and/or (4) People with severe
 immunodeficiency due to symptomatic HIV/AIDS or other causes, or who have a thymus
 20 disorder.

[0067] Moreover, the cost of production and safety of live attenuated virus vaccines is also
 limited. Virus like particles (VLP) on the other hand provide a cheaper, safer and more effective
 vaccine platform for many viral diseases. The success of the VLP vaccine against Papilloma
 virus (HPV) exemplifies the success of this platform. Currently there are no VLP vaccines
 25 against YFV approved for human use.

[0068] The present inventors developed a VLP platform for YFV related Zika virus and shown
 that this platform can provide an economical, safe and highly effective vaccine for use in
 humans. Our technology is built on 4 main innovations.

[0069] A vector system was developed in which the structural proteins of YFV (CprME) are co-expressed with a Zika NS2B3 protein via a single vector by utilizing IRES sequence upstream for the NS2B3 coding region. (Vector design to be patented).

[0070] The inventors generated a consensus sequence of the published YFV sequences and used the consensus sequence translation to codon optimize the sequence for high expression of the proteins. (Sequence to be patented).

[0071] The inventors used a lentiviral system to generate stable cell lines that constitutively express and secrete YFV CprME VLPs into the culture supernatant. (Method and stable cell lines to be patented).

[0072] The production and purification of the VLPs from these stable cell lines was further optimized.

[0073] Yellow Fever Virus consensus Amino acid sequence (SEQ ID NO:5)

MSGRKAQGRTLGVNMVRRGVRSLSNKIKQKTKQIGNRPGPSRGVQGFIFFFLFNILTGKKLTAH
LKKLWRMLDPRQGLAVLRKVVRVASLMRGLASRKRRSNEMAMVPLLLGLLALSGGVTLVRKN
RWLLLNVTAE DLGKTFSVGTGNCTTNILEAKYWCPDSMEYNCPNLSPREEPDDIDWCYGVENV
RVAYGRCDVGRSKRSRRAIDLPTHE NHGLKTRQEKWMTGRMGERQLQKIERWLVRNPFFAVTA
LAIAYLVGNNTTQRVVIALLVLA VGPAZSAHCIGITDRDFIEGVHGGTWVSATLEQDKCVTVMA
PDKPSLDISLQTVAIDGPAEARKVCYSAVLTHVKINDKCPSTGEAHLAEENDGDNACKRTYS DR
GWGNGCGLFGKGSIVACAKFTCAKSMSLFEVDQTKIQYVIRAQLHVGAKQENWNTDIKTLKFDA
LSGSQEA EFTGYGKATLE CQVQTA VDFGNSYIAEMEKDSWIVDRQWAQDLTLPWQSGSGGIWRE
MHHLVEFEP PHAATIRVLALGNQEGSLKTALTGAMRVTKDENDNNLYKLHGHHVSCRVKLSALT
LKGTSYKMCTDKMSFVKNP TDTGHGTVVMQVKVPKGAPCKIPVIVADDLTA AVNKGILVTVNPI
ASTNDDEV LIEVNPPFGDSYII VGTGDSRLTYQWHKEGSSIGKLFTQTMKGAERLAVMGDAAWD
FSSAGGFFTSVGKGIHTVFGSAFQGLFGGLSWITKVIMGAVLIWVGINTRNMTMSMSMILVGVI
MMFLSLGVGAD

[0074] Yellow Fever Virus consensus and codon optimized DNA sequence (SEQ ID NO:6)

ATGTCCGGAAGGAAGGCACAGGGAAGAACCCTGGGCGTGAACATGGTGCGGAGAGGCGTGCGGT
CCCTGTCTAATAAGATCAAGCAGAAGACAAAGCAGATCGGAAACAGGCCTGGACCATCTCGCGG
CGTGCAGGGCTTCATCTTCTTTTTCCTGTTTAATATCCTGACCGGCAAGAAGCTGACAGCCCAC
CTGAAGAAGCTGTGGAGGATGCTGGACCCCAGACAGGGACTGGCCGTGCTGAGGAAGGTGAAGA

GGGTGGTGGCAAGCCTGATGAGGGGACTGGCAAGCAGGAAGAGGCGCTCCAACGAGATGGCCAT
GGTGCCTCTGCTGCTGCTGGGACTGCTGGCCCTGTCTGGAGGAGTGACCCCTGGTGAGGAAGAAC
CGCTGGCTGCTGCTGAATGTGACAGCCGAGGATCTGGGCAAGACCTTCAGCGTGGGCACAGGCA
ACTGCACCACAAATATCCTGGAGGCCAAGTACTGGTGCCCTGACTCCATGGAGTATAACTGTCC
5 AAATCTGTCTCCCAGAGAGGAGCCTGACGATATCGATTGCTGGTGTTACGGCGTGAGAAATGTG
CGGGTGGCCTATGGCAGATGTGACGCCGTGGGCCGGTCTAAGAGAAGCCGGAGAGCCATCGATC
TGCCAACCCACGAGAACCACGGCCTGAAGACCAGACAGGAGAAGTGGATGACAGGCCGGATGGG
CGAGAGACAGCTGCAGAAGATCGAGAGGTGGCTGGTGCGCAACCCCTTCTTCGCAGTGACCGCC
CTGGCAATCGCATACCTGGTGGGCAACAATACCACACAGAGGGTGGTCATCGCCCTGCTGGTGC
10 TGGCAGTGGGACCAGCATATAGCGCCCCTGTCATCGGCATCACCGACAGAGATTTTCATCGAGGG
AGTGACCGGAGGAACCTGGGTGTCCGCCACACTGGAGCAGGACAAGTGCGTGACCGTGATGGCC
CCCGACAAGCCTTCCCTGGATATCTCTCTGCAGACAGTGGCAATCGACGGACCAGCCGAGGCCA
GAAAGGTGTGCTACTCTGCCGTGCTGACCCACGTGAAGATCAATGATAAGTGTCCAAGCACAGG
AGAGGCACACCTGGCAGAGGAGAACGACGGCGATAATGCCTGCAAGAGGACCTATTCCGACCGG
15 GGCTGGGGAAACGGATGTGGACTGTTTGGCAAGGGCTCTATCGTGGCCTGCGCCAAGTTCACCT
GTGCCAAGAGCATGTCCCTGTTTGGAGTGGATCAGACAAAGATCCAGTACGTGATCAGGGCACA
GCTGCACGTGGGAGCAAAGCAGGAGAACTGGAATACCGACATCAAGACACTGAAGTTCGATGCC
CTGTCTGGCAGCCAGGAGGCCGAGTTTACCGGCTACGGCAAGGCCACACTGGAGTGCCAGGTGC
AGACCGCCGTGGACTTCGGCAATAGCTATATCGCCGAGATGGAGAAGGACTCCTGGATCGTGGA
20 TCGCCAGTGGGCTCAGGATCTGACACTGCCATGGCAGTCCGGATCTGGAGGAATCTGGAGGGAG
ATGCACCACCTGGTGGAGTTTGGAGCCCCCTCACGCAGCAACCATCAGAGTGCTGGCCCTGGGCA
ATCAGGAGGGAAGCCTGAAGACCGCCCTGACAGGAGCCATGAGGGTGACAAAGGACGAGAACGA
TAACAATCTGTACAAGCTGCACGGAGGACACGTGTCTTGACAGGGTGAAGCTGAGCGCCCTGACC
CTGAAGGGCACATCTTACAAAATGTGCACCGACAAGATGAGCTTCGTGAAGAACCCAACCGATA
25 CAGGCCACGGCACAGTGGTCATGCAGGTGAAGGTGCCAAAGGGCGCCCCCTGTAAGATCCCTGT
GATCGTGGCCGACGATCTGACCGCCGCCGTGAATAAGGGCATCCTGGTGACCGTGAACCCCATC
GCCTCCACAAATGACGATGAGGTGCTGATCGAGGTGAACCCACCCTTTGGCGACTCCTACATCA
TCGTGGGCACCGGCGATTCTAGGCTGACATATCAGTGGCACAAGGAGGGCAGCTCCATCGGCAA
GCTGTTTACCCAGACAATGAAGGGAGCAGAGCGCCTGGCCGTGATGGGCGACGCCGCTGGGAT
30 TTTTCTAGCGCCGGCGGCTTTTTTACCAGCGTGGGCAAGGGCATCCACACAGTGTTTGGGAAGCG
CCTTCCAGGGACTGTTTGGAGGACTGTCCTGGATCACCAAAGTGATCATGGGCGCCGTGCTGAT

CTGGGTGGGCATCAACACCCGGAATATGACAATGAGCATGTCCATGATCCTGGTGGGCGTGATC
ATGATGTTCCCTGTCCCTGGGCGTGGGCGCCGAC

[0075] FIGS. 2A and 2B show as follows. FIG. 2A shows a schematic of a lentiviral vector expressing Yellow Fever Virus (YFV) CprME along with the protease NS2B-3 for production of capsid containing virus like particles. FIG. 2B shows supernatants harvested from a 293T cell line stably expressing the YFV CprME proteins in the presence of the NSB2-3 protease that is required for release of VLPs from cells. Expression of E protein was analyzed by western blotting using specific antibodies. Lanes 1 and 2 represent supernatants harvested at different days of culture and C represents Control supernatants from 293Y cells. M-molecular weight.

[0076] Example III. Chikungunya Virus (CHIKV) Virus Like Particles for vaccines.

[0077] The present inventors have developed a VLP platform for the related arbovirus, Zika virus, using stable cell lines that constitutively secrete VLPs and demonstrated that this platform can provide an economical, safe and highly effective vaccine especially for use in humans. A similar stable cell line method was used to generate CHIKV VLP secreting cell line. The present invention provides three substantial improvements and advantages over the prior art. The development of the CHIKV VLP is the subject of a co-pending patent application by the present inventors and is incorporated herein in its entirety.

[0078] The inventors generated a consensus sequence of 478 CHIKV sequences from year 2006 onwards to represent the most current CHIKV isolates. The artificial consensus sequence and translation was codon optimized to drive high expression of the proteins. The use of the consensus sequence provides a vaccine that is most relevant to current outbreaks.

[0079] The inventors used a lentiviral system to generate stable cell lines that constitutively express CHIKV structural proteins and secrete the VLPs in the supernatant.

[0080] Finally, the inventors optimized production and purification of the VLPs from these stable cell lines.

[0081] Chikungunya Virus Consensus Sequences used in the vaccine, Amino acid sequence: (SEQ ID NO:7).

[0082] MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPRPQRKAGQLAQLISAVNKLTMRV
VPQQKPRKNRKNKKQKQKQAPRNNTNQKKQPPKKKPVQKKKKPGRRRERMCMKIEN

DCIFEVKHEGKVTGYACLVGDKVMKPAHVKG TIDNADLAKLAFKRSSKYDLECAQIPV
 HMKSDASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNKGRVVAI
 VLGGANEGARTALSVVTWNKDIVTKITPEGAEWLSLAIPVMCLLANTTFPCSRPPCTPC
 CYEKEPEKTLRMLLEDNVMSPGYQQLQASLTCSPRRQRRSIKDHFN VYKATRPYLAHC
 5 PDCGEGHSCHSPVALERIRNEATDGTLKIQVSLQIGIKTDDSHDWTCLRYMDNHMPAD
 AERAGLFVRTSAPCTITGTMGHFILARCPKGETLTVGFTDGRKISHSCTHPFHHDPPVIG
 REKFHSRPQHGRELPCSTYAQSTAATAEEIEVHMPPDTPDRTLMSQQSGNVKITVNSQT
 VRYKCNCGDSSEGLTTTDKVINNCKVDQCHAAVTNHKKWQYNSPLVPRNAEFGDRK
 GKVHIPFPLANVTCTRVKARNPTVTYGKNQVIMLLYPDHP TLLSYRNMGEENYQEEW
 10 VTHKKEIRLTVPTEGLEVTWGNNEPYKYWPQLSTNGTAHGHPHEIILYYELEYPTMTA
 VVLSVASFILLSMVGVAVGMMCMCARRRCITPYELTPGATVPFLLSLICCIRTAKAATYQ
 EAAVYLWNEQQPLFWMQALIPLAALIVLCNCLRLLPCCCKMLTFLAVLSVGAHTVSAY
 EHVTVIPNTVGVVPYKTLVNRPGYSPMVLEMELLSVTLEPTLSLDYITCEYKTVIPSPYVK
 CCGTAECKDKSLPDYSCKVFTGVYPFMWGGAYCFCDTENTQLSEAHVEKSESCKTEFA
 15 SAYRAHTASASAKLRVLYQGN NITVAAYANGDHA VTKDAKFIVGPMSSAWTPFDNK
 IVVYKGDVYNMDYPPFGAGRPGQFGDIQSRTPESEDVYANTQLVLQRPSAGTVHVPYS
 QAPSGFKYWLKERGASLQHTAPFGCQIATNPVRAMNCAVGNMPISIDIPDAAFTRVVD
 APSLTDMSCEVSACTHSSDFGGVAIIKYAASKKKGKCAVHSMTNAV TIREAEIEVEGNSQ
 LQISFSTALASAEFRVQVCSTQVHCAA ECHPPKDHIVNYPASHTTLGVQDISATAMSWV
 20 QKITGGVGLVVAVAALILIVVLCVSFSRH

[0083] Codon Optimized Chikungunya Virus Consensus Sequences used in the vaccine, DNA sequence (SEQ ID NO:8).

[0084] ATGGAGTTCATCCCCACACAGACCTTTTATAACCGGAGATACCAGCCCAGGC
 CTTGGACCCACGCCCAACAATCCAGGTCATCAGGCCTCGGCCAAGACCACAGAGG
 25 AAGGCAGGACAGCTGGCACAGCTGATCAGCGCCGTGAATAAGCTGACCATGCGCG
 TGGTGCCCCAGCAGAAGCCTCGGAAGAACAGAAAGAATAAGAAGCAGAAGCAGAA
 GCAGCAGGCCCAAGGAACAATACCAACCAGAAGAAGCAGCCCCCAAGAAGAAG
 CCTGTGCAGAAGAAGAAGCCAGGCAGGCGCGAGCGCATGTGCATGAAGATCG
 AGAATGATTGCATCTTCGAGGTGAAGCACGAGGGCAAGGTGACCGGCTACGCCTGT
 30 CTGGTGGGCGACAAAGTGATGAAGCCCGCCACGTGAAGGGCACAATCGACAACG
 CCGATCTGGCCAAGCTGGCCTTCAAGAGGAGCTCCAAGTATGATCTGGAGTGCGCC

CAGATCCCCGTGCACATGAAGAGCGACGCCTCCAAGTTTACCCACGAGAAGCCTGA
GGGCTACTATAATTGGCACCACGGAGCAGTGCAGTACTCTGGAGGCAGGTTACCA
TCCCTACAGGAGCAGGCAAGCCAGGCGACAGCGGCAGACCCATCTTTGATAATAAG
GGAAGAGTGGTGGCAATCGTGCTGGGAGGAGCAAACGAGGGCGCCAGAACCGCCC
5 TGAGCGTGGTGACATGGAATAAGGATATCGTGACCAAGATCACACCTGAGGGAGC
AGAGGAGTGGTCTCTGGCAATCCCAGTGATGTGCCTGCTGGCCAACACCACATTCC
CATGTAGCCGGCCACCATGCACCCCATGCTGTTACGAGAAAGAGCCTGAGAAGACA
CTGAGAATGCTGGAGGACAATGTGATGTCCCCTGGCTACTATCAGCTGCTGCAGGC
CTCTCTGACCTGTAGCCACGGAGACAGAGGCGCTCTATCAAGGATCACTTTAACG
10 TGTATAAGGCCACAAGGCCTTACCTGGCACA CTGTCCAGACTGCGGAGAGGGACAC
TCTTGCCACAGCCCAGTGGCCCTGGAGCGGATCAGAAATGAGGCCACCGATGGCAC
ACTGAAGATCCAGGTGAGCCTGCAGATCGGCATCAAGACCGACGATTCCCACGACT
GGACAAAGCTGCGCTACATGGACAACCACATGCCAGCCGATGCAGAGAGGGCAGG
ACTGTTCGTGAGAACCAGCGCCCCCTGTACAATCACCGGCACAATGGGCCACTTCA
15 TCCTGGCAAGGTGCCCCAAAGGGAGAGACCCTGACAGTGGGCTTTACCGATGGCCGC
AAGATCTCTCACAGCTGTACACACCCTTTCCACCACGACCCTCCAGTGATCGGCCGC
GAGAAGTTTCACTCCCGGCCACAGCACGGAAGAGAGCTGCCCTGCTCTACCTATGC
ACAGAGCACCGCCGCCACAGCCGAGGAGATCGAGGTGCACATGCCCCCTGACACC
CCCGATCGGACACTGATGTCCCAGCAGTCTGGCAACGTGAAGATCACCGTGAATAG
20 CCAGACAGTGAGATACAAGTGTA ACTGCGGCGACTCTAGCGAGGGCCTGACCACA
ACCGATAAAGTGATCAACAATTGTAAGGTGGACCAGTGCCACGCCGCCGTGACCAA
CCACAAGAAGTGGCAGTATAATTCCCCACTGGTGCCCAGGAACGCCGAGTTCGGCG
ATCGCAAGGGCAAGGTGCACATCCCTTTTCCACTGGCCAATGTGACCTGCAGGGTG
CCTAAGGCCCGCAATCCAACCGTGACATACGGCAAGAACCAGGTCATCATGCTGCT
25 GTATCCTGACCACCCAACACTGCTGAGCTACAGGAACATGGGCGAGGAGCCTAATT
ATCAGGAGGAGTGGGTGACCCACAAGAAGGAGATCCGCCTGACCGTGCCAACAGA
GGGCCTGGAGGTGACATGGGGCAACAATGAGCCCTATAAGTACTGGCCTCAGCTGT
CCACCAACGGAACAGCACACGGACACCCACACGAGATCATCCTGTACTATTACGAG
CTGTACCCTACCATGACAGCCGTGGTGCTGAGCGTGGCCTCCTTCATCCTGCTGTCC
30 ATGGTGGGAGTGGCAGTGGGAATGTGCATGTGCGCACGGAGAAGGTGCATCACCC
CATATGAGCTGACCCCCGGCGCCACAGTGCCTTTTCTGCTGTCTCTGATCTGCTGTA

TCCGGACCGCCAAGGCCGCCACATATCAGGAGGCCGCCGTGTACCTGTGGAACGAG
CAGCAGCCCCTGTTCTGGATGCAGGCCCTGATCCCTCTGGCCGCCCTGATCGTGCTG
TGCAATTGCCTGAGACTGCTGCCTTGCTGTTGCAAGATGCTGACCTTTCTGGCCGTG
CTGTCCGTGGGCGCCACACAGTGTCTGCCTACGAGCACGTGACCGTGATCCCCAA
5 TACAGTGGGCGTGCCTTACAAGACCCTGGTGAACCGGCCAGGCTATTCTCCCATGG
TGCTGGAGATGGAGCTGCTGAGCGTGACCCTGGAGCCAACACTGTCCCTGGATTAT
ATCACCTGTGAGTACAAGACAGTGATCCCCAGCCCTTACGTGAAGTGTTGCGGCAC
CGCCGAGTGTAAGGACAAGTCCCTGCCAGATTATTCTTGCAAGGTGTTACAGGCG
TGTATCCCTTTATGTGGGGCGGCGCCTACTGTTTCTGCGACACCGAGAACACACAGC
10 TGTCCGAGGCCACGTGGAGAAGTCCGAGTCTTGCAAGACCGAGTTTGCCCTCTGCC
TACAGAGCCCACACAGCAAGCGCCTCCGCCAAGCTGAGAGTGCTGTACCAGGGCA
ACAATATCACCGTGGCCGCCTATGCCAATGGCGACCACGCCGTGACAGTGAAGGAT
GCCAAGTTCATCGTGGGACCCATGTCCTCTGCCTGGACCCCATTTGACAATAAGATC
GTGGTGTACAAGGGCGACGTGTATAACATGGATTACCCACCCTTCGGCGCAGGCAG
15 GCCTGGACAGTTTGGCGATATCCAGAGCCGCACCCCAGAGTCCGAGGACGTGTATG
CCAACACACAGCTGGTGCTGCAGAGGCCAAGCGCCGGCACCGTGACAGTGCCATAC
TCCCAGGCCCCCCTCTGGCTTCAAGTATTGGCTGAAGGAGAGGGGAGCATCCCTGCA
GCACACCGCACCATTTGGCTGTCAGATCGCCACAAATCCCGTGAGAGCCATGAACT
GCGCCGTGGGCAATATGCCAATCAGCATCGACATCCCCGATGCCGCCTTCACCAGA
20 GTGGTGGACGCCCCTTCCCTGACAGATATGAGCTGTGAGGTGTCCGCCTGCACCCA
CAGCTCCGACTTTGGCGGCGTGCCATCATCAAGTACGCCGCCTCTAAGAAGGGCA
AGTGTGCCGTGCACAGCATGACCAACGCCGTGACAATCCGGGAGGCCGAGATCGA
GGTGGAGGGCAATAGCCAGCTGCAGATCTCTTTCAGCACCGCCCTGGCCTCCGCCG
AGTTTAGAGTGCAGGTGTGCTCTACACAGGTGCACTGTGCCGCCGAGTGCCACCCT
25 CCAAAGGATCACATCGTGAACCTATCCAGCATCCCACACAACCCTGGGAGTGACAGGA
CATCTCTGCCACCGCCATGAGCTGGGTGCAGAAGATCACAGGAGGAGTGGGACTGG
TGGTGGCAGTGGCCGCCCTGATCCTGATCGTGGTGCTGTGCGTGTCTTCTCTAGAC
AC

[0085] FIG. 3A is a schematic of a lentiviral vector expression that includes CHKV structural
30 proteins for production of capsid containing virus like particles. FIG. 3B is a Western Blot
showing the culture supernatants harvested from 293T cells stably transfected and expressing

the CHKV structural proteins (293T-CHKV-Lenti) and analyzed for CHKV E1-E2 protein expression. Lanes 1, 2 and 3 represent supernatants harvested from different days and C represents control supernatants from 293T cells. M-molecular weight markers.

[0086] FIGS. 4A and 4B show the establishment of a single cell clones expressing CHKV Structural proteins. In FIG. 4A, the CHKV structural proteins were cloned into the lentiviral vector pLenti6/5-D-Topo and used to produce lentiviral particles containing the CHKV-E1/E2. 293T cells were then transduced with the above lentiviral particles and cells selected by culturing in the presence of blasticidin. Bulk selected cells were confirmed for E1/E2 protein expression via western blotting. Subsequently, cells were plated in 96 well plates using limiting dilution and clones arising from single viable cells selected. In FIG. 4B, the culture supernatants were harvested from 293T single cell clones and concentrated by ultracentrifugation. Expression of the E1/E2 proteins in the supernatants was determined by western blotting.

[0087] FIG. 5 is a flow cytometry analysis of single cell clones expressing the CHKV-E1/E2. Six different single cell clones of 293T cells expressing the CHKV-E1/E2 proteins were stained using the CHKV E protein antibody followed by flow cytometry analysis. The bulk selected cell line was used as control. The CH-6, CH-3 and CF-5 cell lines show good CHKV E protein expression. The flow cytometry is in the same order as the table below the graph.

[0088] In certain embodiments, the present invention includes the combination of separately manufactured arbovirus VLPs that are combined following Table 1, to combine into a vaccine that includes multiple different arboviruses. These VLPs can be co-packaged into individual or separate containers that are used to immunize individuals that live, or are going to travel to, geographic areas with the prevalence of certain arboviruses. In other embodiments, the various vectors taught herein can be co-expressed in the same cells, again following the teachings of Tables 1 and 2, to generate multivalent arbovirus VLPs in a single cell. By stably transfecting cells with one or more vectors that express multiple arbovirus proteins that are replication deficient but that form multivalent arbovirus VLPs, which may be made in the cells and released mechanically, chemically and/or electrically, or that are preferable secreted by the stably transfected cells.

[0089] FIGS. 6A to 6F show the characterization of JEV, YFV and CHKV protein expression and VLP release. FIG. 6A: 293T cells were transfected with JEV CprME expression vector.

Cells were analyzed for E protein and Capsid protein expression by immunofluorescence microscopy. FIG. 6B: 293T cells were transfected with JEV CprME expression vector alone or the JEV CprME vector along with Zika NS2B-3 expression plasmid. Culture supernatants were analyzed for E protein and Capsid protein expression by western blotting. FIG. 6C: 293T cells were transfected with YFV CprME expression vector and analyzed for E protein and Capsid protein expression by microscopy. FIG. 6D: 293T cells were transfected with YFV CprME expression vector alone or the YFV CprME vector along with Zika NS2B-3 expression plasmid. Culture supernatants were analyzed for E protein and Capsid protein expression by western blotting. FIG. 6E: 293T cells were transfected with CHIKV expression vector and analyzed for E protein expression by microscopy. FIG. 6F: 293T cells were transfected with CHIKV expression vector. Culture supernatants were analyzed for E1-E2 protein expression by western blotting.

[0090] FIGS. 7A to 7D show a strategy for JEV, YFV, Zika and CHIKV RVP generation and titration. FIG 7A: 293T cells were transfected with JEV CprME expression vector along with the WNV sub-genomic replicon WNRepG/Z. Culture supernatants were harvested 48h post transfection and titrated in Vero cells using two fold dilutions of the virus stock. Number of GFP positive cells were determined 72hrs post infection using automated microscopy. FIG 7B: 293T cells were transfected with YFV CprME expression vector along with the WNV sub-genomic replicon WNRepG/Z. Culture supernatants were harvested 48h post transfection and titrated in Vero cells as above. FIG 7C: 293T cells were transfected with Zika CprME expression vector along with the WNV sub-genomic replicon WNRepG/Z. Culture supernatants were harvested 48h post transfection and titrated in Vero cells as above. FIG 7D: 293T cells were transfected with the CHIKV E3-E2-E1 expression vector along with the HIV NL.Luc R-/E-. Culture supernatants were harvested 48h post transfection and titrated in Vero cells.

[0091] FIGS. 8A to 8D show the neutralization of RVP infection using disease specific sera. Vero cells were infected with FIG. 8A: JEV RVPs, FIG. 8B: YFV RVPs, FIG. 8C: ZIKV RVPs or FIG. 8D: CHIKV RVPs in the presence of serial dilutions of virus specific sera or control sera. Infection was determined 72hrs post infection via automated microscopy. Data is represented as percent infection normalized to control sera as mean $\pm$ SD of triplicate observations.

[0092] FIGS. 9A to 9C show the generation and characterization of bicistronic lentiviral vectors expressing arboviral structural proteins. FIG. 9A: JEV CprME was cloned into a lentiviral vector that included an IRES sequence followed by the Zika NS2B-3 protease. 293T cells were transfected with the JEV CprME construct alone or the bicistronic JEV lentiviral construct. Culture supernatants were harvested and VLPs analyzed for JEV E protein and Capsid protein secretion via western blotting. FIG. 9B: YFV CprME was cloned into a lentiviral vector as above and VLP secretion determined in the culture supernatants by western blotting for E and Capsid protein. FIG. 9C: CHIKV C-E3-E2-E1 genes were cloned into a lentiviral vector and VLP secretion determined in the culture supernatants by western blotting for E1/E2 protein.

[0093] FIGS. 10A to 10C show the generation of stable cell lines secreting JEV, YFV and CHIKV VLPs. 293T cells were transduced with FIG. 10A: JEV, FIG. 10B: YFV, or FIG. 10C: CHIKV structural protein containing lentivirus particles. Transduced cells were bulk selected with blasticidin followed by limiting dilution single cell cloning. For each virus, several single cell clones were characterized for Envelope protein staining on the cell surface via flow cytometry and VLP secretion into the culture supernatants via western blotting.

[0094] FIGS. 11A and 11B show the characterization of stable single cell clones for Env and capsid protein expression. FIG. 11A: JEV-JD12, YFV-YF9 and CHIKV-CH3 cell lines were stained for E and capsid protein expression. Cells were analyzed by fluorescence microscopy and images acquired. FIG. 11B: the stable cell lines were cultured in the presence or absence of blasticidin for a period of 30 days. Culture supernatants were analyzed for E protein expression by western blotting.

[0095] FIGS. 12A and 12B show the VLP purification and schematic of mice immunization studies. FIG. 12A: single cell cultures producing Zika, YFV, CHIKV and JEV VLPs were propagated in 5 layer flasks. Culture supernatants were concentrated via ultracentrifugation, pooled and analyzed for E protein expression via western blot. FIG. 12B: Balb/c mice were divided into 8 groups (N=6 mice/group) and immunized with the monovalent vaccine or different bivalent or tetravalent combinations as depicted in the table. Alum (2% alhydrogel) was used as adjuvant. Mice received a single booster dose at day 14 and were sacrificed at ~44 days post primary immunization.

[0096] FIGS. 13A to 13D show the neutralization efficacy of VLP vaccine combinations. Sera obtained from mice immunized with different VLP vaccine combinations were analyzed generation of neutralizing antibody response. Reporter virus based assays were used to determine the efficacy of neutralizing antibodies, curves were fit and EC50 titers determined. Data for (13A) JEV, (13B) CHIKV, (13C) YFV and (13D) ZIKV EC50 titers is shown.

[0097] FIGS. 14A to 14D show the quality of antibody response generated in VLP immunized mice. FIG. 14A: CHIKV-E3-E2-E1 stable cell line was radiolabeled with [³⁵S]Met-Cyst and cell lysates immunoprecipitated with pooled sera from indicated groups of immunized mice. Complexes were resolved on an SDS-PAGE gel followed by fluorography. FIG. 14B: YFV-CprME stable cell line was radiolabeled with [³⁵S]Met-Cyst and cell lysates immunoprecipitated with pooled sera from relevant groups of immunized mice. FIG. 14C: JEV-CprME stable cell line was radiolabeled with [³⁵S]Met-Cyst and cell lysates immunoprecipitated with pooled sera from indicated groups of immunized mice. FIG. 14D: JEV-CprME stable cell line was radiolabeled with [³⁵S]Met-Cyst and cell lysates immunoprecipitated with pooled sera from indicated groups of immunized mice.

[0098] FIGS. 15A to 15C shows the adaptation of stable cell lines for growth in suspension culture. FIG. 15A: JEV, YFV, ZIKV and CHIKV stable cell lines were adapted to grow in suspension culture. After complete adaptation, cells were stained for E protein expression via flow cytometry. FIG. 15B: VLP production from the adapted suspension cell lines was determined by western blotting for the E protein. FIG. 15C: Morphology of suspension cell lines via brightfield microscopy and comparison with their adherent counterparts.

[0099] It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, kit, reagent, or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve methods of the invention.

[0100] It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such

equivalents are considered to be within the scope of this invention and are covered by the claims.

[0101] All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[0102] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0103] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. In embodiments of any of the compositions and methods provided herein, “comprising” may be replaced with “consisting essentially of” or “consisting of”. As used herein, the phrase “consisting essentially of” requires the specified integer(s) or steps as well as those that do not materially affect the character or function of the claimed invention. As used herein, the term “consisting” is used to indicate the presence of the recited integer (e.g., a feature, an element, a characteristic, a property, a method/process step or a limitation) or group of integers (e.g., feature(s), element(s), characteristic(s), property(ies), method/process steps or limitation(s)) only.

[0104] As used herein, the term “or combinations thereof” refers to all permutations and combinations of the listed items preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is

important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0105] As used herein, words of approximation such as, without limitation, “about”, “substantial” or “substantially” refers to a condition that when so modified is understood to not necessarily be absolute or perfect but would be considered close enough to those of ordinary skill in the art to warrant designating the condition as being present. The extent to which the description may vary will depend on how great a change can be instituted and still have one of ordinary skill in the art recognize the modified feature as still having the required characteristics and capabilities of the unmodified feature. In general, but subject to the preceding discussion, a numerical value herein that is modified by a word of approximation such as “about” may vary from the stated value by at least ± 1 , 2, 3, 4, 5, 6, 7, 10, 12 or 15%.

[0106] All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

[0107] To aid the Patent Office, and any readers of any patent issued on this application in interpreting the claims appended hereto, applicants wish to note that they do not intend any of the appended claims to invoke paragraph 6 of 35 U.S.C. § 112, U.S.C. § 112 paragraph (f), or equivalent, as it exists on the date of filing hereof unless the words “means for” or “step for” are explicitly used in the particular claim.

[0108] For each of the claims, each dependent claim can depend both from the independent claim and from each of the prior dependent claims for each and every claim so long as the prior claim provides a proper antecedent basis for a claim term or element.

What is claimed is:

1. A nucleic acid encoding two or more consensus, code optimized, or both consensus and codon optimized, multivalent flavivirus nucleic acid sequences that express one or more flavivirus proteins inserted into a lentiviral vector capable of forming Virus Like Particles (VLPs).
5
2. The nucleic acid of claim 1, wherein a cell line makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine.
3. The nucleic acid of claim 1, wherein the nucleic acids are selected from at least one of
10 nucleic acid SEQ ID NOS:2, 6, or 8.
4. The nucleic acid of claim 1, wherein the two or more nucleic acids are inserted into the lentiviral vector.
5. The nucleic acid of claim 1, wherein the nucleic acid express two or more proteins selected from SEQ ID NO:1, 5, or 7.
- 15 6. The nucleic acid of claim 1, wherein the nucleic acid is transfected into a cell line, a human cell line, or a 293T or Vero cell line.
7. The nucleic acid of claim 1, wherein the nucleic acid is stably transduced in a cell line.
8. The nucleic acid of claim 1, wherein the flavivirus proteins are selected from at least one of: Zika virus, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne
20 Encephalitis Virus, West Nile Virus, St Louis Encephalitis Virus.
9. The nucleic acid of claim 1, wherein the nucleic acid is defined further as a vector.
10. The nucleic acid of claim 1, wherein the nucleic acid is defined further as a bicistronic lentiviral vector.
11. A method of making a multivalent flavivirus Virus Like Particles (VLP) comprising:
25 inserting two or more nucleic acids that encode at least one flavivirus or arbovirus protein into a lentiviral backbone vector;

generating a lentivirus by transfecting a first cell line with the lentiviral backbone vector and isolating the lentivirus therefrom;

transducing a second cell line with the lentivirus;

culturing the transduced cell line under conditions in which the multivalent flavivirus

5 Virus Like Particles (VLP) are released from the cell line; and

isolating the multivalent flavivirus Virus Like Particles (VLP) from a culture supernatant.

12. The method of claim 11, wherein a cell line makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent
10 vaccine.

13. The method of claim 11, wherein the two or more nucleic acids are selected from at least one of nucleic acid SEQ ID NOS:2, 6, or 8.

14. The method of claim 11, the two or more nucleic acid is a consensus, a codon optimized, or both a consensus and codon optimized nucleic acid and expresses one or more proteins of
15 SEQ ID NO:1, 5, or 7.

15. The method of claim 11, wherein the first, the second, or both the first and second cell line is a human cell line, a 293T, or Vero cell line.

16. The method of claim 11, wherein the two or more nucleic acid are defined further as inserted into a vector or vectors.

20 17. The method of claim 11, wherein at least one of the nucleic acids is defined further as a bicistronic lentiviral vector.

18. The method of claim 11, further comprising generating two or more VLPs that express proteins from different flavivirus, the express proteins from different flavivirus strains, or express proteins from different flavivirus clades, wherein each of the VLPs is made in a
25 different cell lines and a vaccine is prepared by mixing the different VLPs produced by different transduced cell lines.

19. The method of claim 18, wherein the mix of VLPs from different transduced cell lines into region-specific multivalent vaccines, wherein a ratio of different flavivirus VLPs is prepared based on the most prevalent flavivirus for such a region.

20. The method of claim 18, wherein the mix of VLPs is bivalent, trivalent, or tetravalent.

5 21. The method of claim 18, wherein the mix of VLPs from different transduced cell lines into region-specific multivalent vaccines is the following:

immunization for a worldwide, a travel, or a military use: Zika, JEV, YFV and Chikungunya virus (CHIKV)(an alphavirus), or YFV and JEV;

for an Asia or Australia JEV, CHIKV and Zika;

10 for a South America or Africa: YFV, CHIKV and Zika; or

for a pacific region: JEV, CHIKV and Zika.

22. A vaccine comprising an isolated and purified multivalent arbovirus Virus Like Particles (VLP) that comprises at least one structural protein from viruses selected from Japanese Encephalitis Virus (JEV), Chikungunya virus (CHIKV), Yellow Fever Virus (YFV), and Zika
15 virus, wherein the vaccine is a combination of two or more different VLPs from arboviruses.

23. The vaccine of claim 22, wherein a cell line transduced with a lentiviral vector that makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine.

20 24. The vaccine of claim 22, wherein the vaccine is optimized for immunization worldwide, Asia and Australia, South America and Africa, or Pacific Region.

25. The vaccine of claim 22, wherein the vaccine is bivalent, trivalent or tetravalent for a flavivirus selected from at least one of: Zika virus, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne Encephalitis Virus, West Nile Virus, St Louis Encephalitis Virus.

25 26. The vaccine of claim 22, wherein the mix of VLPs from different transduced cell lines into region-specific multivalent vaccines is the following:

immunization for a worldwide, a travel, or a military use: Zika, JEV, YFV and CHIKV, or YFV and JEV;

for an Asia or Australia JEV, CHIKV and Zika;

for a South America or Africa: YFV, CHIKV and Zika; or

for a pacific region: JEV, CHIKV and Zika.

27. The vaccine of claim 22, further comprising one or more vaccines selected from at least
5 one of: influenza A vaccine, an influenza B vaccine, an influenza A (H1N1) vaccine, a hepatitis
A vaccine, a hepatitis B vaccine, a haemophilus influenzae Type B (HiB) vaccine, a measles
vaccine, a mumps vaccine, a rubella vaccine, a polio vaccine, a human papilloma virus (HPV)
vaccine, a tetanus vaccine, a diptheria vaccine, a pertussis vaccine, a bubonic plague vaccine, a
yellow fever vaccine, a cholera vaccine, a malaria vaccine, a smallpox vaccine, a pneumococcal
10 vaccine, a rotavirus vaccine, a varicella vaccine or a meningococcus vaccine.
28. A cell line transformed with a nucleic acid vector comprising a nucleic acid sequence
that is a consensus, a codon optimized, or both a consensus and codon optimized nucleic acid
that encodes one or more multivalent arbovirus proteins in a lentiviral vector.
29. The cell line of claim 28, wherein the one or more multivalent arbovirus proteins are
15 flavivirus proteins are expressed by a nucleic acid sequence codon optimized for expression in
human cells.
30. The cell line of claim 28, wherein the one or more multivalent arbovirus proteins are
flavivirus proteins are expressed by a nucleic acid sequence codon optimized for expression in
human cells of SEQ ID NO:2, 6, or 8.
- 20 31. The cell line of claim 28, wherein the one or more multivalent arbovirus proteins have
the amino acid sequence of SEQ ID NO:1, 5, or 7.
32. The cell line of claim 28, wherein the cell line is stably transfected with the nucleic acid
vector, is grown in a suspension, or both.
- 25 33. A purified multivalent flavivirus Virus Like Particles (MV-VLP) comprising the two or
more proteins selected from different flaviviruses and the two or more proteins are from a
portion of amino acid sequences SEQ ID NO:1, 5, or 7.

34. The purified MV-VLP of claim 33, wherein a cell line transduced with a lentiviral vector that makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine.

35. The purified MV-VLP of claim 33, wherein the MV-VLPs are produced in a stably transduced cell line.

36. The purified MV-VLP of claim 33, wherein the MV-VLPs are produced in a stably transduced human cell line.

37. The purified MV-VLP of claim 33, wherein the MV-VLPs are produced in a stably transduced 293T or Vero cell line.

38. The purified MV-VLP of claim 33, wherein the MV-VLPs are isolated from at least one of: a culture supernatant, or the cell lines.

39. The purified MV-VLP of claim 33, further comprising one or more vaccines selected from at least one of: influenza A vaccine, an influenza B vaccine, an influenza A (H1N1) vaccine, a hepatitis A vaccine, a hepatitis B vaccine, a haemophilus influenzae Type B (HiB) vaccine, a measles vaccine, a mumps vaccine, a rubella vaccine, a polio vaccine, a human papilloma virus (HPV) vaccine, a tetanus vaccine, a diphtheria vaccine, a pertussis vaccine, a bubonic plague vaccine, a yellow fever vaccine, a cholera vaccine, a malaria vaccine, a smallpox vaccine, a pneumococcal vaccine, a rotavirus vaccine, a varicella vaccine or a meningococcus vaccine.

40. An immunogenic composition comprising at least one codon optimized multivalent Virus Like Particles (VLP) that comprises at least one structural protein from two or more viruses selected from Japanese Encephalitis Virus (JEV), Chikungunya virus (CHIKV), Yellow Fever Virus (YFV), and Zika virus.

41. The immunogenic composition of claim 40, wherein a cell line transduced with a lentiviral vector that makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine.

42. The immunogenic composition of claim 40, further comprising an adjuvant.

43. The immunogenic composition of claim 40, wherein the composition comprises at least two VLPs comprising different flavivirus E proteins.

44. A method of generating an immune response to one or more flaviviruses in a subject, the method comprising administering to the subject an effective amount of an immunogenic composition comprising at least one codon optimized multivalent flavivirus Virus Like Particles (VLP) that comprises at least one structural protein from two or more flaviviruses selected from
5 Zika virus, Yellow Fever Virus, Japanese Encephalitis Virus, Dengue1-4 Virus, Tick Borne Encephalitis Virus, West Nile Virus, or St Louis Encephalitis Virus.
45. The method of claim 44, wherein a cell line transduced with a lentiviral vector that makes a virus-specific VLP, and the virus-specific VLPs are purified and then mixed in different combinations to make a multivalent vaccine.
- 10 46. The method of claim 44, wherein the composition is administered mucosally, intradermally, subcutaneously, intramuscularly, or orally.
47. The method of claim 44, wherein the immune response vaccinates the subject against multiple serotypes or clades of one or more flaviviruses.
48. The method of claim 44, wherein the subject is a human.
- 15 49. The method of claim 44, further comprising one or more vaccines selected from at least one of: influenza A vaccine, an influenza B vaccine, an influenza A (H1N1) vaccine, a hepatitis A vaccine, a hepatitis B vaccine, a haemophilus influenzae Type B (HiB) vaccine, a measles vaccine, a mumps vaccine, a rubella vaccine, a polio vaccine, a human papilloma virus (HPV) vaccine, a tetanus vaccine, a diphtheria vaccine, a pertussis vaccine, a bubonic plague vaccine, a
20 yellow fever vaccine, a cholera vaccine, a malaria vaccine, a smallpox vaccine, a pneumococcal vaccine, a rotavirus vaccine, a varicella vaccine or a meningococcus vaccine.
50. The method of claim 44, wherein the composition is administered in a multiple dose schedule.
51. A nucleic acid vector comprising a lentiviral vector comprising one or more engineered
25 restriction nuclease sites and a codon optimized Zika virus NC2B-3 protein downstream from an internal ribosomal entry site (IRES), wherein a flaviviral insert comprising one or more flavivirus protein sequences can be inserted into the vector at the restriction sites, wherein the vector produces a purified multivalent flavivirus Virus Like Particles (MV-VLP) when in a cell.

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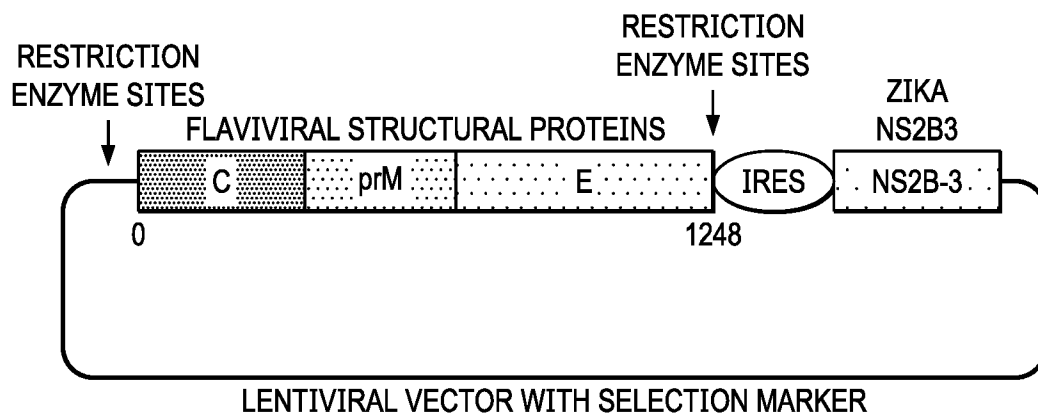


FIG. 1A

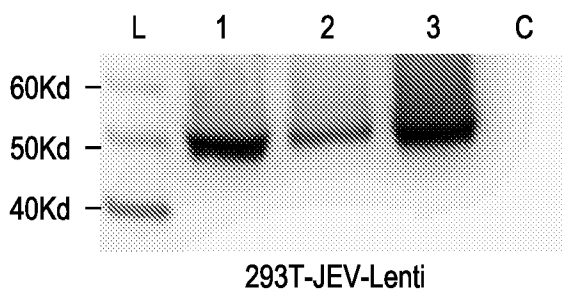


FIG. 1B

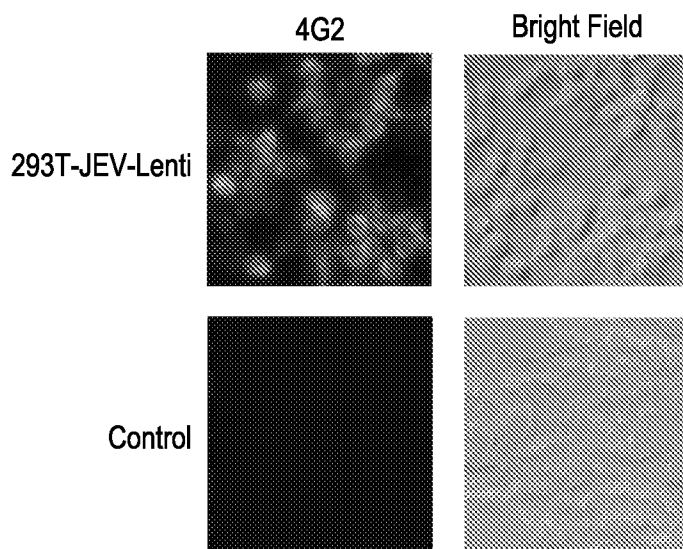


FIG. 1C

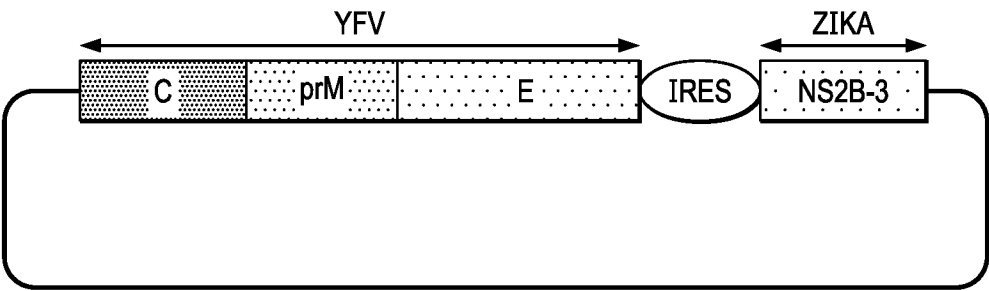


FIG. 2A

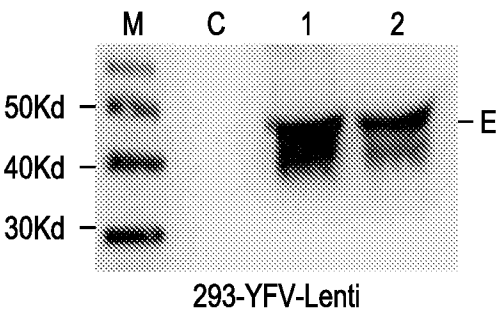


FIG. 2B

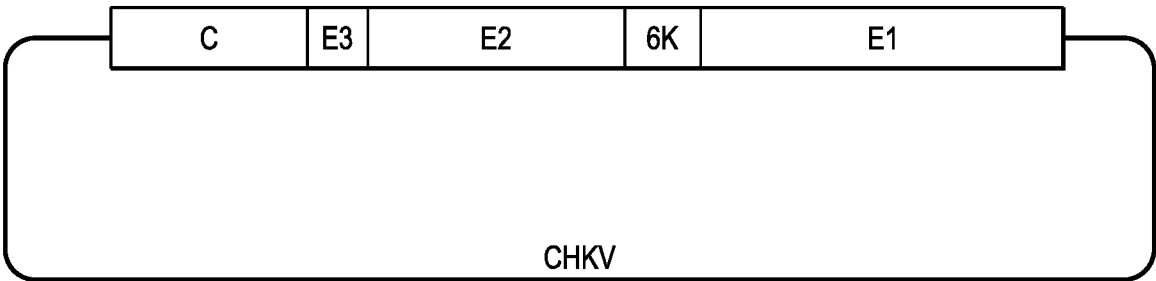


FIG. 3A

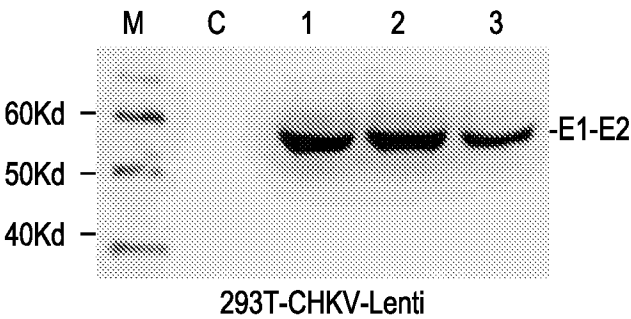


FIG. 3B

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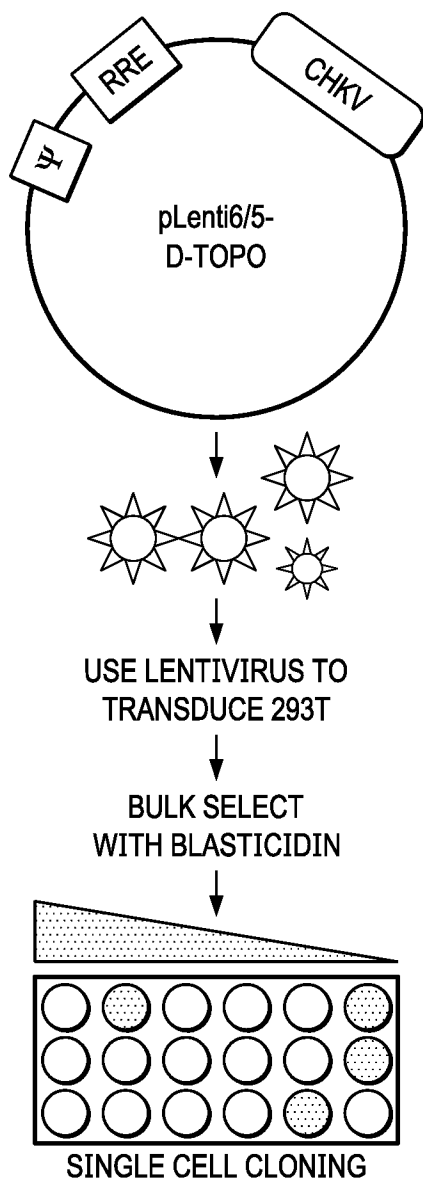


FIG. 4A

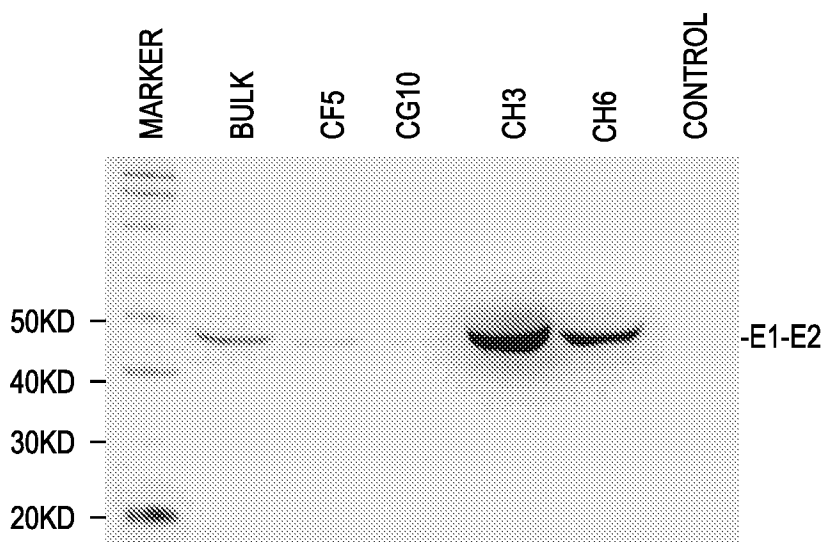
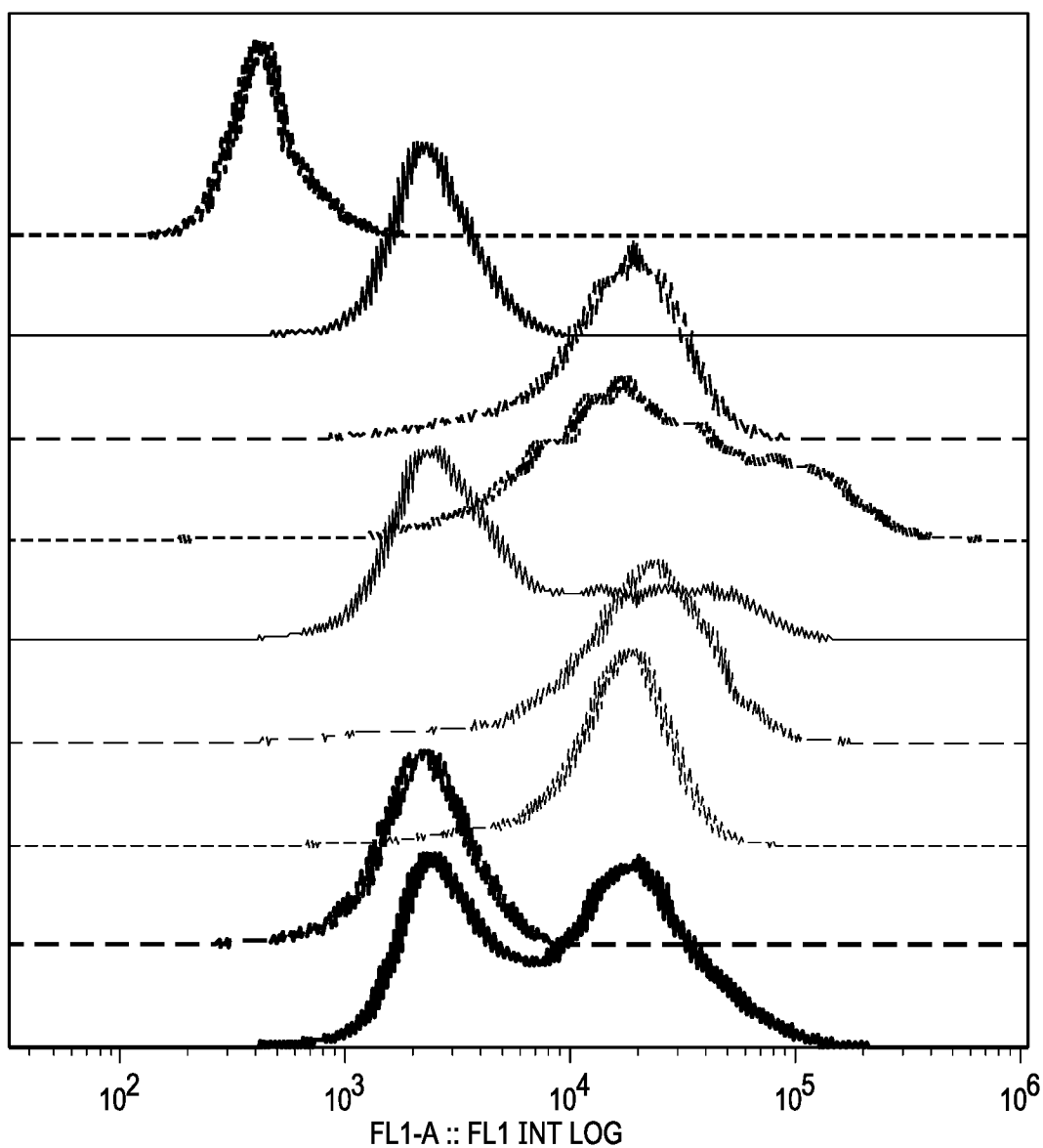


FIG. 4B

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FIG. 5



	SAMPLE NAME	MEAN: FL1-A
☐	CHIKV LENTI CELL LINES 293 UNSTAINED 00005526 542.LMD	476
☐	CHIKV LENTI CELL LINES 293 CHIKV 00005525 541.LMD	2629
☐	CHIKV LENTI CELL LINES CLONE CH-6 00005521 537.LMD	18307
☐	CHIKV LENTI CELL LINES CLONE CH-3 00005528 544.LMD	43722
☐	CHIKV LENTI CELL LINES CLONE CH-2 00005522 538.LMD	12521
☐	CHIKV LENTI CELL LINES CLONE CG-10 00005527 543.LMD	23979
☐	CHIKV LENTI CELL LINES CLONE CF-5 00005520 536.LMD	17417
☐	CHIKV LENTI CELL LINES CLONE CF-1 00005523 539.LMD	2449
☐	CHIKV LENTI CELL LINES BULK 00005524 540.LMD	15544

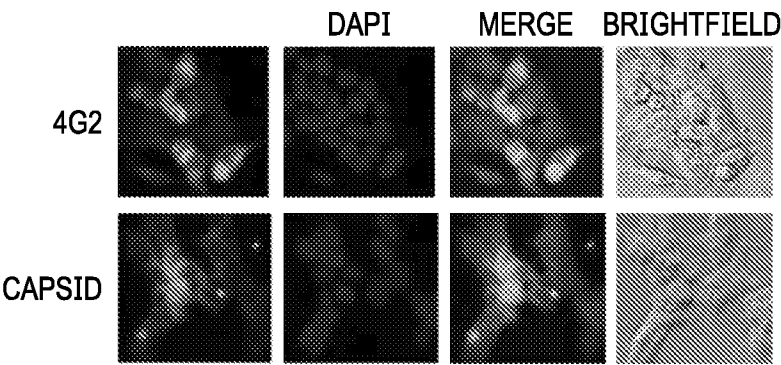


FIG. 6A

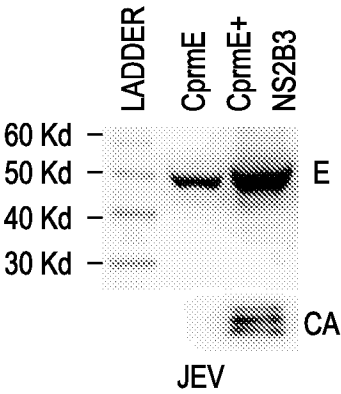


FIG. 6B

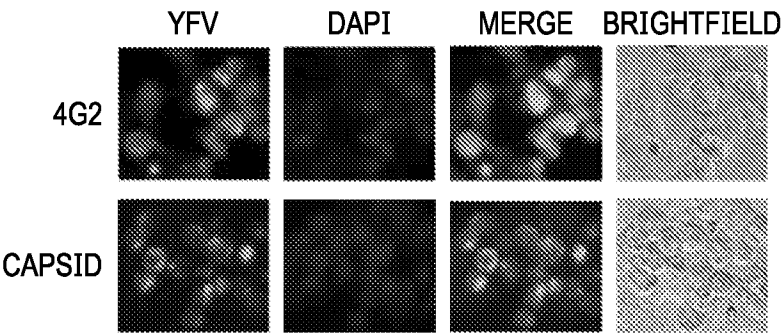


FIG. 6C

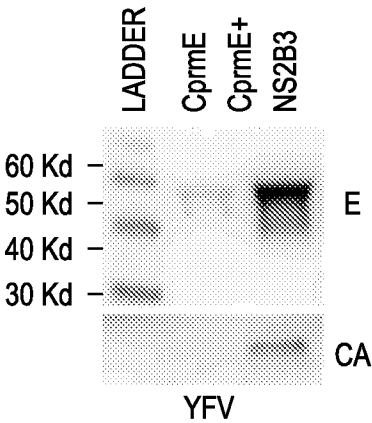


FIG. 6D

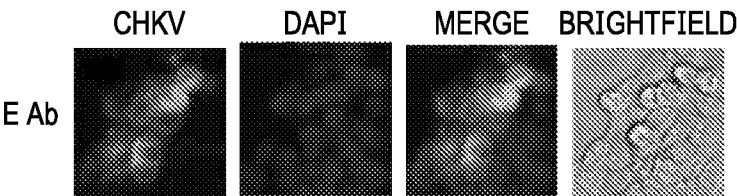


FIG. 6E

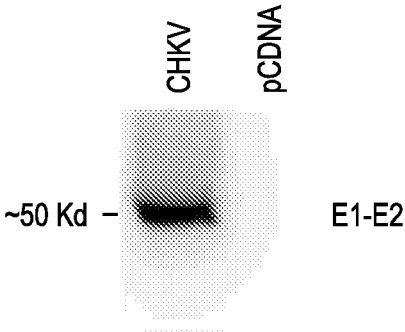


FIG. 6F

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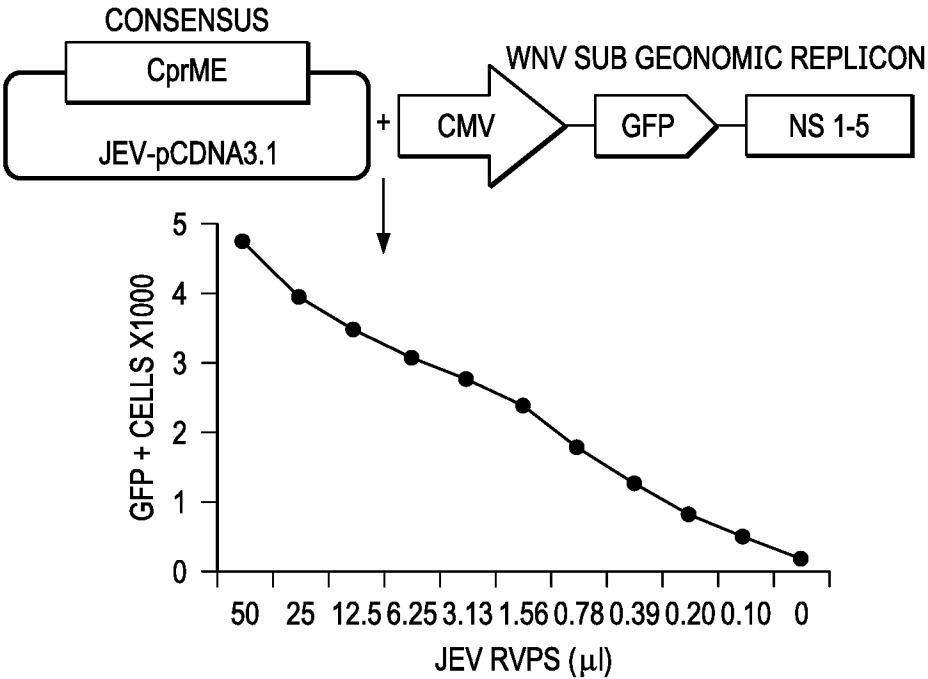


FIG. 7A

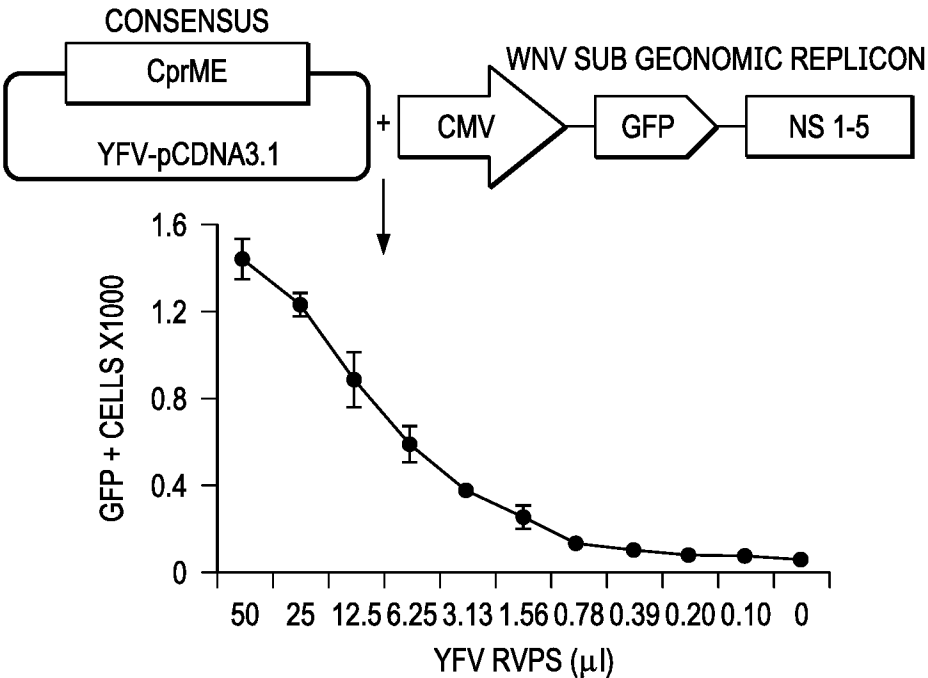


FIG. 7B

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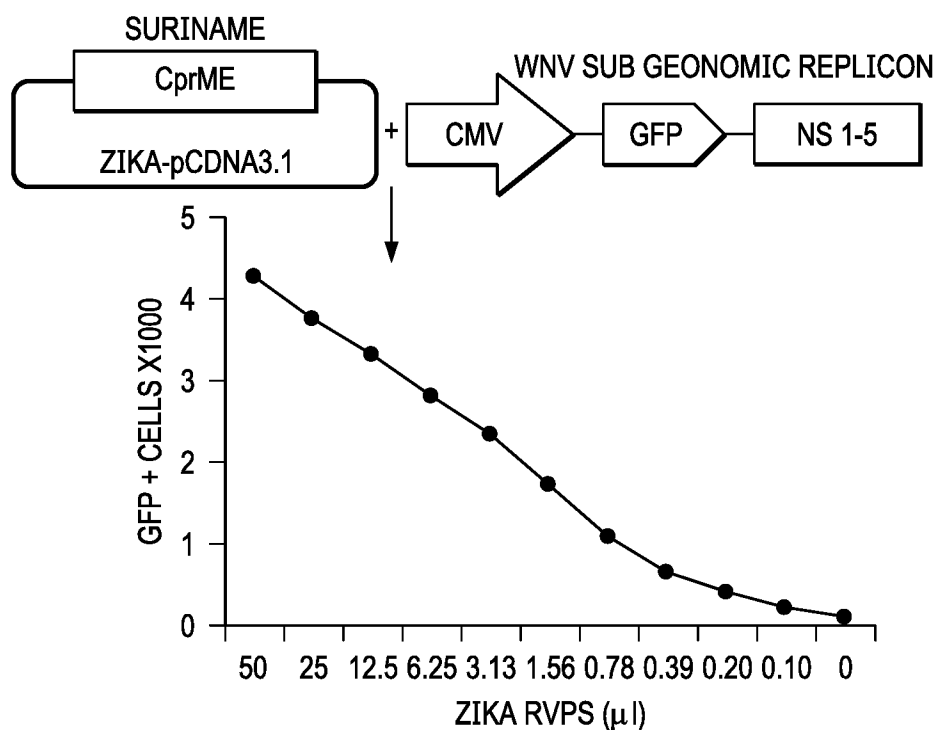


FIG. 7C

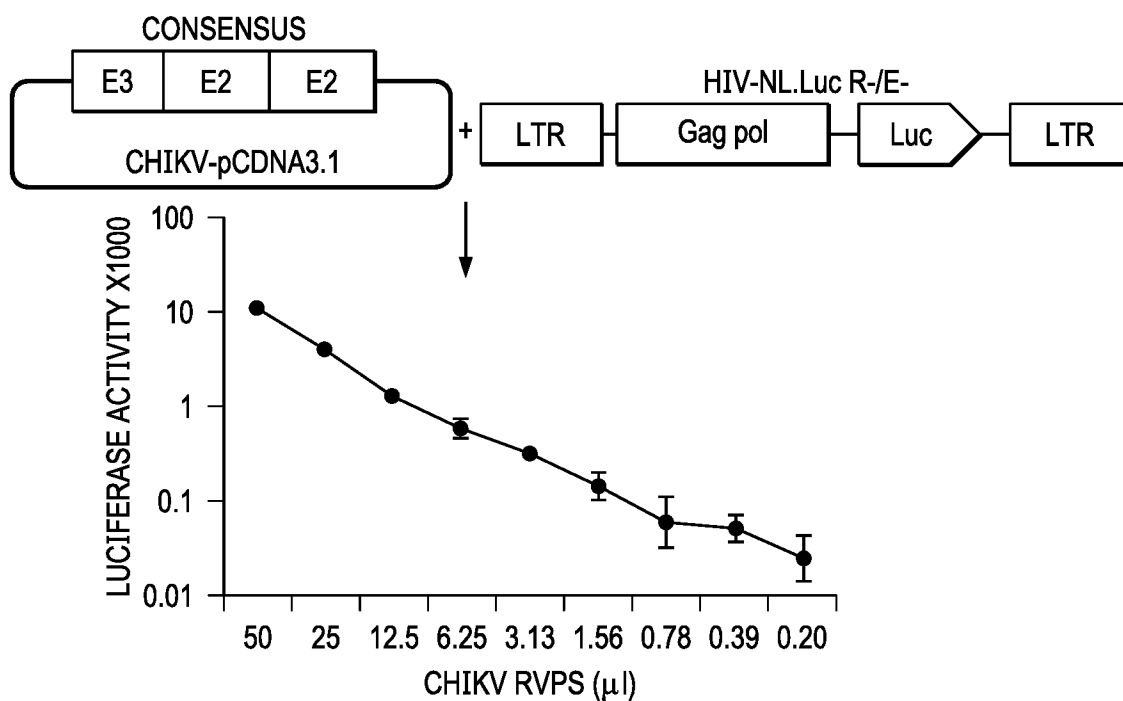


FIG. 7D

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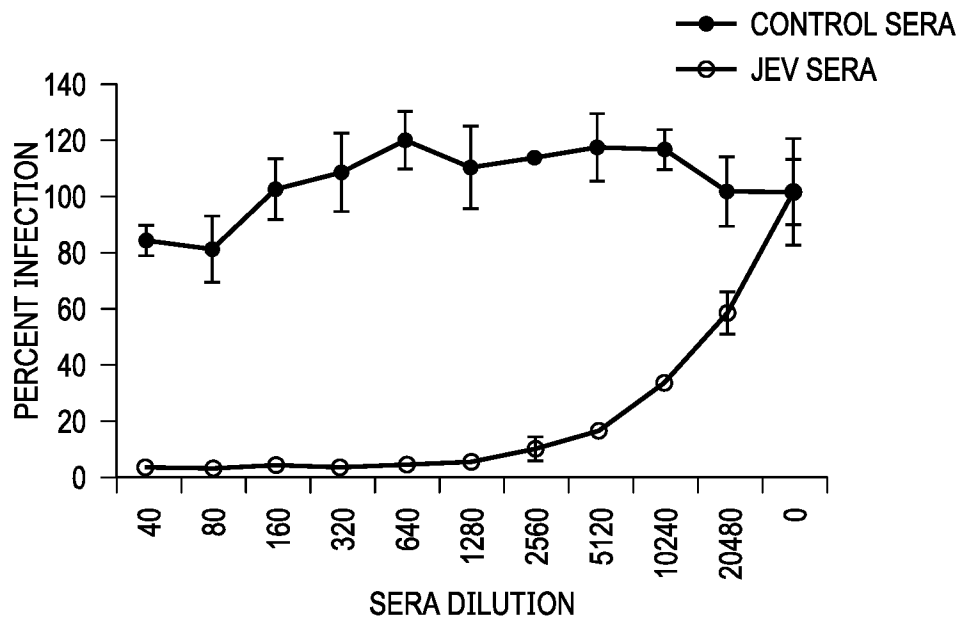


FIG. 8A

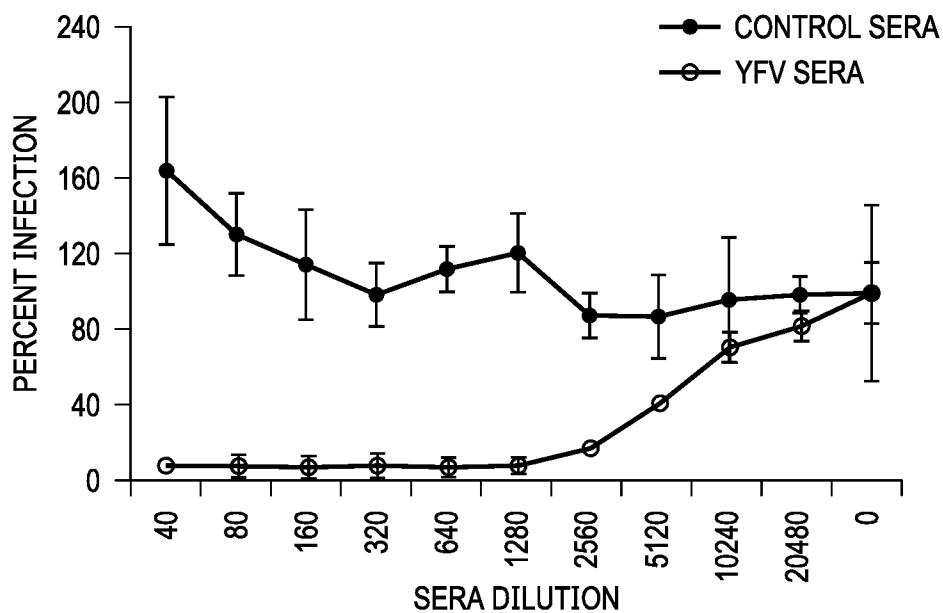


FIG. 8B

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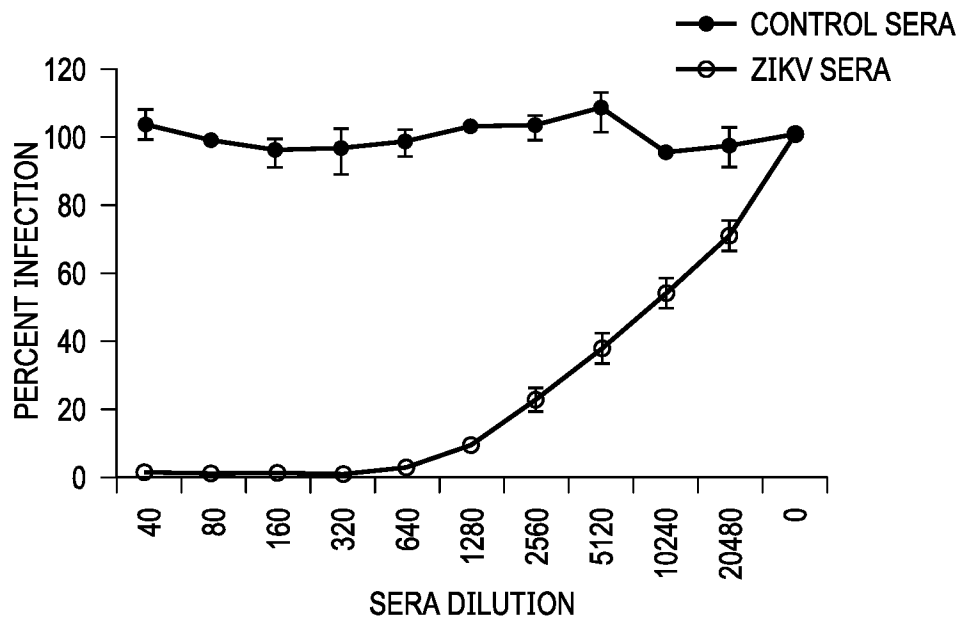


FIG. 8C

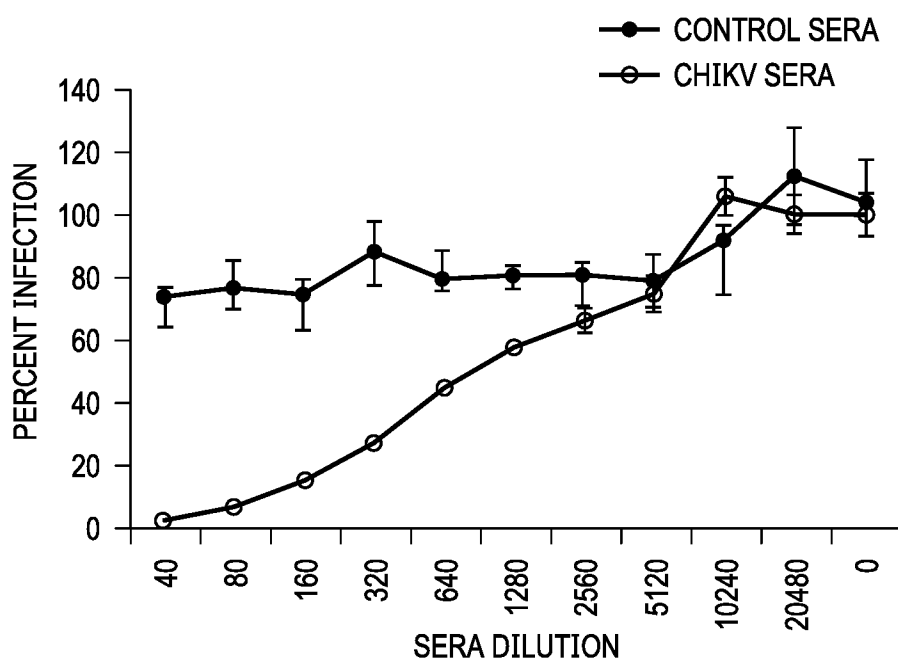


FIG. 8D

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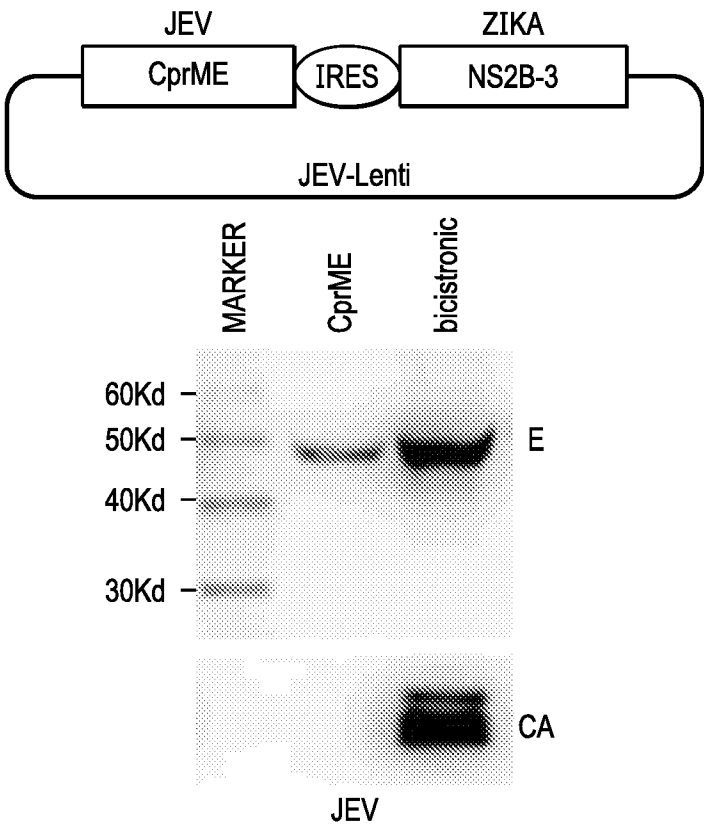


FIG. 9A

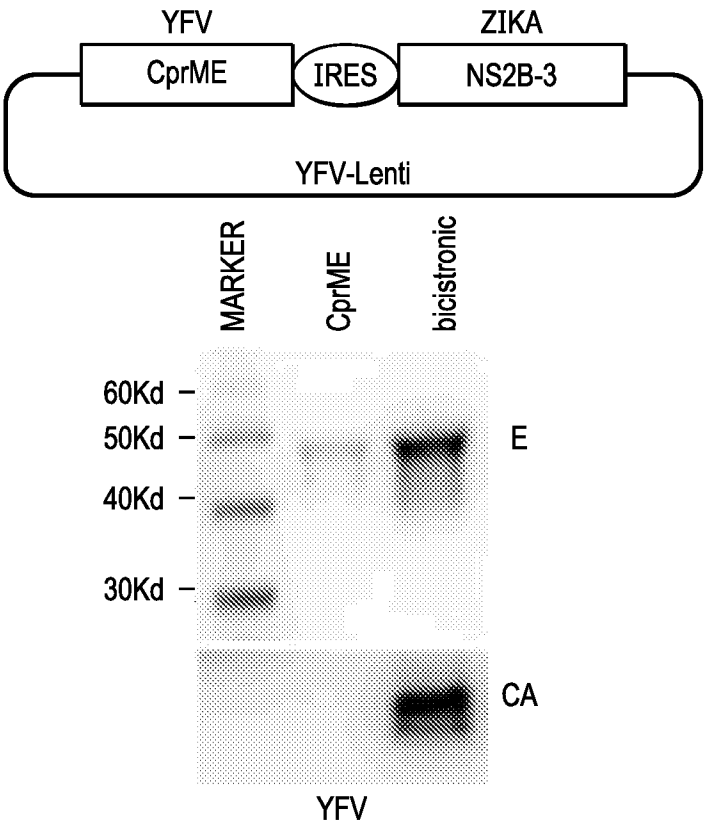


FIG. 9B

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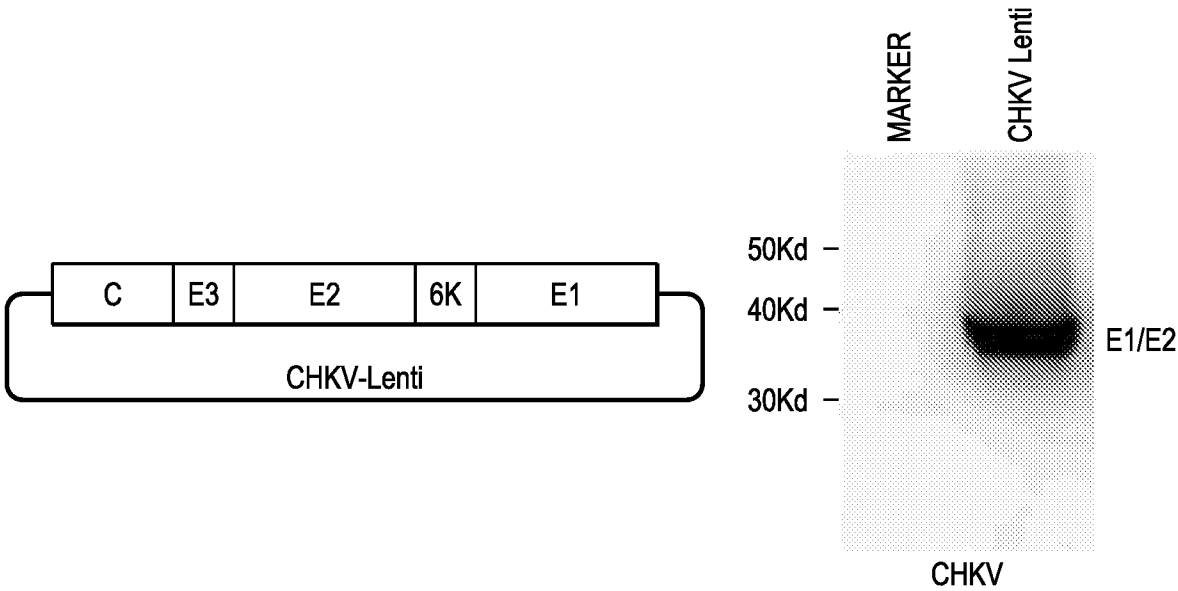
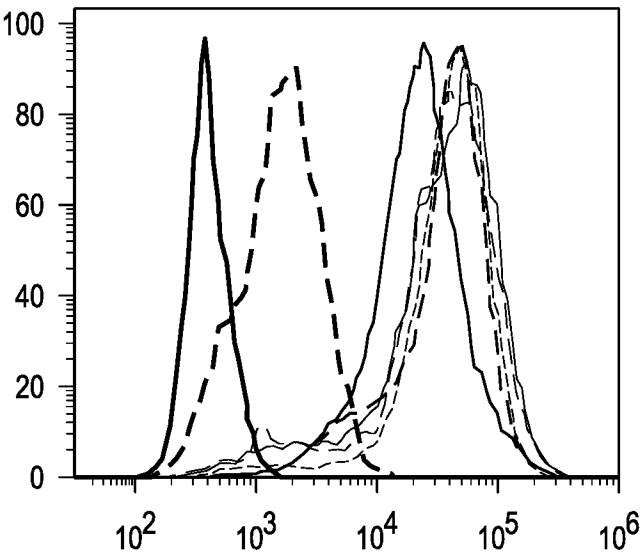
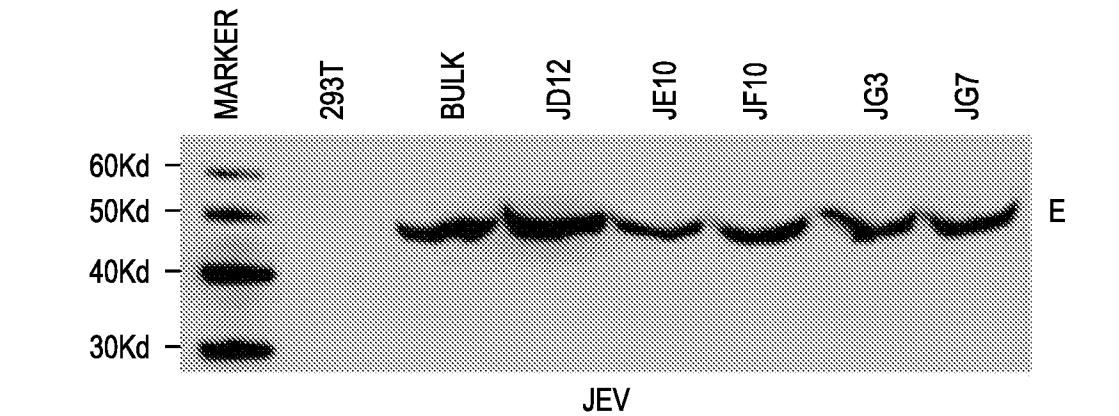
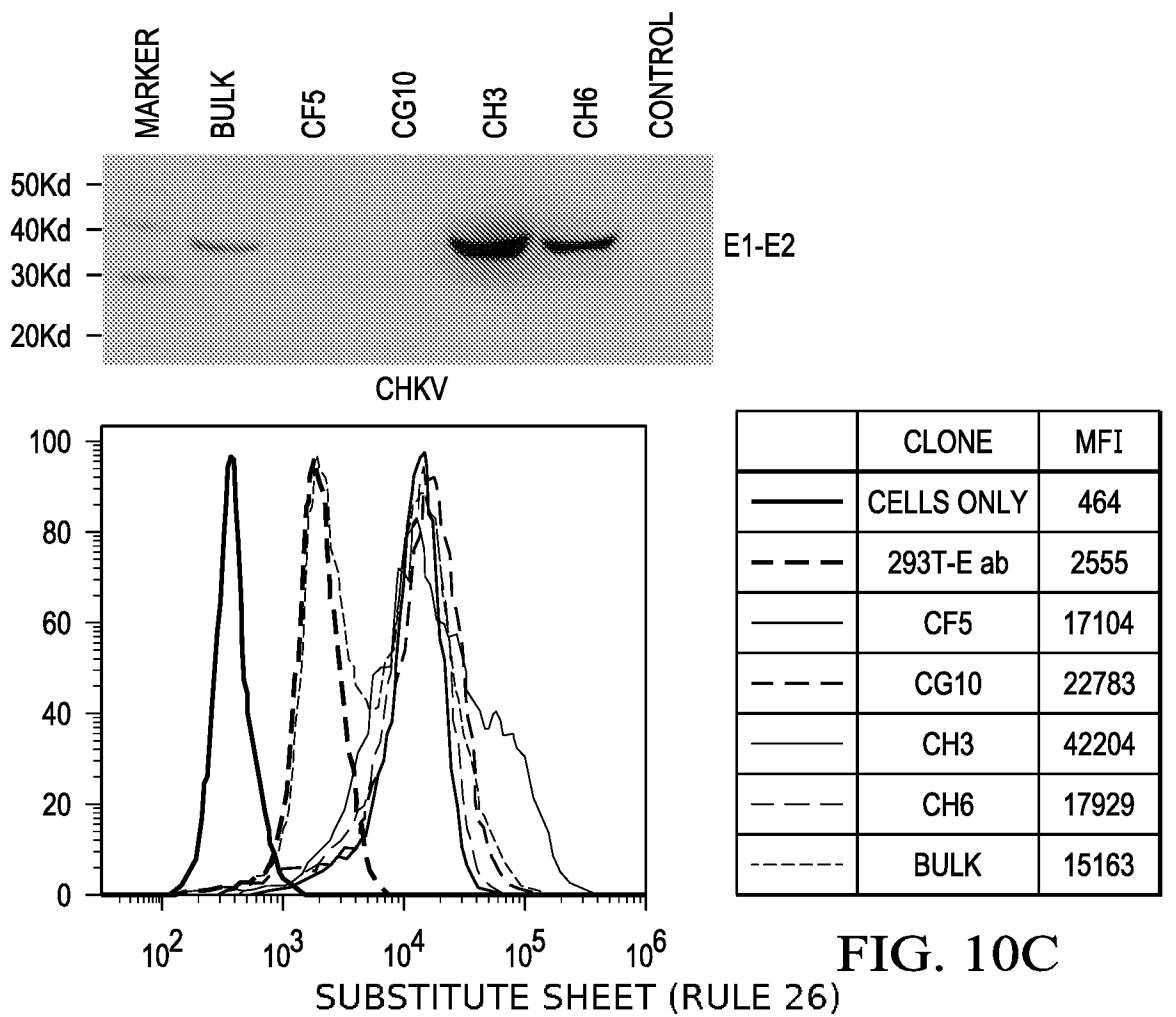
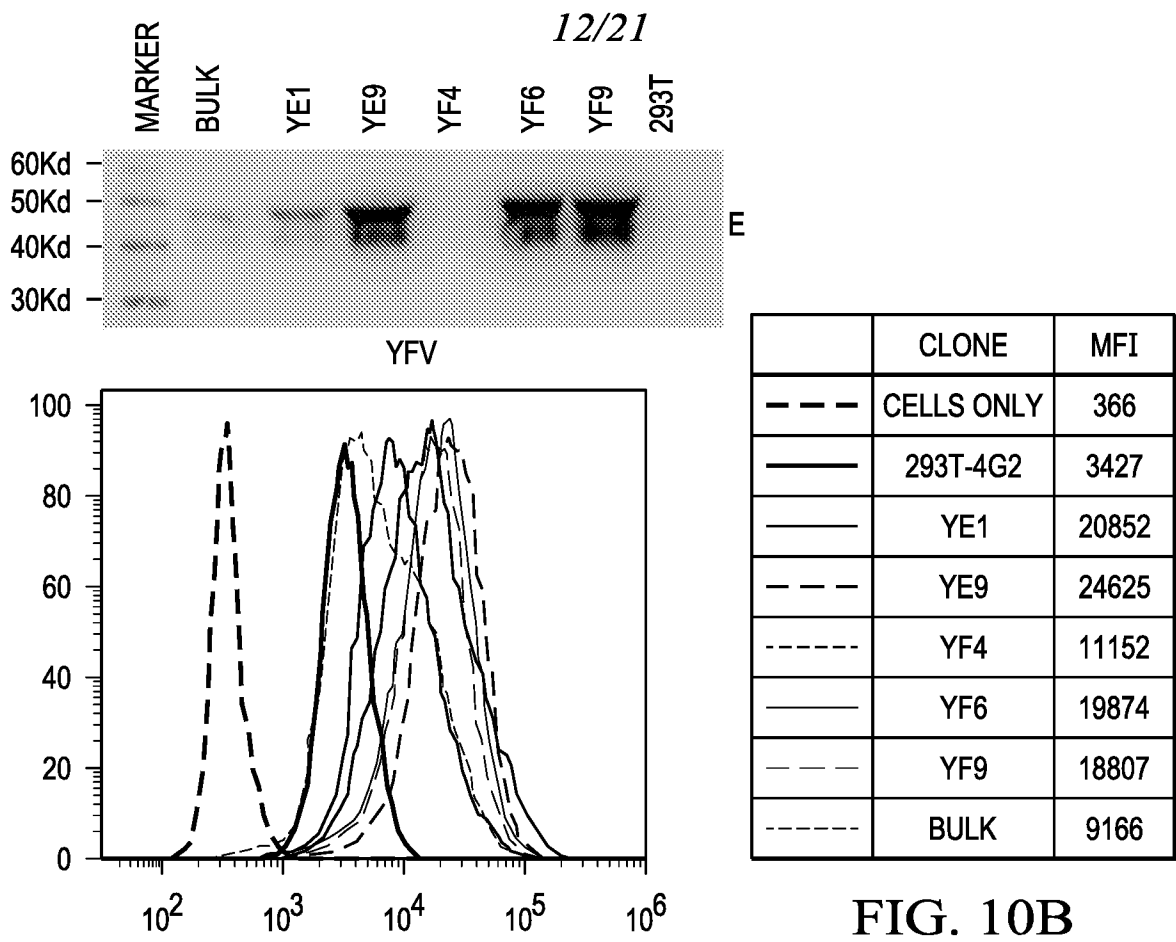


FIG. 9C



	CLONE	MFI
—	CELLS ONLY	453
- - -	293T-4G2	2676
—	JD12	33851
- - -	JF10	50024
—	JE10	57075
- - -	JG7	54074
- - -	JG3	53597

FIG. 10A



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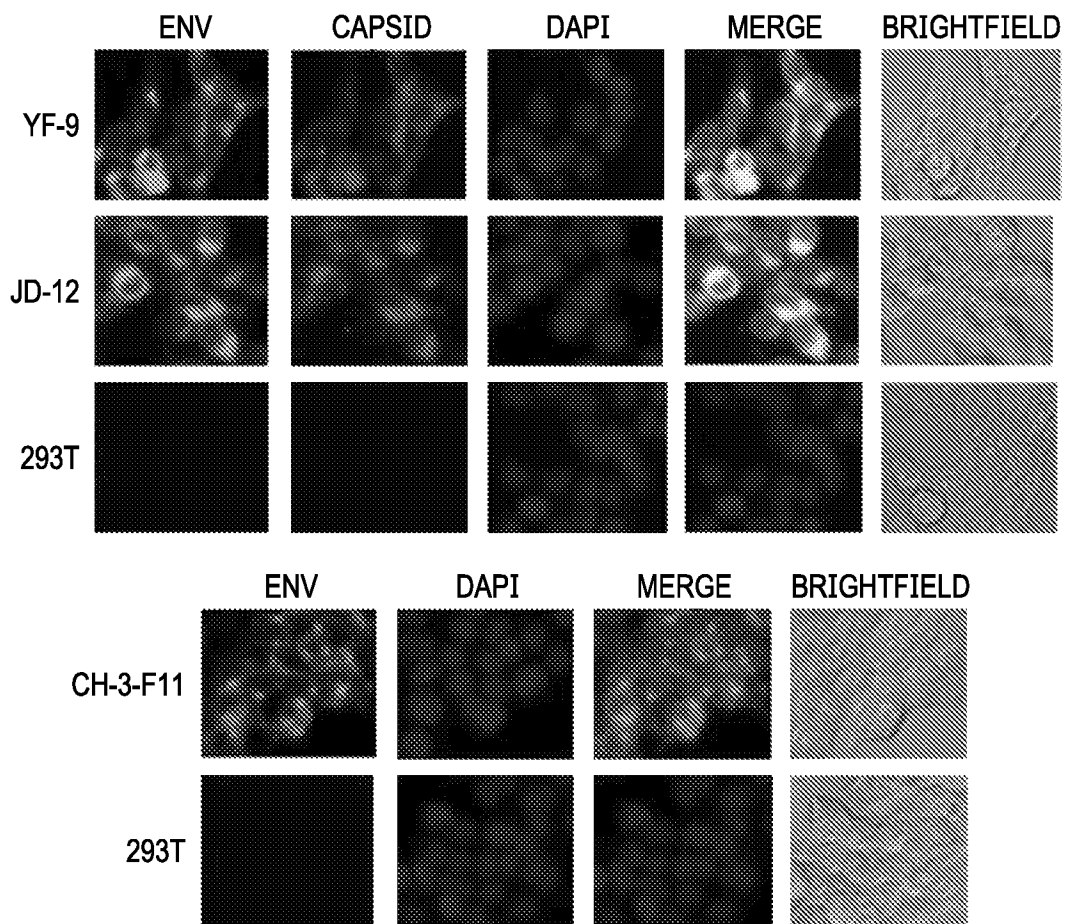


FIG. 11A

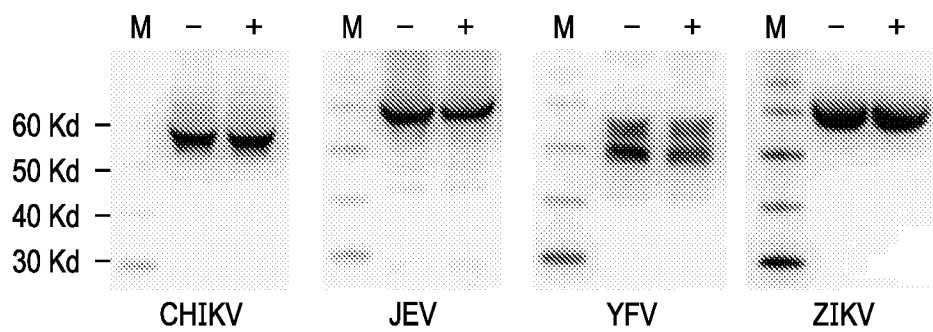


FIG. 11B

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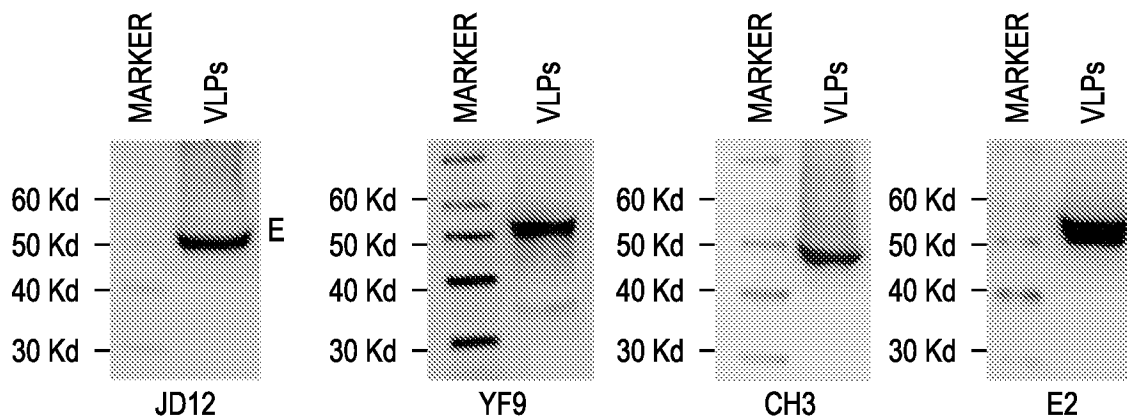
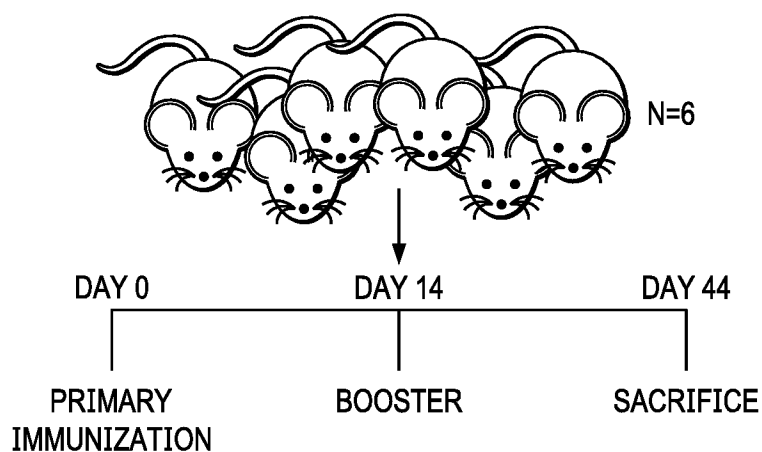


FIG. 12A



GROUP	INJECTION	ROUTE	VOLUME	AMOUNT
1	ZIKA	i.m.	100μl	1:1 MIX WITH ALUM
2	JEV	i.m.	100μl	1:1 MIX WITH ALUM
3	YFV	i.m.	100μl	1:1 MIX WITH ALUM
4	CHKV	i.m.	100μl	1:1 MIX WITH ALUM
5	JEV+CHKV	i.m.	100μl	1:1 MIX WITH ALUM
6	YFV+CHKV	i.m.	100μl	1:1 MIX WITH ALUM
7	ZIKV+YFV+CHKV+JEV	i.m.	100μl	1:1 MIX WITH ALUM
8	PBS	i.m.	100μl	1:1 MIX WITH ALUM

FIG. 12B

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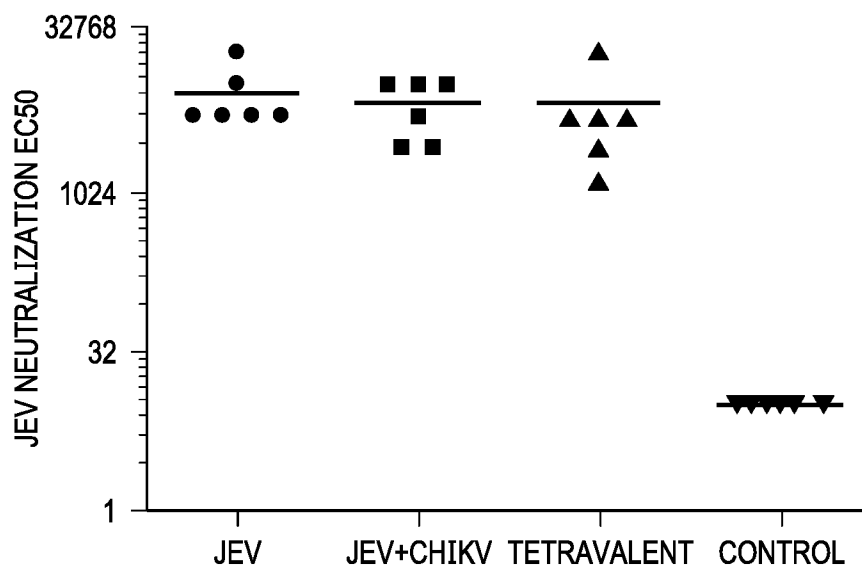


FIG. 13A

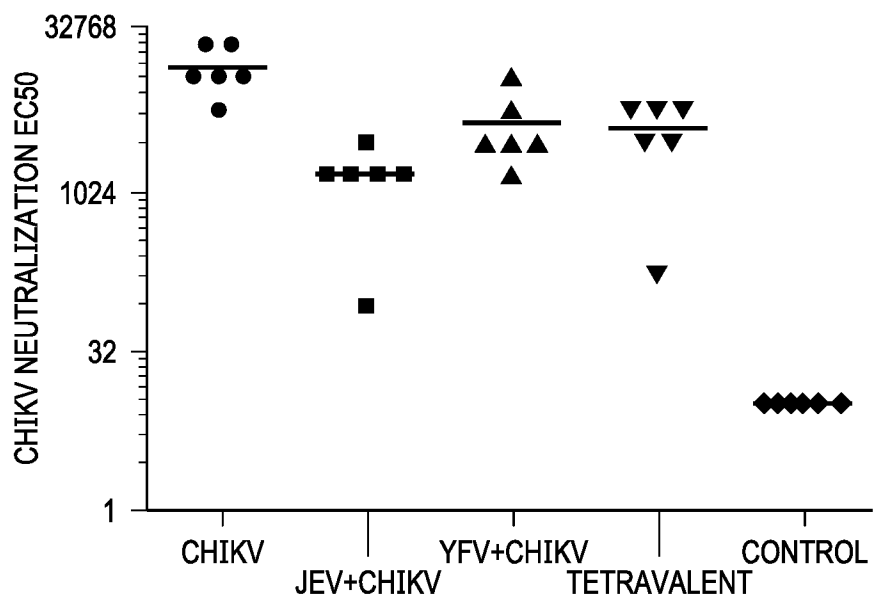


FIG. 13B

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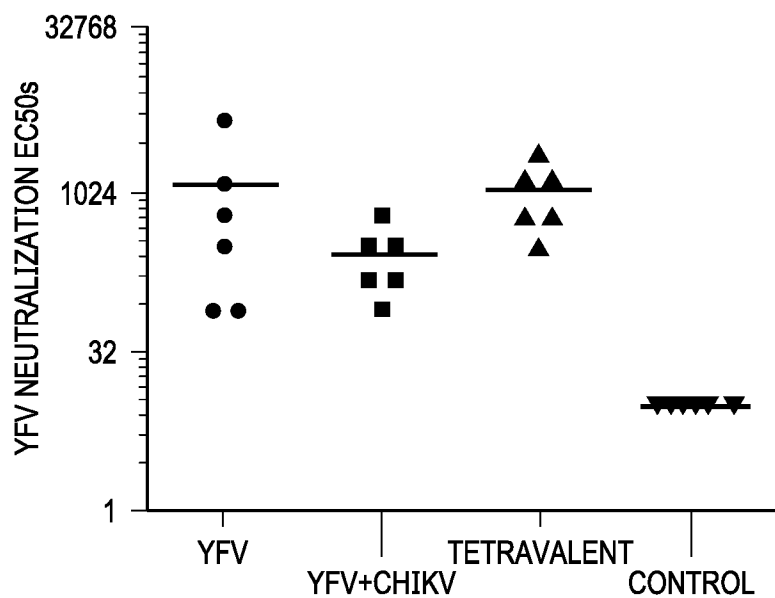


FIG. 13C

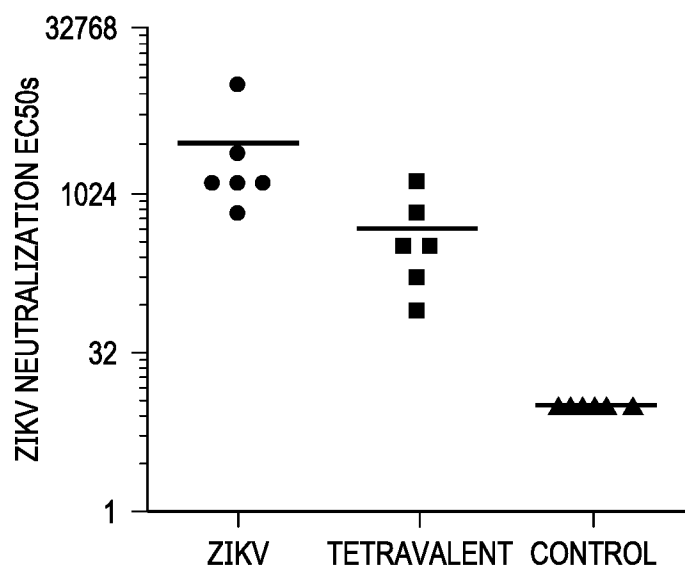


FIG. 13D

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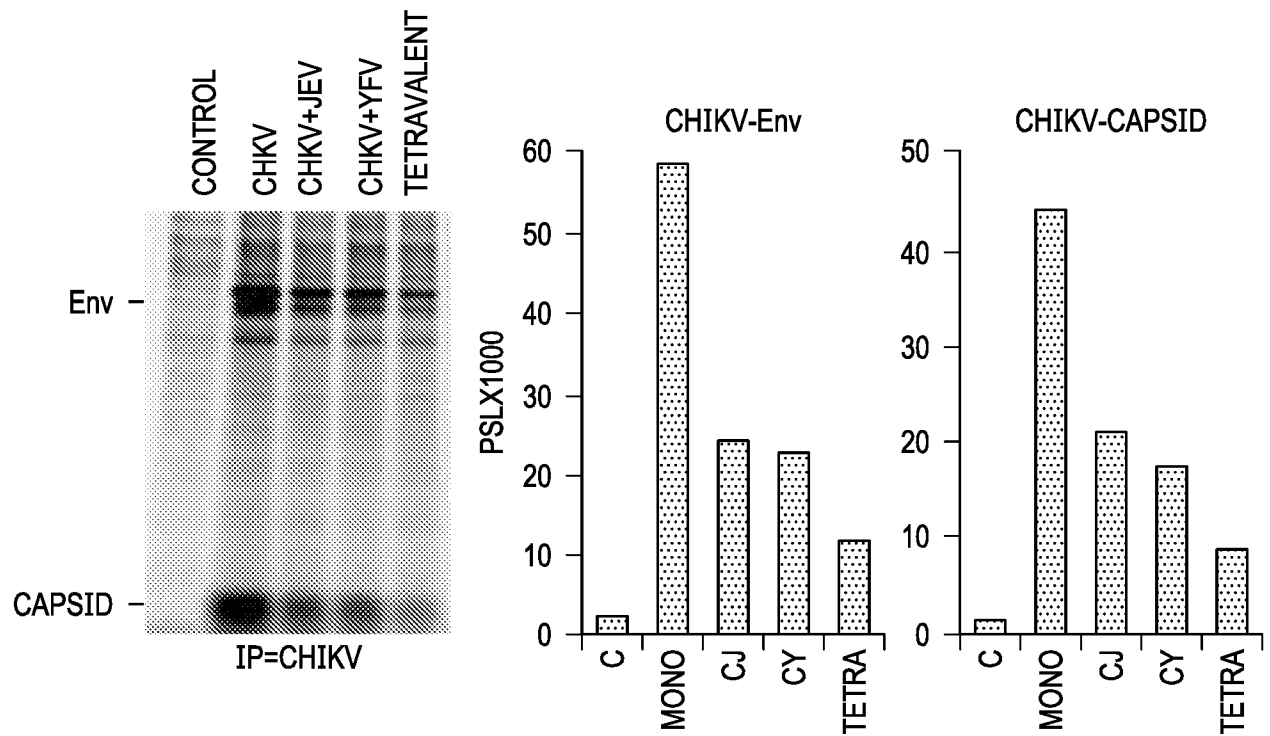


FIG. 14A

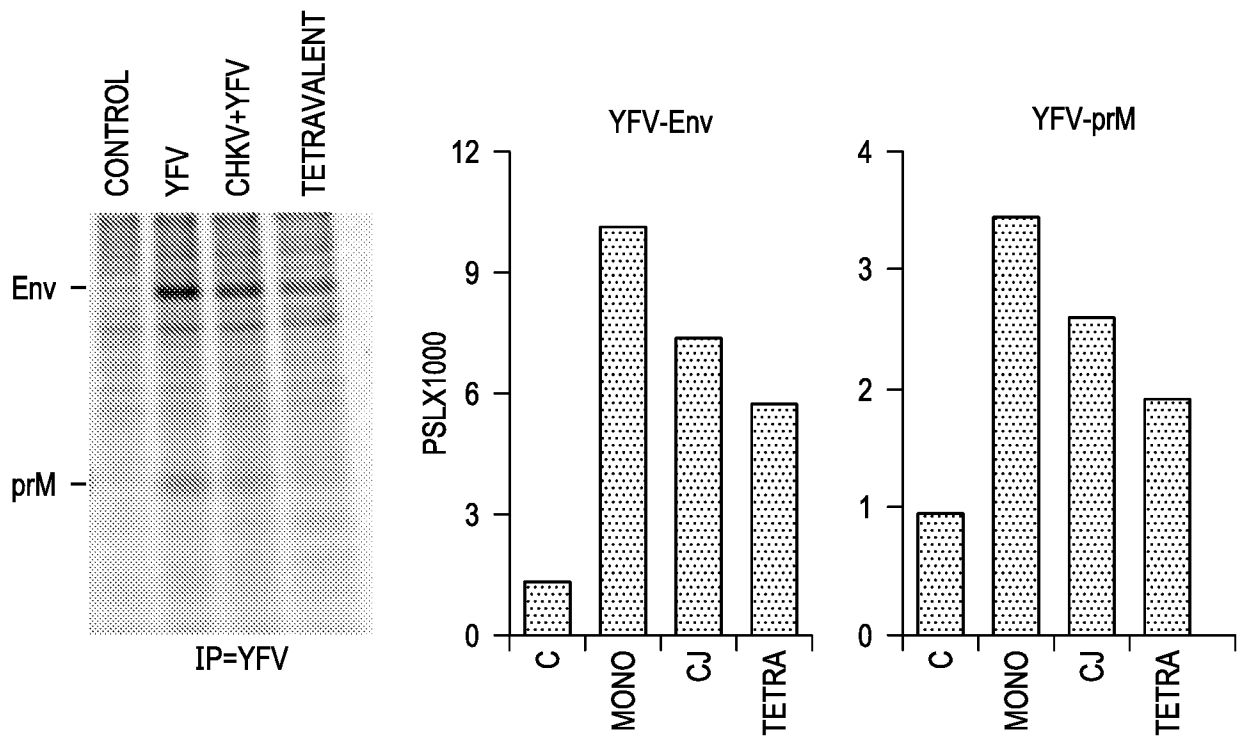


FIG. 14B

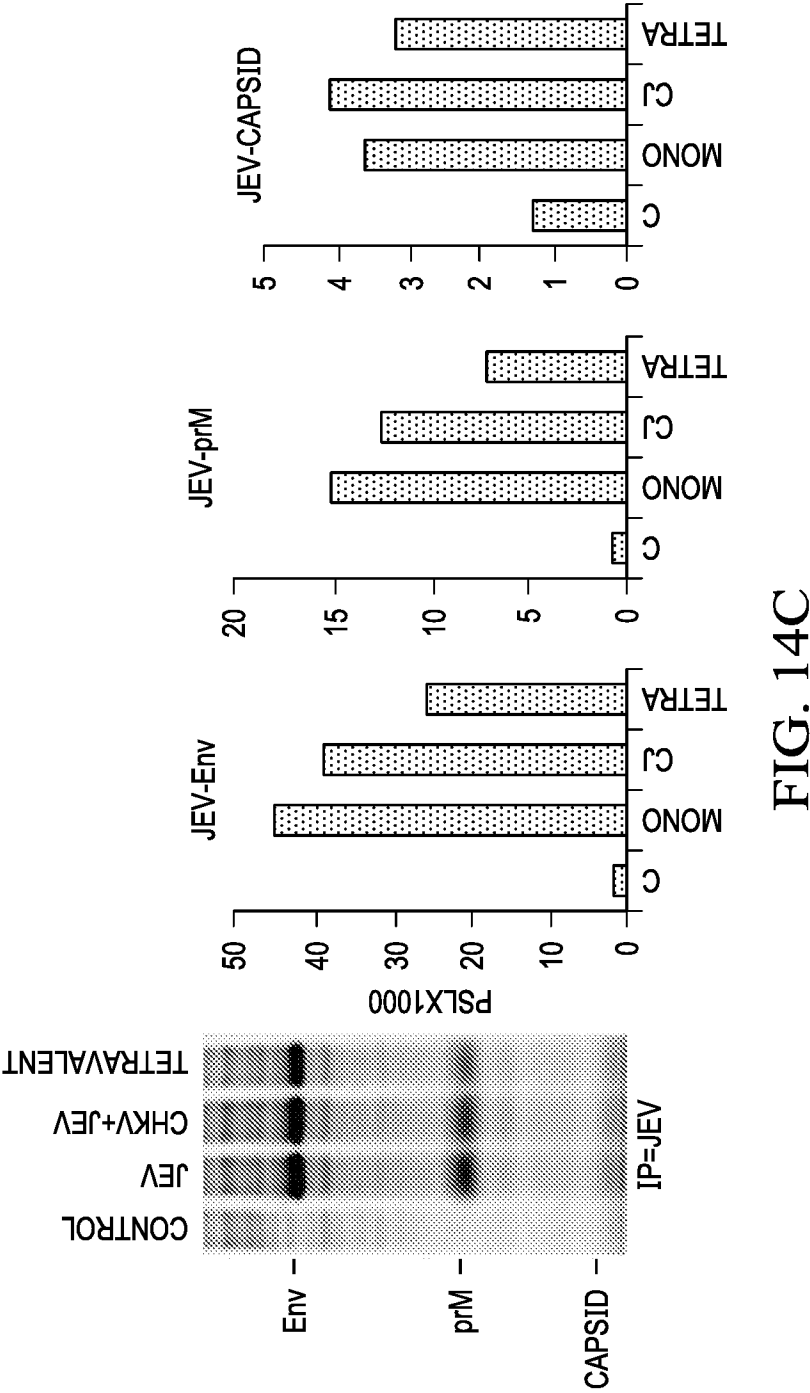


FIG. 14C

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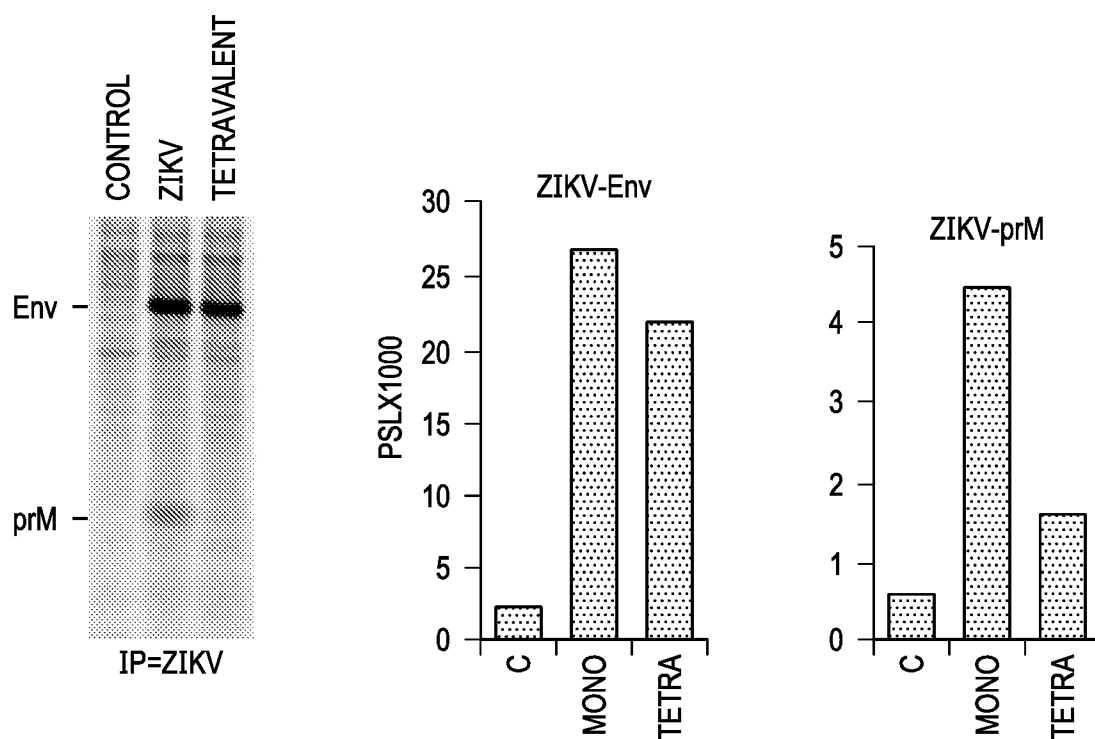


FIG. 14D

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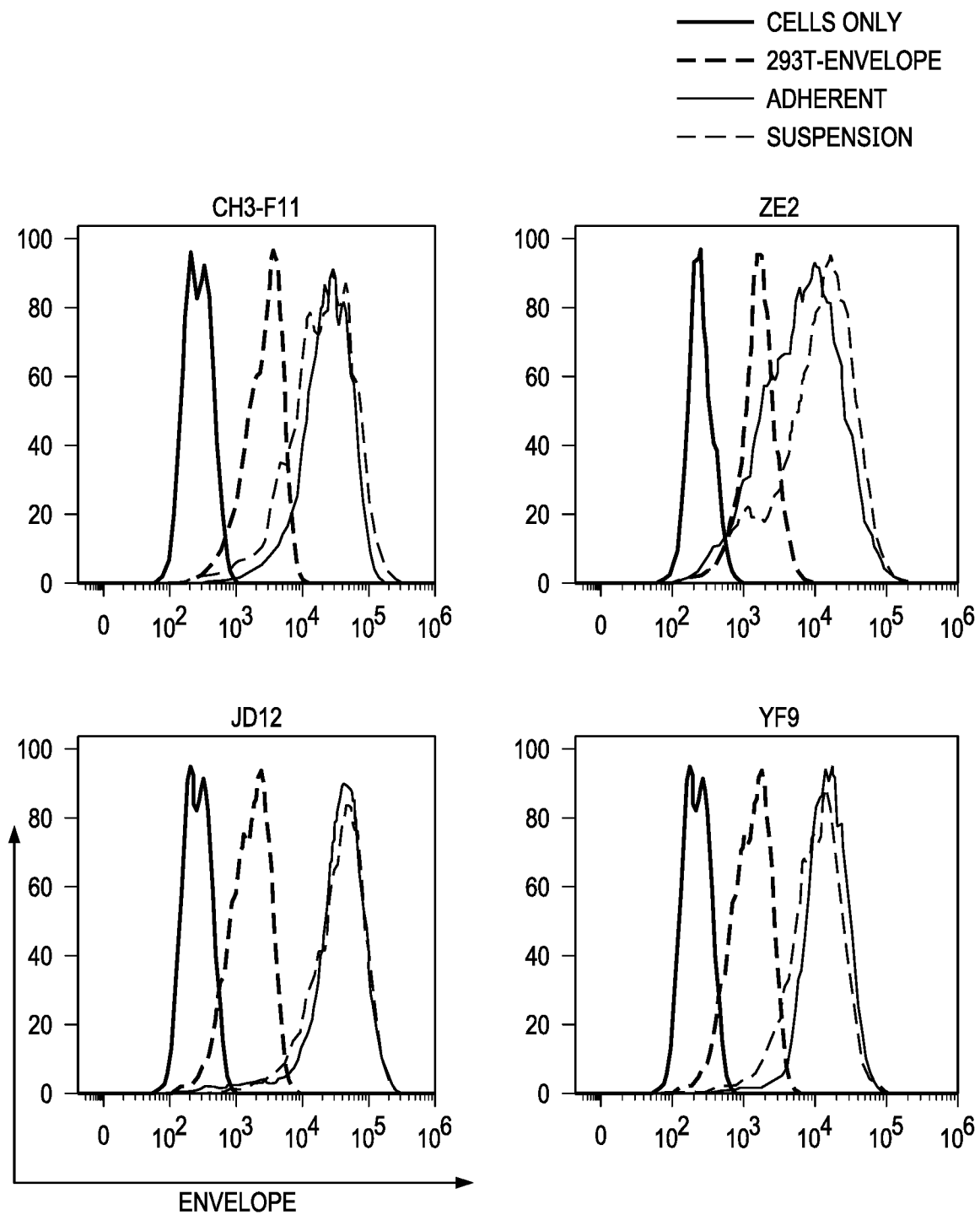


FIG. 15A

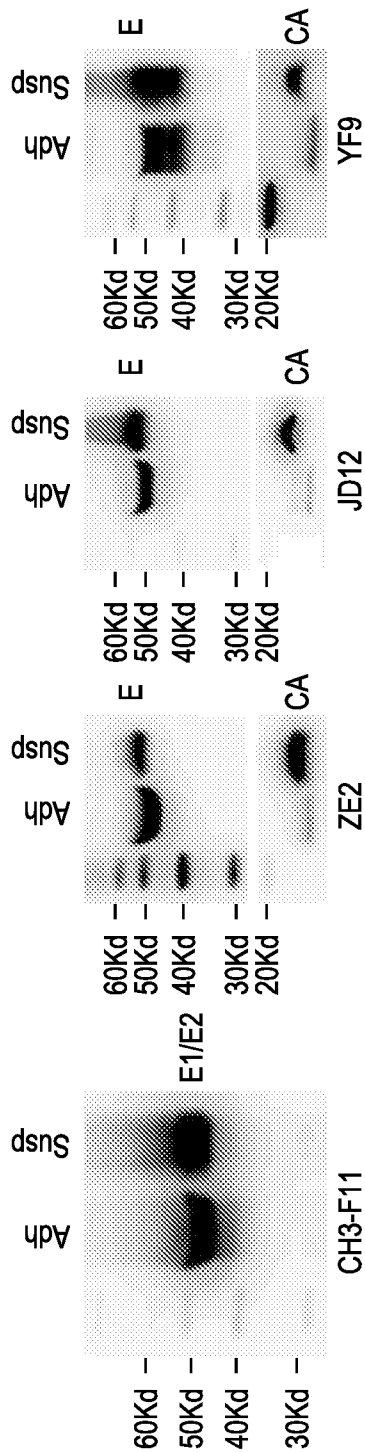


FIG. 15B

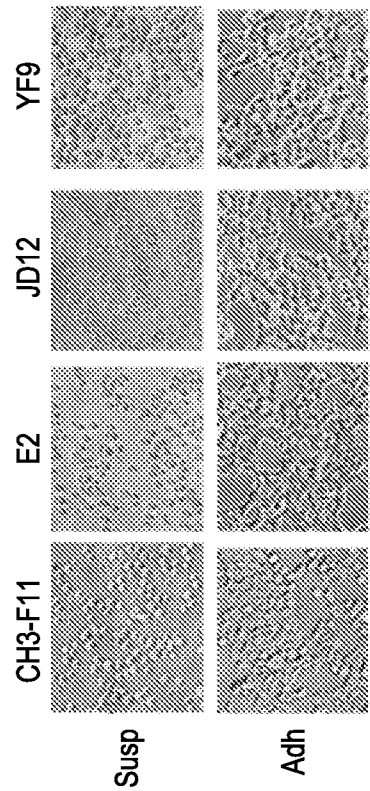


FIG. 15C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/056645

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/12; C07K 14/005; C07K 14/18; C07K 19/00; C12N 5/10; C12N 7/00 (2019.01)
 CPC - C07K 14/005; C12N 15/86; C12N 2770/24111; C12N 2770/24134 (2019.08)

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)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/202.1; 424/218.1; 435/325; 435/235.1; 536/23.72; 536/23.1 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/152526 A1 (THE USA, AS REPRESENTED BY THE SECRETARY, DEPT. OF HEALTH AND HUMAN SERVICES et al) 23 August 2018 (23.08.2018) entire document	1, 2, 4, 6-9, 28, 29, 32
--		10
Y		
X	US 2018/0177859 A1 (TECHNOVAX, INC.) 28 June 2018 (28.06.2018) entire document	22-27, 40-50
--		11, 12, 15-21
Y		
Y	US 2006/0200869 A1 (NALDINI et al) 07 September 2006 (07.09.2006) entire document	10, 17, 51
Y	DE WISPELAERE et al. "A Lentiviral Vector Expressing Japanese Encephalitis Virus-like Particles Elicits Broad Neutralizing Antibody Response in Pigs," PLoS Negl Trop Dis, 05 October 2015 (05.10.2015), Vol. 9, e0004081, Pgs. 1-20. entire document	11, 12, 15-21
Y	ZANOTTO et al. "The Challenges Imposed by Dengue, Zika, and Chikungunya to Brazil," Front Immunol, 28 August 2018 (28.08.2018), Vol. 9, Pgs. 1-6. entire document	19
Y	BOIGARD et al. "Zika virus-like particle (VLP) based vaccine," PLoS Negl Trop Dis, 08 May 2017 (08.05.2017), Vol. 11, e0005608, Pgs. 1-20. entire document	51
P, X	GARG et al. "Capsid containing virus like particle vaccine against Zika virus made from a stable cell line," Vaccine, 10 October 2019 (10.10.2019), Vol. 37, Pgs. 7123-7131. entire document	1-51
A	GARG et al. "Development of Virus-Like-Particle Vaccine and Reporter Assay for Zika Virus," J Virol, 27 September 2017 (27.09.2017), Vol. 91, e00834-17, Pgs. 1-16. entire document	1-51



Further documents are listed in the continuation of Box C.



See patent family annex.

- * Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

20 December 2019

Date of mailing of the international search report

16 JAN 2020

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PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/056645

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OOI et al. "A Guide to Transient Expression of Membrane Proteins in HEK-293 Cells for Functional Characterization," Front Physiol, 19 July 2016 (19.07.2016), Vol. 7, Pgs. 1-15. entire document	1-51
A	"FreeStyle™ 293-F Cells: User Guide," ThermoFisher, 07 June 2017 (27.06.2017), Pgs. 1-2. Retrieved from the Internet: < https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0007834-FreeStyle293-F-Cell-UG.pdf > on 20 December 2019 (20.12.2019). entire document	1-51

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/056645

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

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

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments:

SEQ ID NOs: 1, 2, and 5-8 were searched.