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Shi et al.

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(54) **REVERSE GENETICS SYSTEM OF ZIKA VIRUS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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PCT Pub. Date: **Nov. 9, 2017**

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(51) **Int. Cl.**

A61K 39/00	(2006.01)
A61K 39/12	(2006.01)
C12N 7/00	(2006.01)
A61K 45/06	(2006.01)
C07H 19/20	(2006.01)
G01N 33/569	(2006.01)
C12Q 1/70	(2006.01)
C12Q 1/6897	(2018.01)

(52) **U.S. Cl.**

CPC ***G01N 33/56983*** (2013.01); ***C12Q 1/6897*** (2013.01); ***C12Q 1/70*** (2013.01); *G01N 2333/185* (2013.01)

(58) **Field of Classification Search**

CPC A61K 2300/00; A61K 39/12; A61K 31/7072; A61K 31/7068; C12N 7/00

See application file for complete search history.

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(Continued)

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(74) *Attorney, Agent, or Firm* — Norton Rose Fulbright US LLP

(57) **ABSTRACT**

Embodiments of the invention are directed to stable full-length cDNA clones of a clinical, Asian lineage ZIKV strain. Certain embodiments of the invention are directed to high-throughput assays for ZIKV and dengue virus (DENV) diagnosis.

19 Claims, 11 Drawing Sheets

Specification includes a Sequence Listing.

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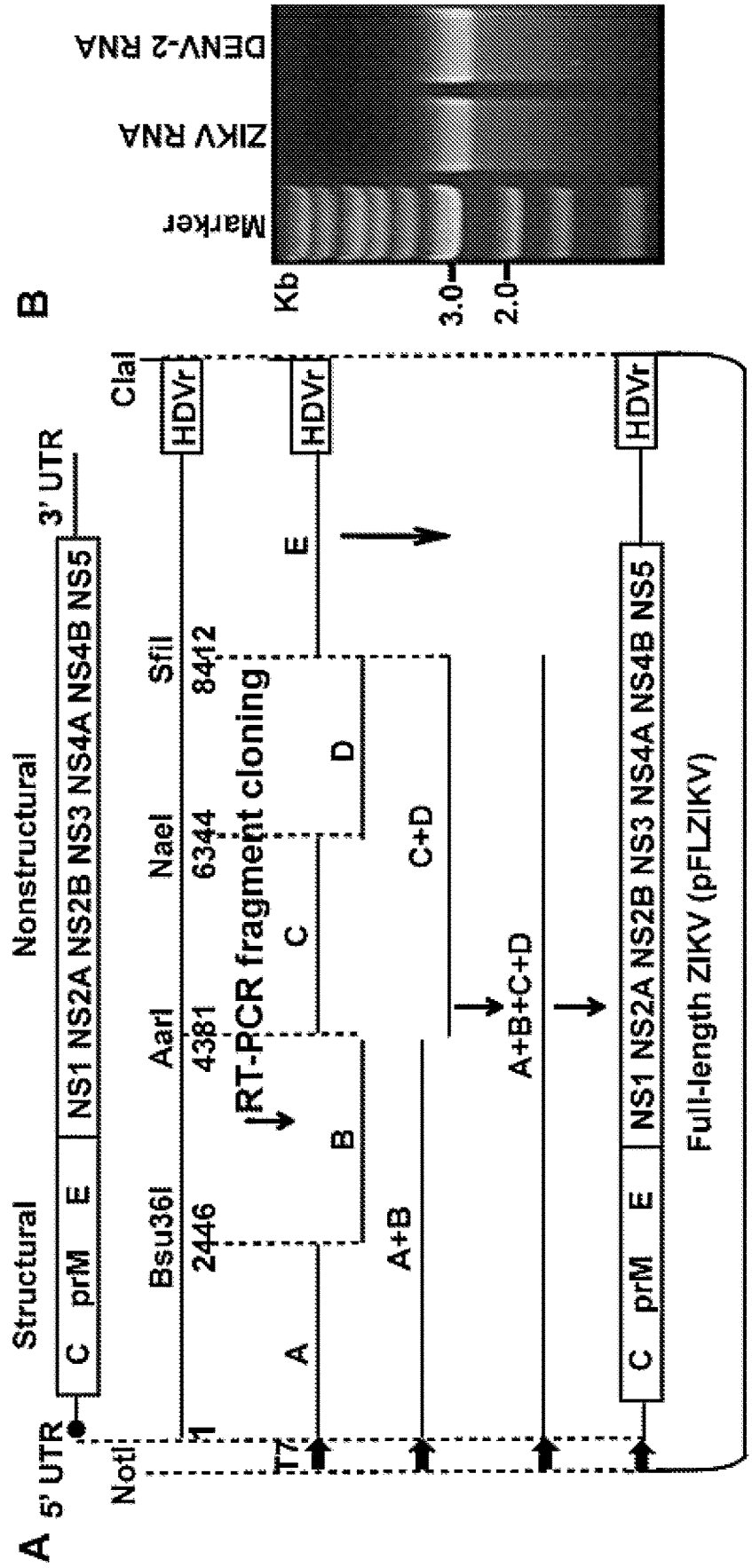
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NotI
1
T7
A
2446
Bsu36I
Structural
C prM E
Nonstructural
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3' UTR
HDVr
ClaI
B
AarI
4381
C
NaeI
6344
D
SfiI
8412
E
RT-PCR fragment cloning
A+B
C+D
A+B+C+D
Full-length ZIKV (pFLZIKV)
B
Kb
3.0
2.0
Marker
ZIKV RNA
DENV-2 RNA

FIG. 1

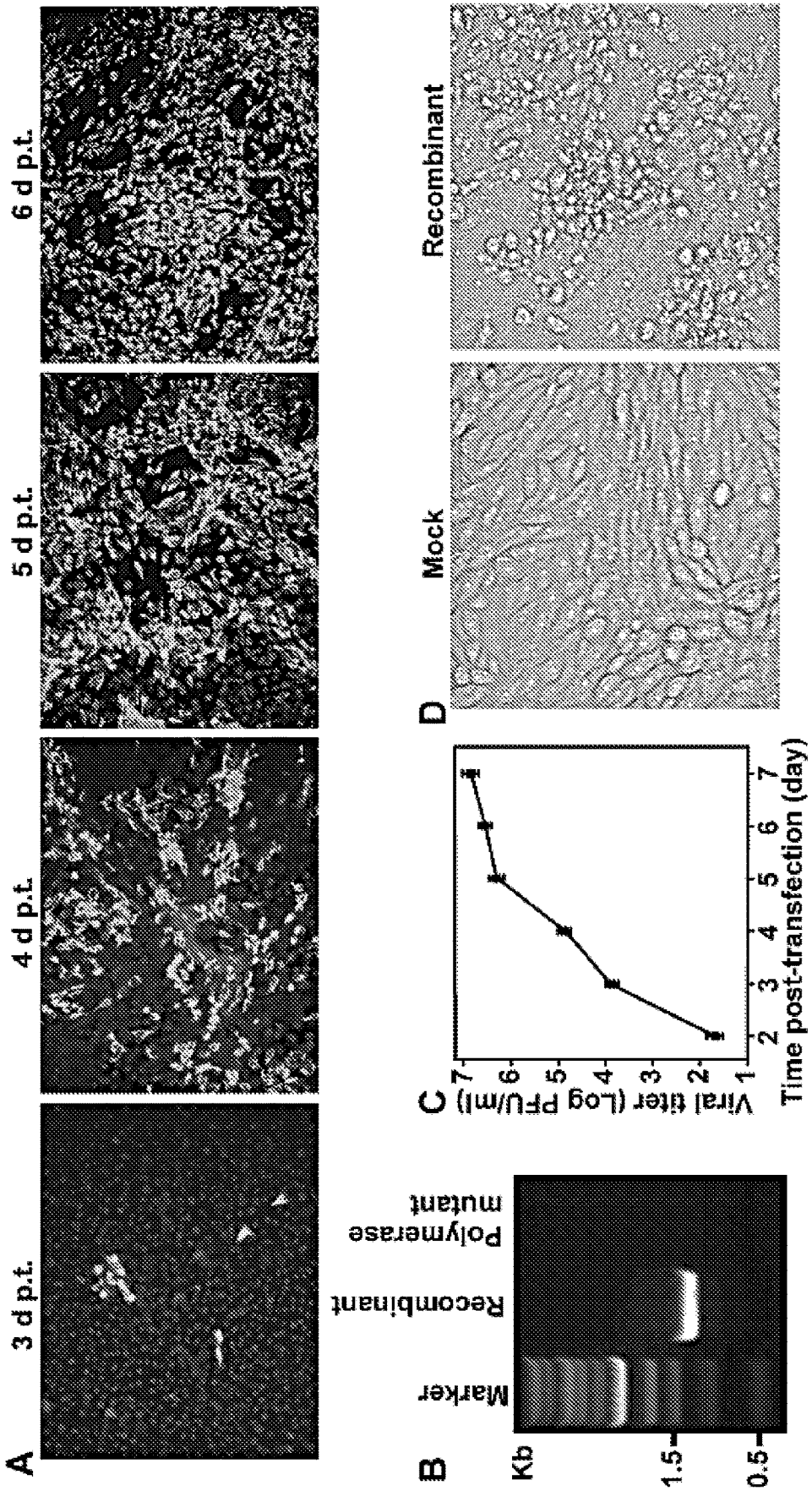
A
3 d p.t.
4 d p.t.
5 d p.t.
6 d p.t.
B
Kb
1.5
0.5
Marker
Recombinant
Polymerase mutant
C
Viral titer (Log PFU/ml)
Time post-transfection (day)
D
Mock
Recombinant

FIG. 2

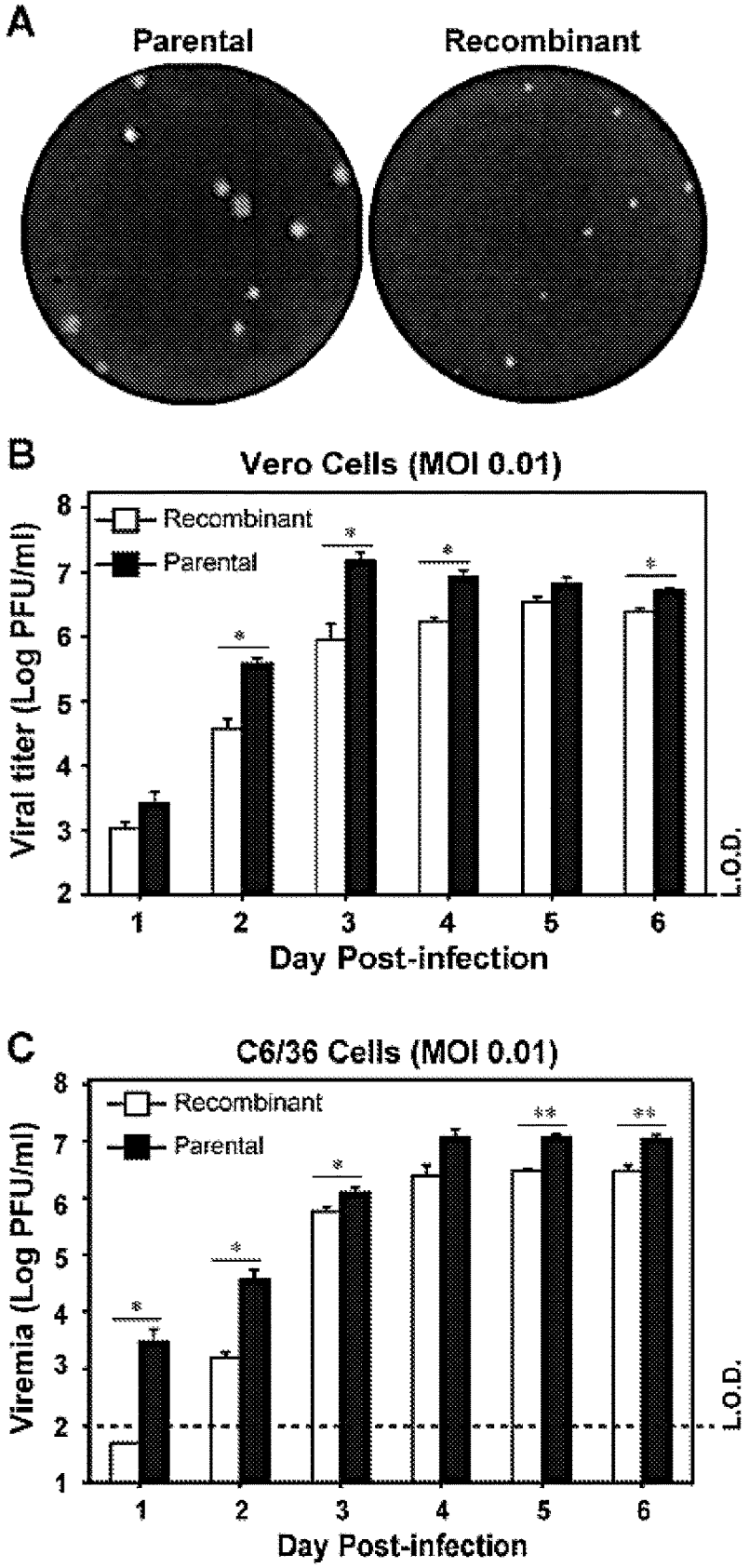
A
Parental
Recombinant
B
Vero Cells (MOI 0.01)
Recombinant
Parental
Viral titer (Log PFU/ml)
L.O.D.
Day Post-infection
C
C6/36 Cells (MOI 0.01)
Recombinant
Parental
Viremia (Log PFU/ml)
L.O.D.
Day Post-infection

FIG. 3

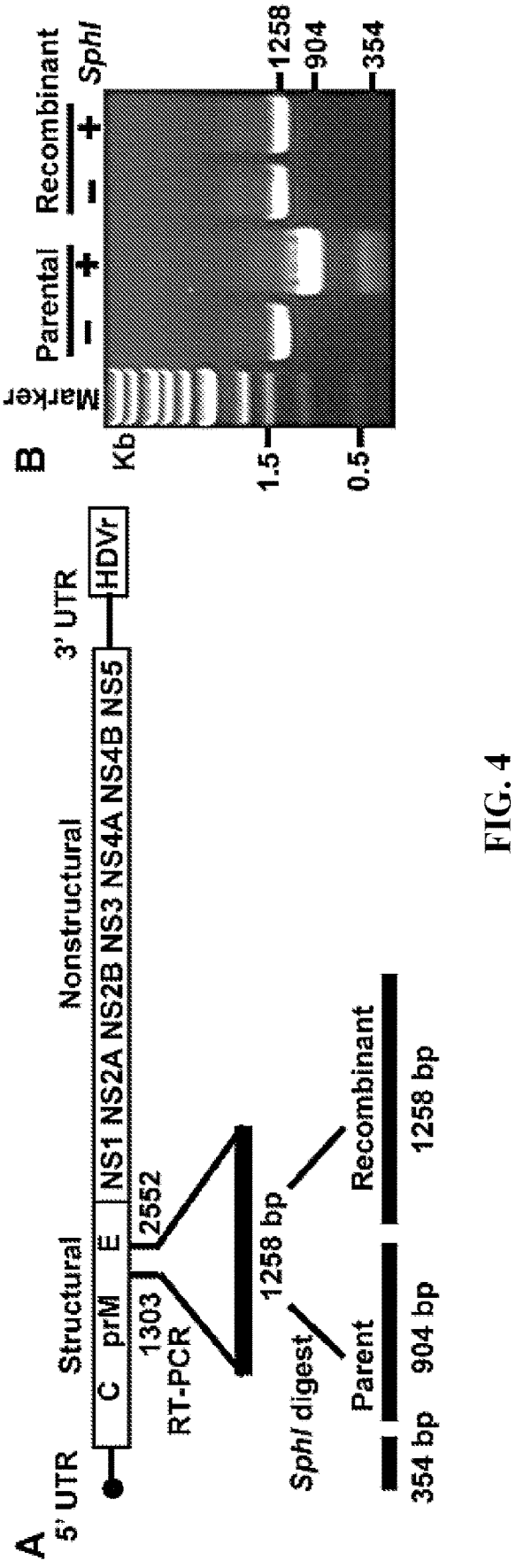
A
5' UTR
Structural
C
prM
E
Nonstructural
NS1 NS2A NS2B NS3 NS4A NS4B NS5
3' UTR
HDVr
1303
2552
RT-PCR
1258 bp
SphI digest
Parent
Recombinant
354 bp
904 bp
1258 bp
B
Marker
Parental
Recombinant
SphI
Kb
1.5
0.5
1258
904
354

FIG. 4

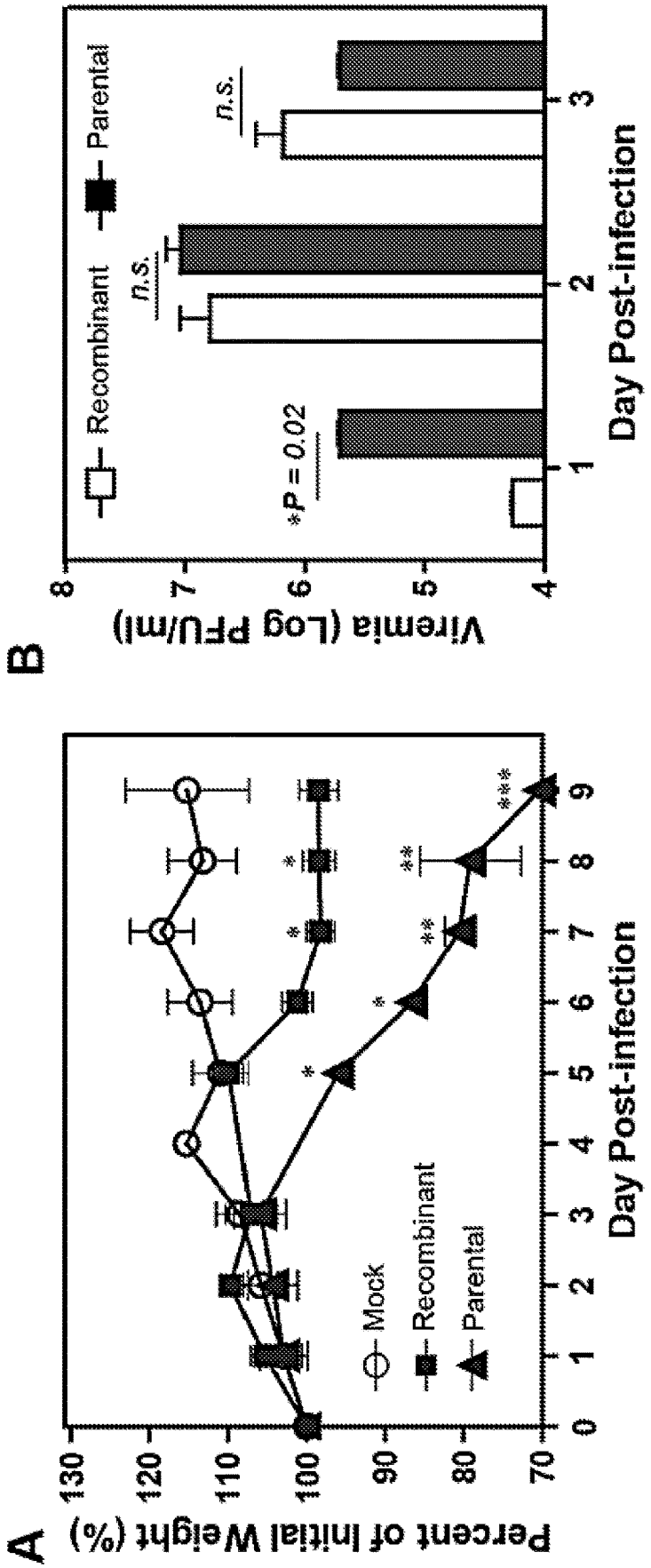
A
Percent of Initial Weight (%)
130
120
110
100
90
80
70
0 1 2 3 4 5 6 7 8 9
Day Post-infection
Mock
Recombinant
Parental
*
**
***
B
Viremia (Log PFU/ml)
8
7
6
5
4
Recombinant
Parental
n.s.
n.s.
*P = 0.02
1 2 3
Day Post-infection

FIG. 5

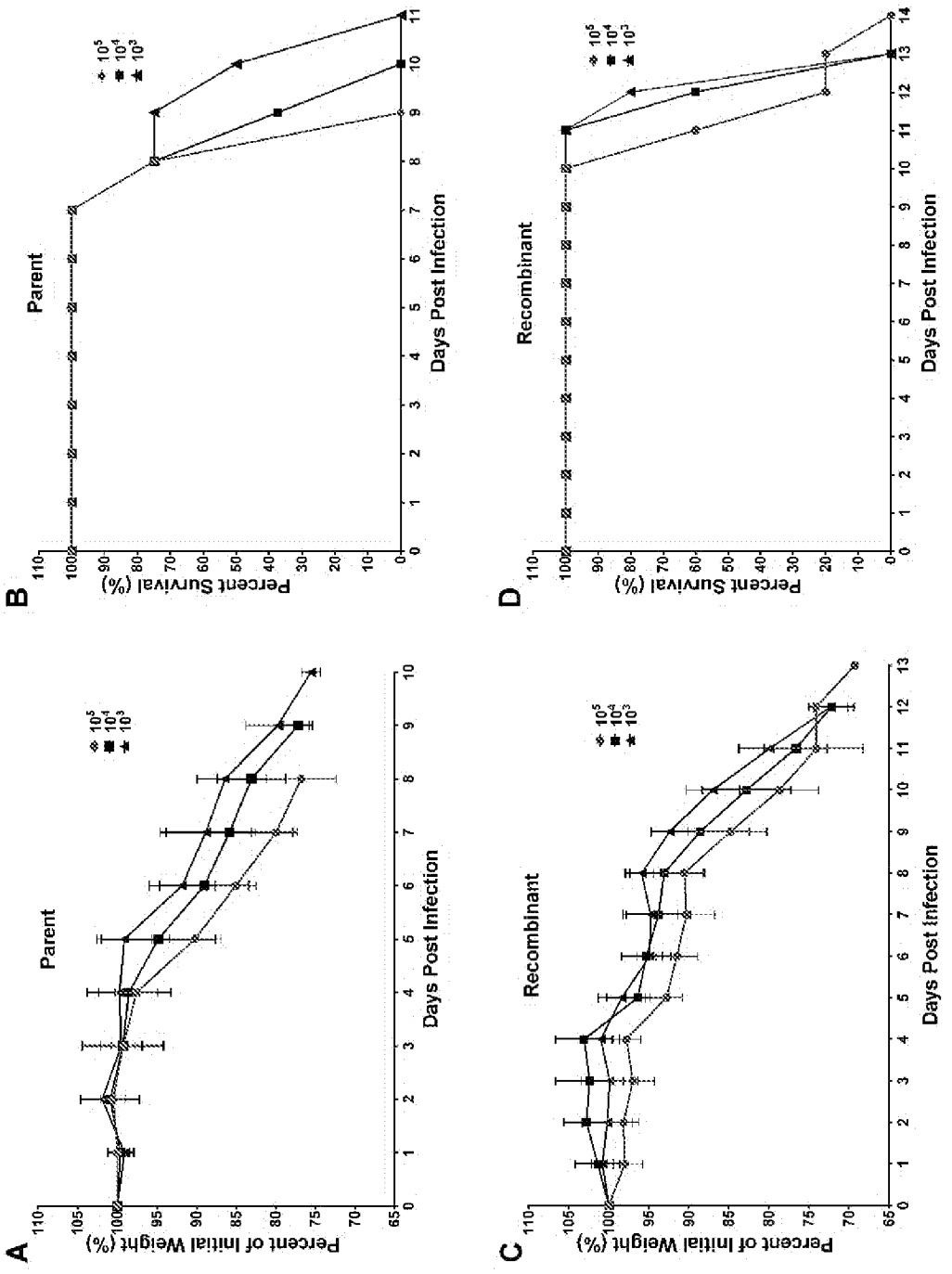
A
Parent
Percent of Initial Weight (%)
Days Post Infection
B
Parent
Percent Survival (%)
Days Post Infection
C
Recombinant
Percent of Initial Weight (%)
Days Post Infection
D
Recombinant
Percent Survival (%)
Days Post Infection

FIG. 6

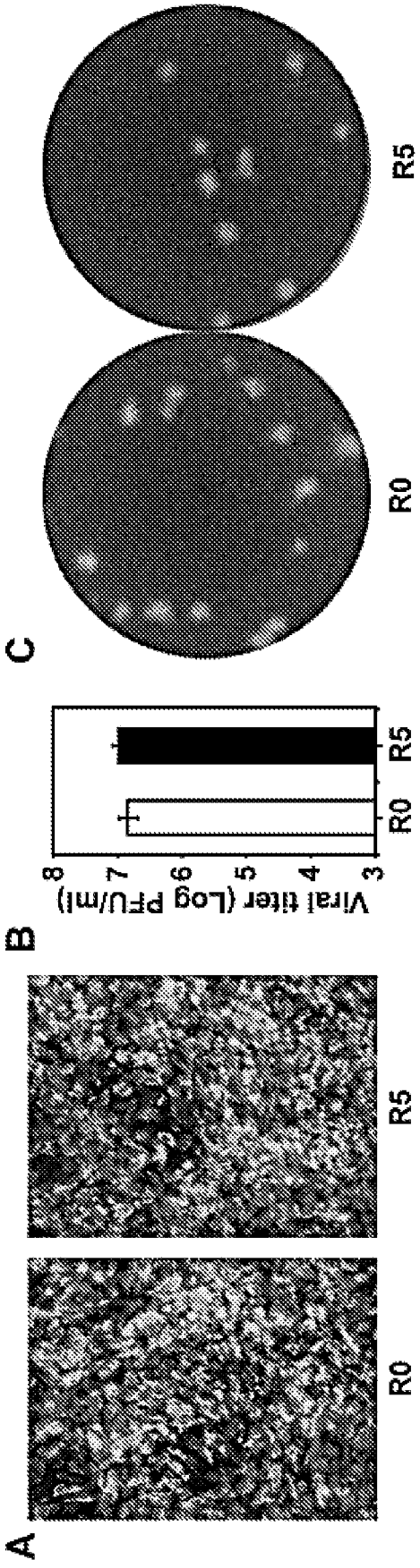

FIG. 7

MKNPKKKSGGFRIVNMLKRGVARVSPFGGLKRLPAGLLLGHGPIRMVLAILAFLRFTAIKPSLGLINRW
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KSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLMCLALGGVLIFLSTAVSADVGCSVDFSKKETRCGTG
VFVYNDVEAWRDRYKYHPDSPRRLAAAVKQAWEDGICGISSVSRMENIMWRSVEGELNAILEENGVQ
LTVVVGSVKNPMWRGPQRLPVPVNELPHGWKAWGKSYFVRAAKTNNSFVVDGDTLKECPLKHRAW
NSFLVEDHGFGVFHTSVWLKVREDYSLECDPAVIGTAAKGKEAVHSDLGYWIESEKNDTWRLKRAHL
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EETCGTRGPSLRSTTASGRVIEEWCCRECTMPPLSFRAKDGCWYGMEIRPRKEPESNLVRSMVTAGST
DHMDHFSLGVLVILLMVQEGLKKRMTTKIIISTSMAVLVAMILGGFSMSDLAKLAILMGATFAEMNTG
GDVAHLALIAAFKVRPALLVSFIFRANWTPRESMLLALASCLLQTAISALEGDLMVPINGFALAWLAIR
AMVVPRTDNITLAILAALTPLARGTLLVAWRAGLATCGGFMLLSLKGKGSVKKNLPFVMALGLTAVR
LVDPINVVGLLLLTRSGKRSWPPSEVLTAVGLICALAGGFAKADIEMAGPMAAVGLLIVSYVVSGKSV
DMYIERAGDITWEKDAEVTGNSPRLDVALDESGDFSLVEDDGPPMREIILKVVLMAICGMNPIAIPFAA
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AVSRGSAKLRWLVERGYLQPYGKVIDLGCGRGGWSYYAATIRKVQEVKGYTKGGPGHEEPMLVQSY
GWNIVRLKSGVDVFHMAAEPCDTLLCDIGESSSSPEVEEARTLRVLSMVGDWLEKRPGAFCIKVLCPY
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FIG. 8

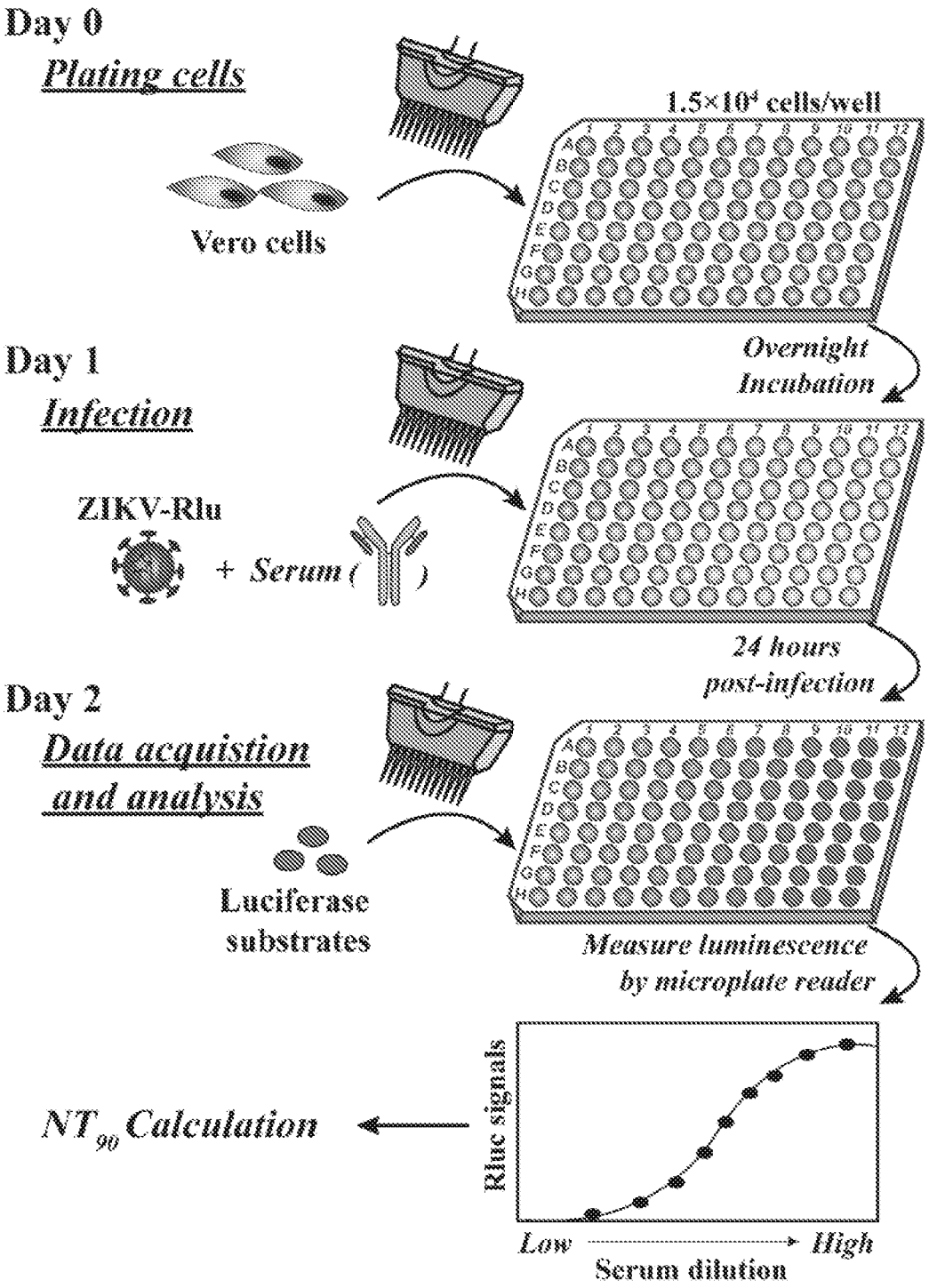
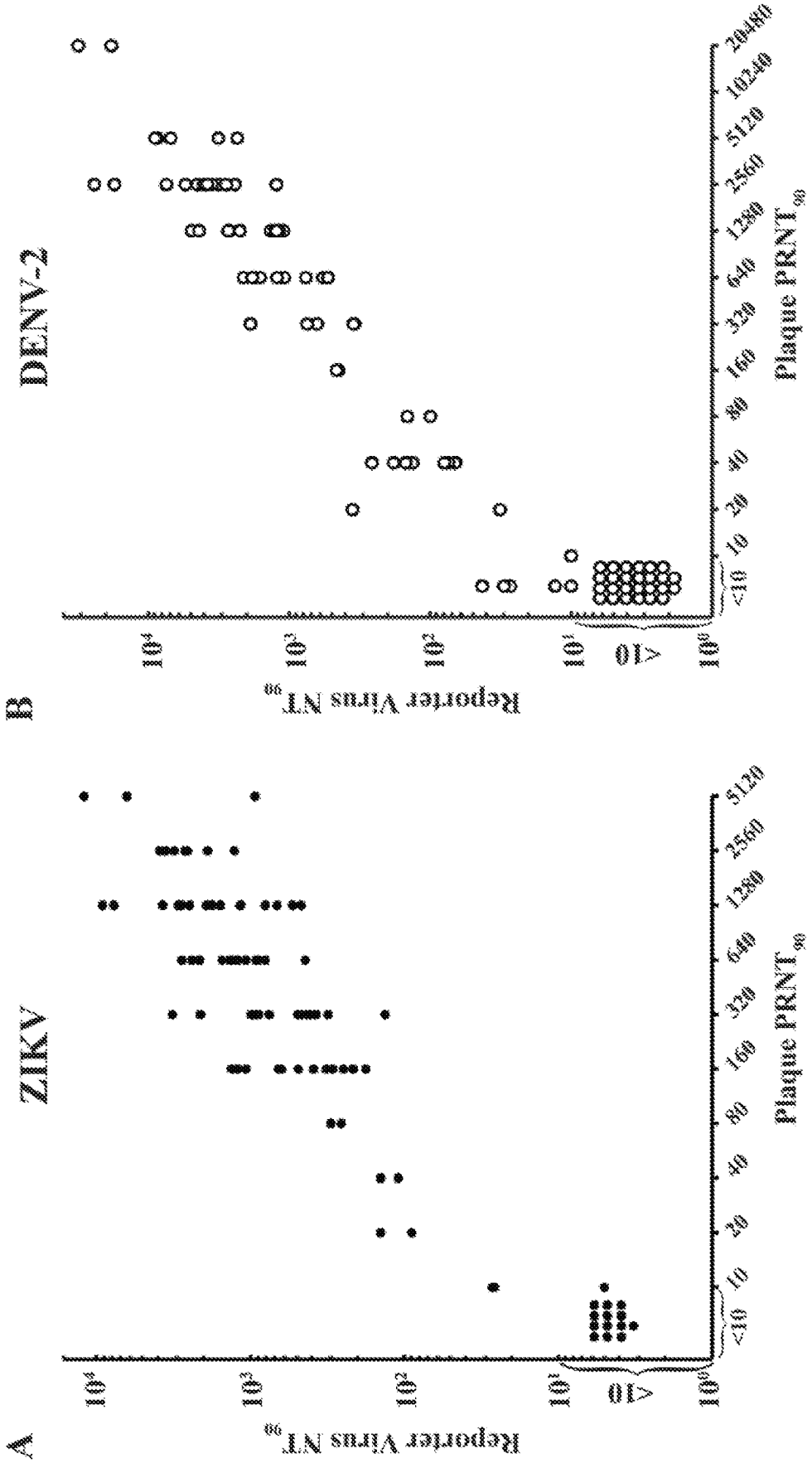

Day 0
Plating cells
1.5×10⁴ cells/well
Vero cells
Overnight
Incubation
Day 1
Infection
ZIKV-Rlu
+ Serum ( )
24 hours
post-infection
Day 2
Data acquistion
and analysis
Luciferase
substrates
Measure luminescence
by microplate reader
NT90 Calculation
Rluc signals
Low
High
Serum dilution

FIG. 9


A
ZIKV
Reporter Virus NT99
Plaque PRNT60
<10
10
20
40
80
160
320
640
1280
2560
5120
B
DENV-2
Reporter Virus NT99
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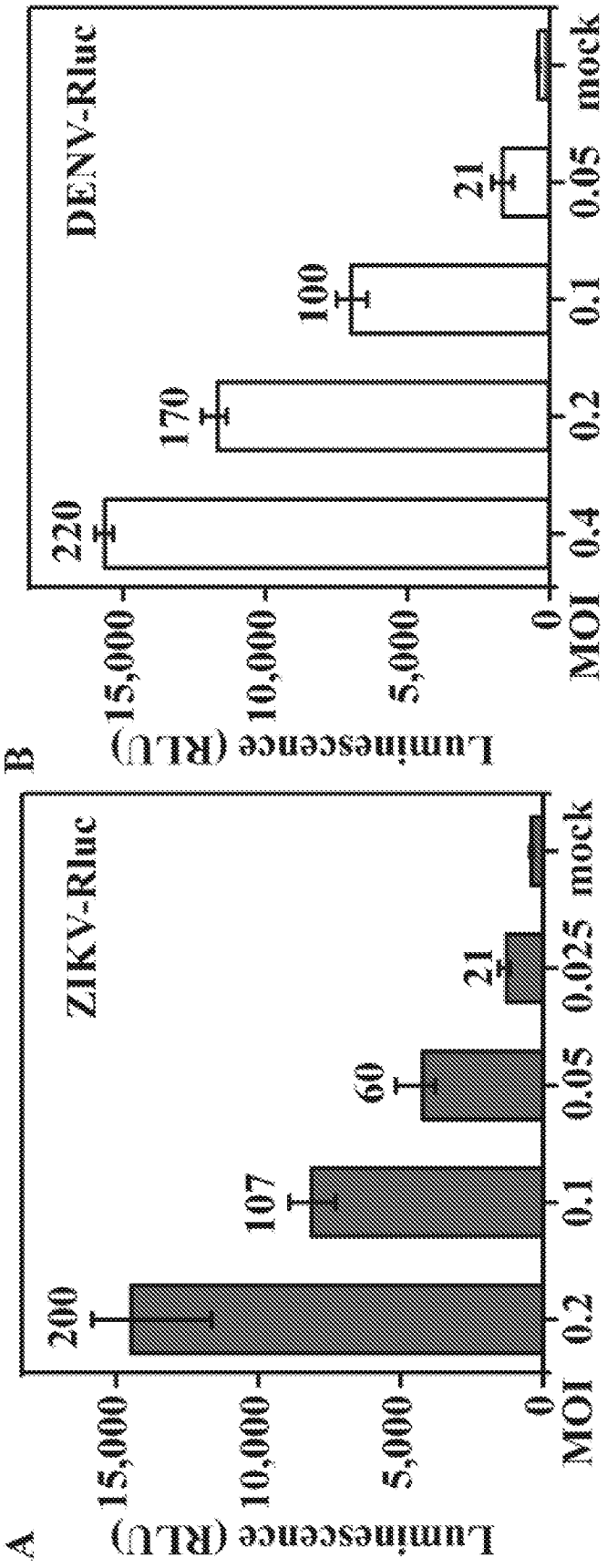
2560
5120
10240
20480

FIG. 10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Luminescence (RLU)
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5,000
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107
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21
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0.1
0.05
0.025
mock
B
DENV-Rluc
Luminescence (RLU)
15,000
10,000
5,000
0
MOI
220
170
100
21
0.4
0.2
0.1
0.05
mock

FIG. 11

REVERSE GENETICS SYSTEM OF ZIKA VIRUS

PRIORITY PARAGRAPH

This Application is a national phase application under 35 U.S.C. § 371 of International Application No. PCT/US2017/030810, filed May 3, 2017 which claims priority to U.S. Provisional Application 62/330,958 filed May 3, 2016 and U.S. Provisional Application 62/455,846, each of which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

This invention was made with government support under AI087856 awarded by the National Institutes of Health. The government has certain rights in the invention.

REFERENCE TO SEQUENCE LISTING

A sequence listing required by 37 CFR 1.821-1.825 is being submitted electronically with this application. The sequence listing is incorporated herein by reference.

BACKGROUND

The current explosive epidemic of Zika virus (ZIKV) in Americas poses a global public health emergency. ZIKV is a member of *Flavivirus* genus within the Flaviviradae family. Flaviviruses have a positive-strand RNA genome of about 11,000 nucleotides. The flaviviral genome encodes three structural proteins (capsid [C], pre-membrane/membrane [prM/M], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form viral particles. The non-structural proteins participate in viral replication, virion assembly, and evasion of the host immune response (Lindenbach et al (2013). Flaviviridae. p. 712-746. In D. M. Knipe and P. M. Howley (ed), Fields virology, 6th., vol. 1. Lippincott William & Wilkins, Philadelphia, Pa.). Like ZIKV, many flaviviruses are significant human pathogens, including yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and dengue virus (DENV). ZIKV is transmitted by *Aedes* spp. mosquitoes, which also transmit YFV and DENV, as well as chikungunya virus. In addition, ZIKV may also be transmitted through sex, blood transfusion, organ transplantation, and potentially through urine or saliva (Musso et al., (2014) *Euro Surveill* 19; Musso et al., (2015) *Emerg Infect Dis* 21, 359-61). Individuals with compromised immunity could be more susceptible to ZIKV infection and disease development (Shan et al., (2016) *ACS Infectious Diseases* 2, 170-72).

Experimental systems, including a reverse genetic system of ZIKV, animal models, and mosquito transmission models, are urgently needed to address these key scientific questions. For animal models, A129 (lacking interferon α/β receptors), AG129 (lacking interferon α/β and γ receptors), and Irf3$^{-/-}$ Irf5$^{-/-}$ Irf$^{-/-}$ triple knockout mice were recently reported to be susceptible to ZIKV infection and to develop neurological diseases (Lazear et al., (2016) A mouse model of Zika virus pathogenesis, *Cell Host & Microbe*; Rossi et al., (2016) Characterization of a Novel Murine Model to Study Zika Virus, *Am J Trop Med Hyg*; Zmurko et al. (2016) The viral polymerase inhibitor 7-deaza-2'-C-methyladenosine is a potent inhibitor of in vitro Zika virus replication and delays

disease progression in a robust mouse infection model. bioRxiv); infection of rhesus macaques with an Asian lineage ZIKV was also reported recently (Dudley et al. (2016). Natural history of Asian lineage Zika virus infection in macaques. bioRxiv). For mosquito infection, one study showed that *A. aegypti* and *A. albopictus* mosquitoes are unexpectedly poor vectors for ZIKV, with disseminated infection rates generally <50% following high titer (10^7 tissue culture infectious dose 50%) oral doses. This suggests the possibility that other mosquito vectors or human-to-human transmission may be contributing to the explosive spread of the virus (Chouin-Carneiro et al., (2016). *PLoS Negl Trop Dis* 10, e0004543).

The potential association of microcephaly and other congenital abnormalities with Zika virus (ZIKV) infection during pregnancy underlines the critical need for a rapid and accurate diagnosis. Due to the short duration of ZIKV viremia in infected patients, a serologic assay that detects antibody responses to viral infection plays an essential role in diagnosing patient specimens. The current serologic diagnosis of ZIKV infection relies heavily on the labor-intensive Plaque Reduction Neutralization Test (PRNT) that requires more than one-week turnaround time and represents a major bottleneck for patient diagnosis.

There is a need for additional reverse genetic systems for Zika virus, as well as additional methods of detecting and diagnosing viral infections such as Zika virus infection.

SUMMARY

Embodiments of the current invention provide additional compositions and methods to overcome the limitations of current methods. The inventors have developed a high-throughput assay for ZIKV and dengue virus (DENV) diagnosis that can attain at least the sensitivity and selectivity of the current PRNT assay. The assays described herein are homogeneous and utilize luciferase viruses to quantify the neutralizing titers in a 96-well format. The inventors have demonstrated that the reporter diagnostic assay of the present invention has a higher dynamic range and maintains the relative specificity of the traditional PRNT assay. Besides the improvement of assay throughput, the reporter virus technology has also shortened the turnaround time to less than two days. Collectively, the results suggest that, along with the viral RT-PCR assay, the reporter virus-based serologic assay could be readily used as a first-line test for clinical diagnosis of ZIKV infection as well as for vaccine clinical trials.

Certain embodiments of the invention are directed to a high-throughput Zika diagnostic assay to measure neutralizing titers of patient specimens using a reporter ZIKV. The assay described herein can have an increased diagnostic dynamic range and a shortened turnaround time from greater than 7 days to less than 2 days in a 96-well format. The assay described herein can also be used in conjunction with viral RT-PCR assays, the reporter serologic assay may serve as the first-line test for diagnosing ZIKV infection. In certain aspects the ZIKV infection is a primary ZIKV infection. As used herein, the term "primary" viral infection refers to a first or original infection, for example, following a first exposure to a virus. In certain aspects a primary ZIKV viral infection presents ZIKV as the only *flavivirus* infection of the subject.

Certain embodiments are directed to an assay for detecting *flavivirus* infection comprising one or more of the following steps: (a) contacting a sample from a subject suspected of having a *flavivirus* infection with a reporter

Zika virus (rZIKV), the rZIKV configured to produce a detectable signal when expressed in viable cell, forming a reporter mixture and incubating the reporter mixture at a temperature of 35 to 40° C.; (b) contacting a host cell monolayer with the reporter mixture under cell growth conditions at about 37° C. forming an inoculated cell monolayer; (c) measuring the reporter signal produced by the inoculated cell monolayer and normalizing the measured signal to a control; and (d) calculating a ZIKV antibody titer of the sample using the reporter signal measurements. In certain instances antibodies present in the sample can bind an neutralize a reporter virus, thus a higher antibody titer results in a lower reporter signal due to reporter virus neutralization. In certain aspects a serial dilution of the sample is contacted with the rZIKV. In a further aspect a plurality of samples are assayed individually. The sample can be a biological sample, such as a blood sample. In certain aspects the sample is from a pregnant subject. The subject can be a mammalian subject, such as a human.

The rZIKV can be a luciferase reporter ZIKV. The luciferase reporter ZIKV expresses a reporter molecule when infecting a cell. In certain aspects the luciferase is *Renilla* luciferase.

The cell monolayer can be a Vero cell monolayer, or other appropriate cell that can be infected by the target virus and express the reporter. In certain aspects the cell monolayers are assayed in a multi-well plate, such as a 96 well microtiter plate. In particular aspects the inoculated cells are incubated for about 12, 24, 36, or 48 hours before measuring the reporter signal.

The assays described herein can be used to detect multiple viruses, such as dengue virus. The assay can further comprising: (e) contacting a sample from a subject suspected of having a *flavivirus* infection with a reporter dengue virus (rDENV), the rDENV configured to produce a detectable signal when infecting a viable cell, forming a reporter mixture and incubating the reporter mixture at a temperature of 35 to 40° C.; (f) contacting a host cell monolayer with the reporter mixture under cell growth conditions at about 37° C. forming an inoculated cell monolayer; (g) measuring the reporter signal produced by the inoculated cell monolayer and normalizing the measured signal to a control; and (h) calculating a DENV antibody titer of the sample using the reporter signal measurements.

In other aspects the assay procedure can further comprising performing virus specific DNA amplification using a second sample from the subject suspected of having a *flavivirus* infection. In certain aspects the DNA amplification is a viral RT-PCR assay.

Embodiments of the invention are directed to stable full-length cDNA clones of a clinical, Asian lineage ZIKV strain. The cDNA clone-derived ZIKV described herein was virulent and caused neurological disease in A129 and AG129 mice. Furthermore, the recombinant virus was highly infectious for *A. aegypti* mosquitoes. These experimental systems are essential to study viral pathogenesis and vector transmission as well as to develop a ZIKV vaccine.

Certain embodiments are directed to a reverse genetic system of Zika virus. This system has three major applications. (1) Vaccine development for both inactivated vaccine and attenuated vaccine. (2) Therapeutics development through reporter virus and high throughput screening. (3) Novel diagnostics development using reporter virus and engineered reporter virus.

In certain aspects the ZIKV nucleic acids can have at least 90, 95, 98, 99, 99.99, 99.991 or 100% sequence identity to SEQ ID NO:1 or any 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 11000 consecutive nucleotide segment thereof, including all values and ranges there between. In certain aspects, a nucleic acid comprises a nucleotide sequence that is at least 90, 95, 98, 99, or 100% identical to all or a part of the non-structural protein coding region of ZIKV (nucleotides 2490 to 10376 of SEQ ID NO:1, or any 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 1000, 2000, 3000, 4000, 5000, 6000, or 7000 consecutive nucleotide segment thereof, including all values and ranges there between). In a further aspect, a nucleic acid comprises a nucleotide sequence that is at least 90, 95, 98, 99, or 100% identical to all or a part of the structural protein coding region of ZIKV (nucleotides 474 to 2489 of SEQ ID NO:1, or any 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10000 consecutive nucleotide segment thereof, including all values and ranges there between).

The ZIKV nucleic acids can be isolated or recombinant nucleic acids (e.g., DNA) or included in a recombinant *flavivirus* replicon, a virus, a *flavivirus*, a viral particle, a *flavivirus* particle, an expression cassette, a host cell, a *flavivirus* vector, and the like. In still a further aspect, an *flavivirus* nucleic acid sequence can comprise a heterologous nucleic acid segment. In certain aspects, the heterologous nucleic acid segment can encode a therapeutic protein, an antigen, a toxin, or a marker (e.g., a reporter protein). In certain aspects the reporter protein is a fluorescent protein, such as a green fluorescent protein.

Certain aspects are directed to an isolated, recombinant, and/or purified ZIKV polypeptide or peptide having at least 90, 95, 98, 99, or 100% amino acid sequence identity to all or part of the amino acid sequence of SEQ ID NO:3 (ZIKV polyprotein). The term "polyprotein" refers to a polypeptide that is post-translationally cleaved to yield more than one polypeptide. "Polypeptide" refers to any peptide or protein comprising a chain or polymer of amino acids joined to each other by peptide bonds. "Polypeptide" refers to both short chains of 100 amino acids or less, commonly referred to as peptides, and to longer chains, generally referred to as proteins. In certain aspects, the isolated and/or purified ZIKV protein has at least 85, 90, 95, 98, 99, or 100% amino acid sequence identity to all or part of the amino acid sequence of an ZIKV non-structural protein. In certain aspects the Zika genome will be mutated to encode one or more amino acids that are associated with microcephaly.

Other embodiments are directed to flaviviruses comprising all or part of the ZIKV nucleic acid sequence of SEQ ID NO: 1. In certain aspects the *flavivirus* is a recombinant *flavivirus*. Certain embodiments are directed to a *flavivirus* having a genome comprising (a) an *flavivirus* nucleic acid segment that is at least 95, 98, 99, or 100% identical to SEQ ID NO:1 and (b) a heterologous nucleic acid segment. In certain aspects, the *flavivirus* is chimeric and comprises segments of a ZIKV *flavivirus* and corresponding segments from a another ZIKV strain or a non-ZIKV *flavivirus*.

As used herein, "control" or "suitable control" is an alternative subject or sample used in an experiment for comparison purposes and included to minimize or distinguish the effect of variables other than an independent variable. A "control" can be positive or negative. A "control" as used herein refers to a control that will allow determination of the presence of a virus or viral infection in a subject. "Control" includes a characteristic or other parameter in a treated sample before administration of a component

described herein or before a detection regimen. "Control" can represent a normal level of the parameter being measured in a subject or sample.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into RNA transcripts. In the context of mRNA and other translated RNA species, "expression" also refers to the process or processes by which the transcribed RNA is subsequently translated into peptides, polypeptides, or proteins.

The term "recombinant" refers to an artificial combination of two otherwise separated segments of nucleic acid, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques.

The term "*flavivirus*" has its conventional meaning, and includes the various species of flaviviruses, including West Nile virus, dengue virus, tick-borne encephalitis virus, yellow fever virus, Zika virus and several other viruses which may cause encephalitis.

The term "*flavivirus* replicon" is used to refer to a nucleic acid molecule expressing *flavivirus* nonstructural protein genes such that it can direct its own replication (amplification).

The term "*flavivirus* replicon particle" refers to a virion or virion-like structural complex incorporating a *flavivirus* replicon.

The term "reporter virus" refers to a virus that is capable of directing the expression of a sequence(s) or gene(s) of interest. The reporter construct can include a 5' sequence capable of initiating transcription of a nucleic acid encoding a reporter molecule or protein such as luciferase, fluorescent protein, Neo, SV2 Neo, hygromycin, phleomycin, histidinol, and DHFR. The reporter virus can be used an indicator of infection of a cell by a certain virus.

The term "expression vector" refers to a nucleic acid that is capable of directing the expression of a sequence(s) or gene(s) of interest. The vector construct can include a 5' sequence capable of initiating transcription of a nucleic acid, e.g., all or part of a *flavivirus*. The vector may also include nucleic acid molecule(s) to allow for production of virus, a 5' promoter that is capable of initiating the synthesis of viral RNA in vitro from cDNA, as well as one or more restriction sites, and a polyadenylation sequence. In addition, the constructs may contain selectable markers such as Neo, SV2 Neo, hygromycin, phleomycin, histidinol, and DHFR. Furthermore, the constructs can include plasmid sequences for replication in host cells and other functionalities known in the art. In certain aspects the vector construct is a DNA construct.

"Expression cassette" refers to a nucleic acid segment capable of directing the expression of one or more proteins or nucleic acids.

Other embodiments of the invention are discussed throughout this application. Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well and vice versa. Each embodiment described herein is understood to be embodiments of the invention that are applicable to all aspects of the invention. It is contemplated that any embodiment discussed herein can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

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."

Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

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."

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.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of the specification embodiments presented herein.

FIG. **1**. Construction of the full-length cDNA clone of ZIKV. (A) The strategy for constructing the full-length cDNA clone of ZIKV. Genome organization, unique restriction sites, and their nucleotide positions are shown. Five cDNA fragments from A to E (represented by thick lines) were synthesized from genomic RNA using RT-PCR to cover the complete ZIKV genome. Individual fragments were assembled to form the full-length cDNA clone of ZIKV (pFLZIKV). The complete ZIKV cDNA is positioned under the control of T7 promoter elements for in vitro transcription. An HDVr ribozyme sequence was engineered at the 3' end of viral genome to generate an authentic 3' end of viral RNA sequence. The numbers are the nucleotide positions based on the sequence of ZIKV strain FSS13025 (GenBank number JN860885). (B) Analysis of RNA transcript from pFLZIKV on a native agarose gel. A 0.8% agarose gel electrophoresis was used to analyze ZIKV RNA transcript along with a genome-length DENV-2 RNA.

FIG. **2**. The RNA transcript from pFLZIKV is infectious. (A) IFA of viral protein expression in cells transfected with full-length ZIKV RNA. Vero cells were electroporated with 10 μg of genome-length ZIKV RNA. From day 3 to 6 post transfection (p.t.), IFA was performed to examine viral E protein expression using a mouse mAb (4G2). Green and blue represent E protein and nuclei (stained with DAPI), respectively. (B) RT-PCR analysis of progeny viral RNA. Viral RNA was extracted from culture supernatant on day 6 p.t. and used as a template for RT-PCR using ZIKV-specific primer pair 1303-F and 2552-ClaI-R (Table 4). As a negative control, a genome-length RNA containing an NS5 polymerase active site mutation (GDD mutated to AAA) was included. (C) Yield of infectious ZIKV after transfection.

Viral titers from culture supernatants at indicated time points were determined by plaque assay. (D) Cytopathic effect on Vero cells on day 6 post transfection.

FIG. **3**. Characterization of parental and recombinant ZIKVs in cell culture. (A) Plaque morphology of parental and recombinant ZIKVs. (B and C) Comparison of growth kinetics in Vero and C6/36 cells, respectively. Vero and C3/36 cells were infected with parental and recombinant virus at an MOI of 0.01. Viral titers were measured at indicated time points using plaque assays on Vero cells. Means and standard deviations from three independent replicates are shown. Statistics were performed using unpaired student's t-test. *significant (p value<0.05); **highly significant (p value<0.01). L.O.D., limitation of detection (100 PFU/ml).

FIG. **4**. A genetic marker was engineered in the recombinant ZIKV. An SphI cleavage site, located in the viral E gene of parental virus, was knocked out in the cDNA clone to serve as a genetic marker to distinguish between recombinant virus and parental virus. A 1258-bp fragment (from nucleotides 1,303 to 2,552) spanning the SphI site was amplified using RT-PCR from RNA extracted from either recombinant virus or parental virus. The RT-PCR fragments were subjected to SphI digestion. The 1258-bp fragment derived from recombinant virus should not be cleavable by SphI; whereas the RT-PCR fragment amplified from parental viral RNA should be cleavable by SphI. (A) Schematic drawing of SphI restriction enzyme analysis. The expected sizes of the digestion products are indicated. (B) Agarose gel analysis of SphI digestion products. Expected digestion pattern as depicted in panel (A) was observed.

FIG. **5**. Comparison of virulence in A129 mice between recombinant and parental viruses. Four-week-old A129 mice were infected with 1×10^5 PFU per individual via the intraperitoneal route. Mock or infected mice (n=5 per group) were monitored for weight loss (A). The viremia at the first three days p.i. was quantified using plaque assay (B). Means and standard deviations are shown. Statistics were performed using unpaired student's t-test. *significant (p value<0.05); **highly significant (p value<0.01); ***extremely significant (p value<0.001).

FIG. **6**. Virulence of parental and recombinant ZIKVs in AG129 mice. Six week-old AG129 mice were inoculated by intraperitoneal injection with 1×10^5 PFU (n=4 for parental virus; n=5 for recombinant virus), 1×10^4 PFU (n=8 for parental virus; n=5 for recombinant virus), or 1×10^3 PFU (n=4 for parental virus; n=5 for recombinant virus) of ZIKV. The infected mice were monitored for weight loss. Mice were euthanized once weight loss exceeded >20%. For each infection dose, weight loss and survival curves are presented. Parental and recombinant viruses and their infection doses are indicated. Values are mean percent weight compared to initial weight.

FIG. **7**. The ZIKV infectious cDNA clone (pFLZIKV) is stable. pFLZIKV was propagated for five rounds of plasmid transformation, bacterial growth, and plasmid purification. Plasmid purified from round 5 was used to transcribe RNA for infectivity test. (A) IFA of viral E protein expression in cells transfected with pFLZIKV RNA from round 0 (R0) and round 5 (R5). Vero cells were electroporated with 10 μg of genome-length RNAs of ZIKV. On day 6 p.t., IFA was performed to examine the E protein expression using a mouse mAb (4G2). Green and blue represent E protein and nuclei staining, respectively. (B) Yields of R0 and R5 ZIKVs on day 6 post-transfection. Culture fluids on day 6 p.t. were measured for infectious viruses using plaque assay on Vero cells. (C) Plaque morphology of R0 and R5 recombinant ZIKVs.

FIG. **8**. Amino acid sequence encoded by SEQ ID NO:1 with amino acid differences of polyprotein as compared to polyprotein encoded by Zika viruses associated with microcephaly highlighted by bold underline.

FIG. **9**. Experimental scheme of reporter virus-based infection assay to measure neutralization titers of specimens. See text for details.

FIG. **10**. Scatter plots of plaque assay-derived $PRNT_{90}$ and reporter assay-derived NT_{90} values for ZIKV and DENV.

FIG. **11**. Optimization of the inoculums of *Renilla* luciferase (Rluc) ZIKV (A) and DENV-2 (B) for the neutralization assay. The experimental scheme is depicted in FIG. **1** and the protocol is detailed in Materials and Methods. Different MOIs of virus inoculum and their luciferase activities at 24 h post-infection are presented. Ratios of the luciferase signals derived from the infections versus the signals from the mock-infected cells are indicated above the bars representing luciferase signals. The average results of three independent experiments are presented.

DESCRIPTION

Since its first isolation in Uganda in 1947 (Dick et al., (1952). *Transactions of the Royal Society of Tropical Medicine and Hygiene* 46, 509-20), ZIKV has predominantly been associated with sylvatic transmission cycles between primates and arboreal mosquitoes in forests, and has for six decades rarely caused human diseases, with only 13 naturally acquired cases reported (Petersen et al., (2016) Zika Virus, *N Engl J Med*). Up to 80% of infected people are asymptomatic. Signs and symptoms of ZIKV infection include fever, lethargy, conjunctivitis, rash, and arthralgia. However, in the past decade, ZIKV has emerged into urban transmission cycles between humans and mosquitoes in the South Pacific and the Americas, and has caused severe diseases, including Guillain-Barré Syndrome and congenital microcephaly (Fauci and Morens, (2016) Zika Virus in the Americas—Yet Another Arbovirus Threat, *N Engl J Med*).

Phylogenetic analysis indicates ZIKV exists as African and Asian lineages. The Asian lineage is responsible for the recent/current epidemics: it caused an epidemic on Yap Island, Micronesia in 2007; it then spread from an unknown source, probably in Southeast Asia, to French Polynesia and other regions of the South Pacific and caused large epidemics in 2013-14; subsequently, ZIKV arrived in the Americas in 2015 and led to millions of human infections (Weaver et al., (2016) *Antiviral Res* 130, 69-80; Weaver et al., (2016) Zika Virus: History, Emergence, Biology, and Prospects for Control, *Antiviral Res*). It is currently not known what has triggered the surge of recent epidemics and severe diseases.

Zika virus (ZIKV) exists as two main lineages: African and Asian. After its discovery in 1947, ZIKV remained obscure with few human cases identified and mild disease symptoms. However, since 2007, the Asian lineage has caused frequent epidemics associated with severe symptoms such as microcephaly and Guillain-Barré syndrome. Unraveling the mechanisms of increased transmissibility and disease severity requires a number of experimental systems, including a reverse genetic system of ZIKV, animal models, and viral vector competence. An infectious cDNA clone of ZIKV using a clinical isolate of Asian lineage (with >99% amino acid identity to the epidemic American strains) is described herein. The RNA transcribed from the cDNA

clone was highly infectious upon transfection into Vero cells, generating recombinant ZIKV with titers of 2-8×10^6 PFU/ml. A genetic marker was engineered into the recombinant virus to differentiate it from the parental and other ZIKV strains. The recombinant virus was virulent in A129 and AG129 mice, and infected mice developed neurological signs that are relevant to human diseases. Furthermore, the recombinant ZIKV was highly infectious for *Aedes aegypti* (the presumed urban American vector) with a dissemination rate of 58% after blood meals containing approximately 10^6 PFU/ml of recombinant virus, suggesting that this mosquito is an efficient vector. Collectively, the reverse genetic system of ZIKV, together with the mouse and mosquito infection models, represent a major advance towards deciphering potential viral determinants of human virulence and urban mosquito transmission. The genetic system will enable rapid development a vaccine using a target-based, rational design.

The current recommendation for diagnosis of ZIKV infection includes three main assays (Musso and Gubler, 2016 *Clin Microbiol Rev* 29, 487-524; Staples et al., 2016 Interim Guidelines for the Evaluation and Testing of Infants with Possible Congenital Zika Virus Infection—United States, 2016. *MMWR Morb Mortal Wkly Rep* 65, 63-67). (i) Detection of viral RNA by RT-PCR. The RT-PCR assay is relatively straightforward and reliable with good sensitivity and specificity (Lanciotti et al., 2008 *Emerg Infect Dis* 14, 1232-39). (ii) Detection of ZIKV-reactive IgM antibodies by an ELISA. One major weakness of the current IgM ELISA test is cross-reactivity with other flaviviruses (such as DENV). This is because the assay uses only viral structural proteins (e.g., E protein) which are the major antigenic proteins known to illicit cross-reactive antibodies. To reduce the assay cross reactivity, one could include viral nonstructural proteins in the ELISA. This idea is based on the rationale that, during *flavivirus* infection, antibody response to viral nonstructural proteins may be more virus-type specific than that to structural proteins. Indeed, several studies reported that *flavivirus* NS1, NS3, and NS5 could be used to improve the specificity of serologic diagnosis (Garcia et al., 1997 *American Journal of Tropical Medicine & Hygiene* 56:466-70; Shu et al., 2000 *Journal of Medical Virology* 62:224-32; Stettler et al., 2016 *Science* 353:823-26; Wong et al. 2003 *J Clin Microbiol* 41:4217-23). In support of this rationale, a multiplex Luminex assay employing ZIKV E, NS1, and NS5 was recently shown to significantly improve the assay specificity (Wong et al., 2017 *E Bio Medicine*). However, it should be pointed out that, although cross reactivity against ZIKV NS1 and NS5 is lower than that against E protein, residual cross reactivity remains to be eliminated for further improvement. This could be achieved through antigen engineering (applicable to both structural and non-structural proteins) to remove the cross-reactive epitopes. The antigen engineering could be rationally guided by protein structures and their epitope profiles. Employment of such virus-specific proteins without cross-reactive epitopes will further improve the assay specificity. (iii) Confirmation of the IgM ELISA-positive specimens using a PRNT assay. Although PRNT remains the "gold standard" for arbovirus serology, the low-throughput nature of the assay limits the number of samples that could be diagnosed in a timely manner. This limitation is particularly pressing in ZIKV diagnosis for pregnant patients.

Embodiments of the invention described herein are directed to a rapid assay to replace the traditional plaque-based PRNT assay. The inventors took advantage of their previously constructed luciferase reporter ZIKV and DENV, and developed a homogeneous neutralization assay in a 96-well format. Validation of the reporter assay using 91 human sera generated diagnostic results equivalent to the traditional PRNT. Importantly, the reporter assay has significantly improved test turnaround time, assay dynamic range, and diagnostic throughput. These improvements have practical implications in clinics by overcoming the bottleneck of test capacity and by achieving test results within 48 hours. Since the current diagnostic algorithm is to confirm the IgM ELISA-positive specimens using PRNT, the reporter assay may be used directly to test neutralization titer of patient samples without prior IgM ELISA. In this way, the reporter assay could serve in conjunction with RT-PCR as the first-line test for ZIKV serologic diagnosis from which physicians would be able to attain the diagnostic results within two days. Furthermore, the reporter assay could be used to specifically measure IgM or IgG neutralization titers when other antibody types have been pre-depleted from the patient sera.

The reporter virus-based neutralization assay can be expanded to other flaviviruses (Zhang et al., 2016 *Virus Res* 211:17-24) as well as to other arboviruses (such as chikungunya virus) that often co-circulate in many tropical and sub-tropical regions. Besides the use in clinical diagnosis, reporter viruses could also be useful for other aspects of research, such as tracking infection in mosquitos and in small animal models (Schoggins et al., 2012 *Proc Natl Acad Sci USA* 109:14610-15), as well as for siRNA/CRISPR library screening or antiviral drug discovery (Puig-Basagoiti et al., 2005 *Antimicrob Agent Chemother* 49:4980-88). For serologic diagnosis, the reporter viruses are superior to trans packaged virus-like particles using reporter replicons (Hanna et al., 2005 *J Virol* 79:13262-74; Harvey et al., 2004 *J Virol* 78:531-38; Khromykh et al., 1998 *J Virol* 72:5967-5977) because once stable reporter viruses have been established, they could be produced in large quantities.

The inventors have developed a reporter ZIKV assay that can replace the current "gold standard" PRNT assay to measure neutralization titers of patient specimens. Since the assay is high throughput and has a turnaround time of less than 48 h, it can be used as the first-line diagnostic test without prior IgM ELISA test. The reporter ZIKV assay can be readily used for clinical diagnosis, serologic surveillance, and monitoring antibody response in vaccine trial. This serologic assay, together with the well-established viral RT-PCR assay, can deliver a rapid diagnosis of ZIKV infection.

EXAMPLES

The following examples as well as the figures are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples or figures represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

An Infectious cDNA Clone of Zika Virus to Study Viral Virulence and Mosquito Transmission

Construction of the Full-Length cDNA Clone of ZIKV.

The inventors chose a clinical ZIKV isolate of Asian lineage to construct the cDNA clone. This ZIKV strain (FSS13025) was isolated from a three-year old patient from Cambodia in 2010 (Heang et al., (2012) *Emerg Infect Dis* 18,

349-351). Viral RNA from Vero cell passage two of the isolate was sequenced (GenBank number JN860885.1) and used as the template to construct the infectious cDNA clone. Five RT-PCR fragments (A to E) spanning the complete viral genome were individually cloned and assembled into the full-length cDNA of ZIKV (named as pFLZIKV; FIG. **1**A). Based on previous experience with infectious clones of other flaviviruses (Li et al. (2014) *J Gen Virol* 95, 806-15; Shi et al., (2002) *J Virol* 76, 5847-56; Zou et al., (2011) *Antiviral Res* 91, 11-19), the inventors chose a low-copy number plasmid pACYC177 (15 copies per *E. coli* cell) to clone fragments A and B as well as to assemble the full-genome cDNA. This plasmid was used because fragments A and B, spanning the viral prM-E-NS1 genes, were toxic to *E. coli* during the cloning procedure; high copy-number vectors containing these fragments were unstable, leading to aberrant deletions/mutations of the inserts (Shi et al., (2002) *J Virol* 76, 5847-56). In contrast, fragments C, D, and E were not toxic to *E. coli*, and could be cloned individually into a high copy-number plasmid pCR2.1-TOPO. A T7 promoter and a hepatitis delta virus ribozyme (HDVr) sequence were engineered at the 5' and 3' ends of the complete viral cDNA for in vitro transcription and for generation of the authentic 3' end of the RNA transcript, respectively. Sequence comparison of the fully assembled pFLZIKV cDNA with the parental virus revealed two synonymous mutations in the E gene (Table 1), one of which was derived from an engineered genetic marker (see below). RNA synthesized from the pFLZIKV plasmid (10,808 nucleotides [nt] long without HDVr) and RNA transcribed from a DENV-2 infectious clone (10,723 nt long; (Zou et al., (2011) *Antiviral Res* 91, 11-19)) migrated similarly on a native agarose gel (FIG. **1**B).

TABLE 1

Sequence differences between the infectious cDNA clone and parental ZIKV[a]

Nucleotide position	Parental strain	cDNA clone	Amino acid change	Location
1655	T	C	Silent (SphI knockout)	E
1865	T	C	None	E

[a]ZIKV strain FSS13025 (GenBank number JN860885.1) was used in the current study. After sequencing this strain FSS13025, we found an error in the current GenBank sequence. The sequence at nucleotide position 798 should be T, not C.

RNA Transcript from ZIKV cDNA Clone is Highly Infectious.

The pFLZIKV RNA transcript was transfected into Vero cells to examine the infectivity of the cDNA clone. The transfected cells were monitored for viral protein expression, RNA synthesis, and virus production. As shown in FIG. **2**A, an increasing number of cells expressed viral E protein from day 1 to 6 post-transfection (p.t.). RT-PCR analysis detected ZIKV RNA in culture media of the transfected cells; as a negative control, no RT-PCR product was detected from the cells transfected with an RNA containing the polymerase active site GDD residues mutated to AAA (FIG. **2**B). Increasing amounts of infectious virus were produced from the wild-type RNA-transfected cells, with peak titers of $1\times10^{6-7}$ plaque-forming units (PFU)/ml on days 5-7 (FIG. **2**C). On days 6-7 p.t., the transfected cells exhibited cytopathic effects (CPE; FIG. **2**D). Full-genome sequencing of the recombinant virus revealed no change other than the two synonymous mutations that originated from pFLZIKV. Collectively, these results demonstrate that the ZIKV cDNA clone is highly infectious.

Comparison of Cell Culture Growth Between Parental and Recombinant Viruses.

The recombinant and parental viruses were compared in cell culture. As shown in FIG. **3**A, the recombinant virus produced homogeneous plaque morphology, whereas the parental virus generated heterogeneous plaque sizes. The difference in plaque morphology was not surprising because the recombinant viruses were derived from a homogenous population of RNA transcripts, whereas the parental virus presumably was composed of a quasi-species. In agreement with this notion, the recombinant virus displayed attenuated replication kinetics in both mammalian Vero and mosquito C6/36 cells (FIG. **3**B and FIG. **3**C), indicating that the replication level of recombinant virus was attenuated in cell culture.

Recombinant ZIKV Retained an Engineered Genetic Marker.

To exclude the possibility that the recovered recombinant virus represented contamination with the parental virus, the inventors engineered a genetic marker into the recombinant virus, in which an SphI cleavage site in the E gene from the parental virus was eliminated (FIG. **4**A). A 1,257-bp fragment spanning nucleotides 1,301-1,252 of viral genome was amplified using RT-PCR from RNAs extracted from the parental and recombinant viruses. The RT-PCR product from the parental virus was readily cleaved by SphI, whereas the RT-PCR product from the recombinant virus was resistant to SphI digestion (FIG. **4**B). These results demonstrate that the recombinant virus was produced from the cDNA-derived RNA transcript. The genetic marker allows differentiation and quantification between the recombinant virus and potentially other ZIKV isolates (which have the SphI site); it could be used to study viral fitness when the recombinant virus serves as an internal standard to gauge viral fitness of other ZIKV strains in a competition assay (Fitzpatrick, et al., (2010) *Virology* 404, 89-95).

The Infectious cDNA Clone of ZIKV is Stable.

Since infectious cDNA clones of flaviviruses are known to be unstable and deleterious for bacterial host (Khromykh and Westaway, (1994) *Journal of Virology* 68, 4580-88; Lai et al., (1991) *PNAS USA* 88, 5139-43; Mandl et al., (1997) *Journal of General Virology* 78, 1049-57; Rice et al., (1989) *New Biologist* 1, 285-96; Sumiyoshi et al., (1992) *Journal of Virology* 66, 5425-31), the stability of pFLZIKV was examined through five rounds of plasmid transformation, bacterial growth, and plasmid purification. Plasmid purified from round 5 was used to transcribe RNA for an infectivity testing. Transfection of the 5^{th} round RNA into Vero cells generated viral E protein-expressing cells (FIG. **7**A) and infectious virus (FIG. **7**B) at levels equivalent to those derived from the original pFLZIKV RNA without passaging (FIGS. **7**A and **7**B). Similar plaque morphology was observed for the original and 5^{th} round RNA-derived recombinant viruses (FIG. **7**C). These results demonstrate the stability of the ZIKV infectious clone.

Virulence in A129 and AG129 Mice.

The inventors compared the virulence of the parental and recombinant ZIKVs in two mouse models: A129 (lacking interferon α/β receptor) and AG129 (lacking interferon α/β and γ receptors). The AG129 mice have recently been reported to be more susceptible to ZIKV-induced disease than the A129 mice (Rossi et al., (2016) Characterization of a Novel Murine Model to Study Zika Virus, Am J Trop Med Hyg). In the A129 mice, intraperitoneal (i.p.) infection with parental virus (10^5 PFU) led to weight loss and disease characterized by hunched posture and ruffled fur; all infected mice were euthanized due to >20% weight loss on day 9

post-infection (p.i.; FIG. **5**A). In contrast, infection with the same inoculum of recombinant ZIKV resulted in less weight loss, but none of the infected mice died (FIG. **5**A). In agreement with these observations, the recombinant virus generated significantly lower viremia than the parental virus on day 1 p.i. in the A129 mice; whereas the differences on days 2 and 3 viremia were not statistically significant between the two viruses (FIG. **5**B). The results suggest that the slower replication kinetics of the recombinant virus may be responsible for its attenuated virulence.

In AG129 mice, i.p. injection of both parental and recombinant ZIKVs ($1\times10^{3-5}$ PFU) led to neurological disease, weight loss, and death (due to >20% weight loss; FIG. **6**). The neurological disease was characterized by hyperactivity, uncoordinated movements, inability to right the body, body spinning, and hind limb paralysis. The kinetics of weight loss was dependent on the viral dose: mice infected with the recombinant virus exhibited slower weight loss and longer survival than those infected with the parental virus (FIG. **6**). These results demonstrate that the recombinant virus is less virulent than the parental virus in vivo; however, infection AG129 mice with the recombinant virus still leads to neurological disease, consistent with evidence that ZIKV causes congenital neurodevelopmental disorders in human fetuses.

Mosquito Infection and Dissemination.

To compare viral fitness between parental and recombinant viruses in mosquitoes, the inventors determined the oral susceptibility of *A. aegypti* using artificial human blood meals containing ZIKV. As summarized in Table 2, the recombinant virus showed higher infection and disseminated infection rates than the parental virus, which may have reflected the slightly higher blood meal titer of the recombinant virus. The overall dissemination rates (number of disseminated mosquitoes/number of infected mosquitoes× 100%) were equivalent between the parental and recombinant viruses, suggesting that the recombinant virus has a wild-type phenotype in *A. aegypti* mosquitoes. These results demonstrate that the recombinant virus is highly infectious for *A. aegypti*, and the disseminated infection rates suggest that this species is an efficient vector for ZIKV.

TABLE 2

Infection and dissemination of Asian lineage ZIKV strain FSS13025 (Cambodia, 2010) in *A. aegypti*

Strain	Blood meal titer (Log_{10} FFU/ml)	Infection rate (%)[a]	Disseminated infection rate (%)[b]	Dissemination rate (%)[c]
FSS13025 parental	6.2	18/42 (43)	11/42 (26)	11/18 (61)
FSS13025 recombinant	6.5	33/42 (78)	19/42 (45)	19/33 (58)

[a]Infection rate = Number of infected mosquitos/number of engorged mosquitos × 100%
[b]Disseminated infection rate = Number of disseminated mosquitos/number of engorged mosquitos × 100%
[c]Dissemination rate (%) = Number of disseminated mosquitos/number of infected mosquitos × 100%

The reverse genetic system described herein, together with the mosquito infection and A129/AG129 mouse models, provide a tractable platform to explore the mechanisms responsible for the explosive epidemics and increased disease severity of ZIKV infection since 2007. A number of non-exclusive mechanisms are possible. (i) ZIKV has undergone adaptive evolution that enhanced mosquito transmission, leading to rapid virus spread and an increased number of human infections. This hypothesis could be tested by comparisons of mosquito infectivity of the older ZIKV strains with recent isolates, followed by using the reverse genetic system to test the effects of recent mutations on mosquito transmission. This mechanism was responsible for the emergence of chikungunya virus, in which a series of mutations in the viral envelope genes enhanced viral transmission by *A. albopictus* through increased infection of epithelial cells in the midgut (Tsetsarkin et al. (2014) *Nature communications* 5, 4084; Tsetsarkin and Weaver, (2011) *PLoS Pathog* 7, e1002412). (ii) The Asian lineage of ZIKV has adapted to generate higher viremia in humans, leading to enhanced cross-placental infection and microcephaly. This hypothesis could be tested by engineering adaptive mutations from the recent isolates into the infectious cDNA clone, generating mutant viruses, and quantifying the mutational effect on viral virulence in the A129/AG129 mouse and on microcephaly development (the animal model for microcephaly remains to be established). (iii) Stochastic introduction of ZIKV into a population (in the Pacific and Americas) lacking herd immunity, leading to greater susceptibility to ZIKV infection and efficient mosquito transmission. Sero-prevalence and its correlation with ZIKV transmission and outbreak frequency need to be established to address this hypothesis. (iv) Previous infection with DENV may exacerbate ZIKV disease severity because the two viruses share approximately 43% amino acids identity and extensive antibody cross-reactivity (Alkan et al. (2015). *J Virol* 89, 11773-85; Lanciotti et al. (2008) *Emerg Infect Dis* 14, 1232-39). This hypothesis could be tested in the AG129 mouse because this mouse is susceptible to infection with both DENV and ZIKV. (v) Human genetic predisposition may account for the severe disease outcomes. Any viral infection is modulated by pro-viral and anti-viral host factors. The interaction between viral and host factors determines the efficiency of infection, pathogenicity, transmission, and epidemic potential. Therefore, variations of critical host factor(s) among infected individuals may contribute to different disease severity.

Compared with the parental virus, the replication efficiency of the recombinant virus was reduced in Vero and C6/36 cells (FIG. **3**). This attenuated replication of recombinant virus in Vero cells was translated to the attenuated virulence in the A129 and AG129 mice (FIG. **5** and FIG. **6**). The differences (in replication and virulence) between the parental and recombinant viruses could be due to the limited genetic heterogeneity of the recombinant virus population and the more genetically diverse quasi-species nature of the parental virus. Interestingly, although the replication of the recombinant virus was reduced in C6/36 cells, it yielded a disseminated infection rate in *A. aegypti* mosquitoes similar to that of the parental virus, indicating that the cell culture system does not necessarily recapitulate in vivo outcomes. Such a discrepancy is not surprising because of the more complex host-virus interactions in vivo.

The infectious cDNA clone of ZIKV will facilitate vaccine development through rational design. Target-based attenuation of ZIKV could be achieved through mutating viral replication components (viral RNA and replication complex) or through ablating viral components needed for evasion of host immune response (Li et al. (2013) *J Virol* 87, 5812-19; Whitehead et al., (2007) *Nat Rev Microbiol* 5, 518-28; Zust et al. (2013) *PLoS Pathog* 9, e1003521). The ZIKV strain used in the current study is appropriate for such attenuated vaccine because of its high sequence similarity to the American epidemic strains. As summarized in Table 3, only 19 amino acid differences were observed between the infectious clone-derived virus described herein and strains

recently isolated from microcephaly fetuses (Calvet et al. (2016). Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study, *The Lancet Infectious diseases*; Faria et al. (2016). Zika virus in the Americas: Early epidemiological and genetic findings. *Science*; Mlakar et al. (2016) N Engl J Med 374, 951-58), representing >99% amino acid identity. In addition, the recombinant virus was attenuated in both A129 and AG129 mice, yet replicated robustly in Vero cells (an approved cell line for vaccine production) to titers above 1×10^6 PFU/ml. Besides its application for vaccine development, the infectious clone can also be used as a reporter ZIKV (e.g., GFP or luciferase), which facilitates the tracking of viral replication in vivo and screening for antiviral inhibitors in a high-throughput manner (Shan et al., (2016) *ACS Infectious Diseases* 2, 170-72).

TABLE 3

Amino acid differences between the infectious cDNA clone and microcephaly ZIKV isolates

Polyprotein position[a]	Associated protein and position	Microcephaly strains[b] KU497555	KU527068	Infectious clone JN860885
106	Capsid: 106 (or anchor C: 2)	Ala	Ala	**Thr**[c]
123	prM: 1	Ala	Ala	**Val**[c]
130	prM: 8	Ser	Ser	**Asn**[c]
139	prM: 17	Asn	Asn	**Ser**[c]
550	E: 260	Thr	Ser	Ser
763	E: 473	Met	Met	**Val**[c]
940	NS1: 146	Lys	Glu	Lys
982	NS1: 188	Val	Val	**Ala**[c]
1027	NS1: 233	Thr	Ala	Thr
1143	NS1: 349	Met	Val	Met
1259	NS2A: 113	Phe	Leu	Leu
1274	NS2A: 128	Leu	Leu	**Pro**[c]
1477	NS2B: 105	Thr	Thr	**Ala**[c]
2086	NS3: 584	His	His	**Tyr**[c]
2509	NS4B: 240	Thr	Iso	Thr
2634	NS5: 114	Val	Val	**Met**[c]
2831	NS5: 311	Val	Glu	Glu
3392	NS5: 872	Val	Val	**Met**[c]
3403	NS5: 883	Met	Met	**Val**[c]

[a]The amino acid position of polyprotein is numbered based on the infectious cDNA clone strain FSS13025 (GenBank number JN860885)
[b]Three ZIKV strains from microcephaly fetuses are listed for sequence comparison: strain Fss13025 (GenBank number KU497555) and Natal RGN (GenBank number KU527068). The GenBank numbers are indicated.
[c]Residues in bold are from the infectious cDNA clone that are consistently different from the two microcephaly strains.

In summary, the current invention provides a multi-component platform to study ZIKV transmission and disease pathogenesis, and to develop countermeasures.

Material and Methods

Cells, Viruses, and Antibodies.

Vero cells were purchased from the American Type Culture Collection (ATCC, Bethesda, Md.), and maintained in a high glucose Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, Calif.) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, Utah) and 1% penicillin/streptomycin (Invitrogen) at 37° C. with 5% CO_2. *A. albopictus* C6/36 (C6/36) cells were grown in RPMI1640 (Invitrogen) containing 10% FBS and 1% penicillin/streptomycin at 28° C. with 5% CO_2. The parental ZIKV Cambodian strain FSS13025 (GenBank number JN860885.1) was isolated in 2010 from the blood of a patient from Cambodia. The following antibodies were used in this study: a mouse monoclonal antibody (mAb) 4G2 cross-reactive with *flavivirus* E protein (ATCC) and goat anti-mouse IgG conjugated with Alexa Fluor 488 (Thermo Fisher Scientific).

cDNA Synthesis and Cloning.

Viral RNA was extracted from viral stocks using QIAamp Viral RNA Kits (Qiagen). cDNA fragments covering the complete genome were synthesized from genomic RNA using SuperScript® III (RT)-PCR using primers (Table 4) according to the manufacturer's instructions (Invitrogen). FIG. **1**A depicts the scheme to clone and assemble the full-genome of ZIKV. Plasmid pACYC177 (New England Biolabs, Ipswich, Mass.) was used to clone fragments B and A+B. Plasmid pCR2.1-TOPO (Invitrogen) was used to clone individual fragment C, D, and E. The full-length genomic cDNA was assembled using plasmid pACYC177. Bacterial strain Top 10 (Invitrogen) was used as the *E. coli* host for construction and propagation of cDNA clones. A standard cloning procedure was used, as previously reported for making WNV (Shi et al., (2002) *J Virol* 76, 5847-56) and DENV (Zou et al., (2011) *Antiviral Res* 91, 11-19) infectious clones. The virus-specific sequence of each intermediate clone was validated by Sanger DNA sequencing before it was used in subsequent cloning steps. The final plasmid containing full-length cDNA of ZIKV (pFLZIKV) was sequenced to ensure no undesired mutations. All restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.).

RNA Transcription and Transfection.

Plasmid pFLZIKV, containing the full length cDNA of ZIKV, was amplified in *E. coli* Top10 and purified using MaxiPrep PLUS (Qiagen). For in vitro transcription, 10 μg of pFLZIKV was linearized with restriction enzyme ClaI. The linearized plasmid was extracted with phenol-chloroform and chloroform, precipitated with ethanol, and re-suspended in 15 μl of RNase-free water (Ambion, Austin, Tex.). The mMESSAGE mMACHINE kit (Ambion) was used to in vitro transcribe RNA in a 20-μl reaction with an additional 1 μl of 30 mM GTP solution. The reaction mixture was incubated at 37° C. for 2 h, followed by the addition of DNase I to remove the DNA template. The RNA was precipitated with lithium chloride, washed with 70% ethanol, re-suspended in RNase-free water, quantitated by spectrophotometry, and stored at −80° C. in aliquots. For transfection, approximately 10 μg of RNA was electroporated to 8×10^6 Vero cells in 0.8 ml of Ingenio® Electroporation Solution (Mirus, Madison, Wis.), in 4-mm cuvettes with the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV and 25 μF, pulsing three times, with 3-second intervals. After a 10-min recovery at room temperature, the transfected cells were mixed with media and incubated in a T-175 flask (5% CO_2 at 37° C.). At different time points post-electroporation (p.t.), recombinant viruses in cell culture media were harvested, clarified by centrifugation at 500×g, stored in aliquots at −80° C., and subjected to analysis.

Indirect Immunofluorescence Assays (IFA).

IFA was performed to detect viral protein expression in ZIKV RNA-transfected Vero cells. Vero cells transfected with viral RNA were grown in an 8-well Lab-Tek chamber slide (Thermo Fisher Scientific, Waltham, Mass.). At indicated time points, the cells were fixed in 100% methanol at −20° C. for 15 min. After 1 h incubation in a blocking buffer containing 1% FBS and 0.05% Tween-20 in PBS, the cells were treated with a mouse monoclonal antibody 4G2 for 1 h and washed three times with PBS (5 min for each wash). The cells were then incubated with Alexa Fluor® 488 goat anti-mouse IgG for 1 h in blocking buffer, after which the cells were washed three times with PBS. The cells were mounted in a mounting medium with DAPI (4', 6-diamidino-2-phenylindole; Vector Laboratories, Inc.). Fluores-

cence images were observed under a fluorescence microscope equipped with a video documentation system (Olympus).

Restriction Enzyme Digestion Analysis to Differentiate Between Parental and Recombinant Viruses.

A restriction endonuclease site for SphI existing in the parental ZIKV was eliminated in the cDNA clone and the resulting recombinant virus. The disappearance of the SphI site was used to distinguish between the parental (with SphI site) and recombinant (without SphI site) viruses. Recombinant virus (harvested from culture media on day 6 p.t.) and parental virus were subjected to RNA extraction using QIAamp Viral RNA Kits (Qiagen). The extracted viral RNAs were used to amplify the 1258-bp fragments spanning the SphI site using primers E-1303V and NS1-2552-ClaI-R (Table 4). The RT-PCR products were digested with SphI and analyzed on a 0.8% agarose gel.

TABLE 4

Oligonucleotides used to construct the full-length cDNA of ZIKV

Primers[a]	Primer sequence (5' to 3')
pACYC-14437-F	gcctacccggaactgagtgtc (SEQ ID NO: 5)
T7-5UTR-F	taatacgactcactatagAGTTGTTGATCTGTGTGAATCAGACTG (SEQ ID NO: 6)
T7-5UTR-R	TCACACAGATCAACAACTctatagtgagtcgtattagcggccgc (SEQ ID NO: 7)
1303-F	GCAAAGGGAGCCTGGTGACATGCGC (SEQ ID NO: 8)
2552-ClaI-R	ccatcgatGACGAACACCCCTGTACCGC (SEQ ID NO: 9)
2402-NotI-F	tctgcggccgcGGGTCTGAATACAAAGAATGG (SEQ ID NO: 10)
XbaI-4438-R	gctctagatatcgatttGGACTGTTTCCAGTGACTTCC (SEQ ID NO: 11)
EcoRI-4130-F	cggaattcACCATTTGTCATGGCCCTGGGACTAAC (SEQ ID NO: 12)
XbaI-6408-R	gctctagatatcgatttCTCTGGCGTCCATCCACCTCGG (SEQ ID NO: 13)
EcoRI-6098-F	cggaattcCAACATTTACCTCCAAGATGGCCTC (SEQ ID NO: 14)
XbaI-8470-R	gctctagatatcgatttCTTACCACAGCCCGCGTGCCAG (SEQ ID NO: 15)
KpnI-8266F	ggggtaccGTAGGTATGGGGGAGGACTGGTCAGAG (SEQ ID NO: 16)
XbaI-11002-R	gctctagatatcgatttcatgataagatacattgatg (SEQ ID NO: 17)
3'UTR-HDVr-F	GGGAAATCCATGGTTTCTggtcggcatggcatctc (SEQ ID NO: 18)
3'UTR-HDVr-R	gagatgccatgccgaccAGAAACCATGGATTTCCCCACACCGGCC (SEQ ID NO: 19)
SphI-1638-F	ATTCCATTACCTTGGCAcGCTGGGGCAGACACC (SEQ ID NO: 20)
SphI-1670-R	GGTGTCTGCCCCAGCgTGCCAAGGTAATGGAAT (SEQ ID NO: 21)

[a]The primers were named after the nucleotide position of viral sequence and polarity. F, viral genome sense; R, complementary sense. Nucleotide numbering is based on ZIKV strain FSS13025 (GenBank number JN860885).
[b]Viral and nonviral sequences are in uppercase and lowercase, respectively. Silent mutation to elliminate SphI restriction site in the E gene is also depicted in lowercase.

Plaque Assay.

Viral samples were ten-fold serially diluted six times in DMEM. For each dilution, 100 μl sample was added to a 12-well plate containing Vero cells at about 90% confluency. The infected cells were incubated for 1 h and swirled every 15 min to ensure complete coverage of the monolayer for even infection. After the incubation, 1 ml of methyl cellulose overlay containing 5% FBS 1% P/S was added to each well and the plate was incubated at 37° C. for four days. Following the incubation, methyl cellulose overlay was removed; the plate was washed twice with PBS, fixed with 3.7% formaldehyde, and incubated at room temperature for 20 min. After remove the fixative, the plate was stained with crystal 1% violet for 1 min. Visible plaques were counted and viral titers (PFU/ml) were calculated.

Replication Curves.

Subconfluent Vero and C6/36 cells in 12-well plates were inoculated with either parental or recombinant ZIKV at an MOI of 0.01 in triplicate wells. Virus stocks were diluted in DMEM containing 5% FBS and 1% penicillin/streptomycin. One hundred microliters of virus was added to each well of the 12-well plates. After 1 h attachment (5% CO_2 at 37° C. for Vero cells and at 28° C. for C6/36 cells), the inocula were removed. The cell monolayers were washed three times with PBS, Afterwards, 1 ml DMEM medium containing 2% FBS and 1% penicillin/streptomycin was added to each well. The plates were incubated for up to 6 days. The medium was collected daily and subjected to plaque assay as described above.

Virulence in Mice.

Both A129 and AG129 mice were used to examine the virulence of parental and recombinant ZIKVs. The details of parental ZIKV infection in A129 mice have been recently reported (Rossi et al., (2016) Characterization of a Novel Murine Model to Study Zika Virus, *Am J Trop Med Hyg*). Briefly, four week-old A129 mice were infected with 1×10^5 PFU via the intraperitoneal route. Five mice per group were used for parental and recombinant viruses. PBS was used to dilute the virus stocks to the desired concentration. The inoculum was back-titrated to verify the viral dose. Mock-infected mice were given PBS by the same route. Mice were weighed and monitored daily for signs of illness (hunched posture, ruffled fur, lethargy, etc.). Mice were bled via the retro-orbital sinus (RO) after being anesthetized every other day. Blood was clarified post collection by centrifugation at 3,380×g for 5 min and immediately stored at −80° C. for storage. Viral titers were determined by plaque assay on Vero cells. Mice were considered moribund if they did not respond to stimuli, were unable to remain upright, or lost 20% or more of their initial weight (consistent with the approved protocol).

The AG129 mice were bred and maintained in animal facilities at the University of Texas Medical Branch

(UTMB). Young adult animals (6 weeks old) were inoculated by intraperitoneal injection with parental or recombinant ZIKV using a range of inocula. Following inoculation, mice were weighed daily and visually monitored to determine the course of infection. Mice exhibiting weight loss of >20% of initial body weight or neurologic disease were euthanized. Euthanized animals were counted as being dead on the following day for analysis. All animal work was completed in compliance with the UTMB policy as approved by the Institutional Animal Care and Use Committee (IACUC).

Experimental Infection of Mosquitoes with ZIKV.

A. aegypti colony mosquitoes derived from the Galveston, Tex. were fed for 30 min on blood meals consisting of 1% (weight/vol) sucrose, 20% (vol/vol) FBS, 5 mM ATP, 33% (vol/vol) PBS-washed human blood cells (UTMB Blood Bank), and 33% (vol/vol) DMEM medium and combined with 1 ml virus offered in Hemotek 2-ml heated reservoirs (Discovery Workshops) covered with a mouse skin. Virus titer in the blood meals ranged from 6.2 to 6.5 $\log_{10}$ FFU/ml. Infectious blood meals were loaded on cartons containing *A. aegypti*. Engorged mosquitoes were incubated at 28° C., 80% relative humidity on a 12:12 h light:dark cycle with ad lib access to 10% sucrose solution for 14 days and then frozen at −20° C. overnight. To assess infection and dissemination, bodies and legs were individually homogenized (Retsch MM300 homogenizer, Retsch Inc., Newton, Pa.) in DMEM with 20% fetal bovine serum (FBS) and 250 μg/ml amphotericin B. Samples were centrifuged for 10 min at 5,000 rpm, and 50 μl of each sample supernatant were inoculated into 96-well plates containing Vero cells at 37° C. and 5% CO_2 for 3 days, when they were fixed with a mixture of ice-cold acetone and methanol (1:1) solution and immunostained as described below. Infection was determined by recovery of virus from the homogenized body and dissemination from the alimentary track into the hemocoel was determined by recovery of virus from the legs. The infection rate was recorded as the fraction of virus-positive bodies divided by the total number of bodies from engorged mosquitos and the disseminated infection rate is the number of virus-positive legs divided by the total number of engorged mosquitoes.

Focus Forming Assays and Immunostaining.

Ten-fold serial dilutions of virus in DMEM supplemented with 2% FBS and 250 μg/ml amphotericin B (Invitrogen, Carlsbad, Calif.) were added onto confluent Vero cell monolayers attached to 96-well Costar (Corning, N.Y.) plates and incubated for 1 h with periodic gentle rocking to facilitate virus adsorption at 37° C. Wells were then overlaid with 150 μl of DMEM supplemented with 2% FBS and 250 μg/ml amphotericin B and incubated undisturbed for 3 days at 37° C. Media overlay was aspirated and cell monolayers were rinsed once with PBS, pH 7.4 (Invitrogen, Carlsbad, Calif.), fixed with a mixture of ice-cold acetone and methanol (1:1) solution and allowed to incubate for 30 min at room temperature. Fixation solution was aspirated and plates were allowed to air dry. Plates were washed thrice with PBS supplemented with 3% FBS, followed by 1 h incubation with ZIKV-specific HMAF (hyper-immune ascitic fluid). Plates were washed thrice followed by an hour-long incubation with a secondary antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, Md.). Detection proceeded with the addition of aminoethylcarbazole substrate (ENZO Life sciences, Farmingdale, Mass.) prepared according to the vendor's instructions.

TABLE 5

Overview for SEQ ID NO: 1

	Nucleotide Location (length bp)	AA Length (MW)	Unique Enzyme sites Name (location)	primer
			NotI (14398)	14222F
5' UTR	1-107 (107)		NheI (53)	Aford
Capsid	108-473 (366)	122 (13.98 Kd)		410V
pr	474-752 (279)	93 18.5		688C
M	753-977 (225)	75 Kd		839V
Envelope	978-2489 (1512)	504 (55 Kd)	AvrII (1533)	1303V 1785V 2255V 2402V
NS1	2490-3545 (1056)	352 (38.7 Kd)	PmlI (3348)	2552C 2707V 3207V 3257V 3285V 3498C
NS2A	3546-4223 (678)	226 (24.5 Kd)	SphI (3861)	3659V 3805F 4130V 4158V
NS2B	4224-4613 (390)	130 (14.3 Kd)		4323F 4438R

TABLE 5-continued

Overview for SEQ ID NO: 1

	Nucleotide Location (length bp)	AA Length (MW)	Unique Enzyme sites Name (location)	primer
NS3	4614-6464 (1851)	617 (67.8 Kd)	NaeI (6344)	4662V 5150V 5618V 6098F 6138V 6303F 6408R
NS4A	6465-6845 (381)	127 (13.97 Kd)		6674V
2K	6846-6914 (69)	23 (2.53 Kd)		
NS4B	6915-7667 (753)	251 (27.6 Kd)		7233V 7597V
NS5	7668-10376 (2709)	903 (99.3 Kd)	AflII (8044) SfiI (8412) EcoRI (9174)	7756V 8316V 8382F 8806V 9363V 9854V 10000C 10310V
3' UTR	10270-10807 (453)			10522C 10808C
HDvr	10808-10874 (67) gggtcggcatggcatctccacctcctcgcggtcc gacctgggctacttcggtaggctaagggagaag (SEQ ID NO: 4)			
pACYC177	10875-14274 (3400 bp)		ClaI(10878)	11168V
T7 promoter	14275-14292 (18) taatacgactcactatag (SEQ ID NO: 3)			

Example 2

Zika Virus Plaque Reduction Neutralization Test (PRNT)

Assay Design.

The inventors chose to infect Vero cells with *Renilla* luciferase ZIKV and DENV-2 in a 96-well format for assay development. Since the goal is to measure the neutralization titters of sera that block virus to infect cells, the inventors limited the infection time to 24 h to avoid multiple rounds of infections. Cell permeable substrate ViviRen was selected to measure luciferase activity because it can penetrate into cells to generate luciferase signals without cell lysis. The inventors first determined the optimal virus inoculum per well (seeded with a nearly confluent monolayer of Vero cells) to achieve a liner range of luciferase signal at 24 h post-infection (p.i.; FIG. **11**). The inventors chose the infection dose of multiplicity of infection (MOI) of 0.1 for the neutralization assay; at this infection dose, the assay consistently generated luciferase signals of 100- to 110-fold higher than that from mock-infected cells (FIG. **11**). FIG. **9** summarizes the optimal assay protocol. Specifically, Vero cells (1.5×10^4 in 50 μl medium without phenol red per well) were seeded in a white opaque 96-well plate. After an overnight culturing, the cells were infected with reporter ZIKV or reporter DENV that had been pre-incubated with serially diluted patient sera at 37° C. for 60 min. At 24 hours post infection, luciferase substrate was added to the infected cells. The plates were quantified for luciferase activities. The dose-responsive curves of luciferase activity were used to calculate the 90% neutralization titer (NT_{90}) of each serum using the Prism Software. The reporter assay is homogeneous (i.e., add cells/virus/substrate and measure luciferase activity without any steps of medium aspiration or washing) and can be completed in less than 48 hours.

Selection of Patient Sera.

A total of 91 human sera were selected to validate the reporter virus-based neutralization assay. These sera were categorized into four groups based on their known ZIKV and DENV $PRNT_{90}$ values which had been previously determined by the traditional plaque assay. The $PRNT_{90}$ values of <, =, and >10 are defined as negative, marginally positive, and positive in neutralizing activities, respectively. As shown in Table 6, group I specimens (n=10; specimens number 1 to 10) were negative in neutralizing ZIKV and DENV. Group II specimens (n=6; specimens number 11 to 16) were negative or marginally positive in neutralizing ZIKV, but positive in neutralizing DENV. Group III specimens (n=23; specimens number 17 to 39) were positive in

neutralizing ZIKV, but negative or marginally positive in neutralizing DENV. Group IV specimens (n=43 patients; specimen number 40-91) were positive in neutralizing both ZIKV and DENV. It is worth pointing out that, due to possible cross-neutralization of antibodies among ZIKV and DENV, patients from group IV could have one of the three possible infections: (i) infections with both ZIKV and DENV, (ii) infection with ZIKV only but with antibodies cross-reactive to DENV, or (iii) infection with DENV only but with antibodies cross-reactive to ZIKV.

TABLE 6

Comparison of neutralization titers from plaque assay ($PRNT_{90}$) and reporter virus assay (NT_{90})*

	Plaque assay		Luciferase assay	
Specimen number	ZIKV	DENV	ZIKV	DENV
1	<10	<10	<10	<10
2	<10	<10	<10	<10
3	<10	<10	<10	<10
4	<10	<10	<10	<10
5	<10	<10	<10	<10
6	<10	<10	<10	<10
7	<10	<10	<10	<10
8	<10	<10	<10	<10
9	<10	<10	<10	<10
10	<10	<10	<10	<10
11	<10	40	<10	66
12	<10	40	<10	74
13	<10	40	<10	79
14	10	40	<10	181
15	10	80	26	99
16	10	160	27	448
17	40	<10	109	<10
18	40	<10	142	<10
19	80	<10	257	<10
20	160	<10	249	<10
21	160	<10	489	10
22	160	<10	661	<10
23	160	<10	1321	<10
24	320	<10	133	<10
25	320	<10	313	43
26	320	<10	407	13
27	320	<10	494	27
28	320	<10	759	13
29	320	<10	991	<10
30	320	10	465	10
31	640	<10	440	<10
32	640	<10	890	<10
33	640	<10	1076	<10
34	640	<10	1316	<10
35	640	<10	1355	<10
36	1280	<10	469	<10
37	1280	<10	532	30
38	1280	<10	803	<10
39	1280	<10	1160	<10
40	20	640	142	1811
41	20	1280	89	1355
42	80	640	300	576
43	160	40	178	144
44	160	40	217	133
45	160	320	214	1886
46	160	320	631	636
47	160	640	292	762
48	160	640	389	531
49	160	640	1215	2116
50	160	2560	322	1239
51	160	2560	1071	3125
52	320	20	949	32
53	320	40	375	149
54	320	40	424	259
55	320	160	757	462
56	320	640	885	1085
57	320	2560	2107	2437
58	320	5120	3217	8561
59	640	640	2395	1223
60	640	640	2785	1614
61	640	1280	804	1158
62	640	1280	906	4897
63	640	1280	925	1098
64	640	1280	2134	4351
65	640	1280	2150	2658
66	640	2560	889	17346
67	640	2560	1207	2803
68	640	2560	1356	4492
69	640	5120	1524	6910
70	1280	20	673	355
71	1280	80	1563	145
72	1280	640	2483	1834
73	1280	1280	1760	1183
74	1280	1280	2804	2705
75	1280	1280	3709	2250
76	1280	2560	1173	5418
77	1280	2560	1925	7430
78	1280	2560	2897	3530
79	1280	2560	9156	24147
80	1280	5120	2937	3174
81	1280	20480	7729	31361
82	2560	320	1279	345
83	2560	320	1892	746
84	2560	320	2654	350
85	2560	1280	3885	1258
86	2560	2560	3545	4016
87	2560	2560	3114	3811
88	2560	20480	2555	18316
89	5120	5120	934	2353
90	5120	1280	6352	1237
91	5120	5120	12068	8925

Comparison of Traditional PRNT and Reporter Virus Assays.

All 91 patient samples were subjected to the reporter ZIKV and DENV assay. Table 6 summarizes the NT_{90} values derived from the reporter assay as well as the $PRNT_{90}$ results derived from the traditional plaque assay. Since the NT_{90} values of the reporter assay were calculated using Prism Software, most of these numbers fell between two serum dilutions sandwiching the 90% inhibition of luciferase signals. Comparison of the neutralization results from the two assays revealed three features. (i) For any given specimen, the relative neutralization titers against ZIKV and DENV are in full agreement between the reporter and plaque assays. FIG. **10** shows the scatter plot of 90% neutralization titters derived from the two assay formats for ZIKV and DENV, suggesting a general concordance between the reporter and plaque assays. (ii) Specimens from groups II and III exhibited virus type-specific neutralizing activities against DENV and ZIKV, respectively, when tested with both plaque and reporter virus assays (Table 6). Such specificity was particularly noteworthy for specimens 36-39 that potently neutralized ZIKV ($PRNT_{90}$ or NT_{90} values of 469-1280) but could not or barely neutralize DENV (all NT_{90} values of <10, except specimen 37 with an NT_{90} of 30). (iii) The neutralization titers derived from the reporter ZIKV and DENV assay were on average 2.5- and 2.4-fold higher than those derived from the corresponding ZIKV and DENV plaque assay, respectively. This observation is in agreement with a recent study reporting that the neutralization titers measured by a single-round infection assay using WNV GFP replicon particles were higher than the traditional plaque assay (Dowd et al., 2016 *Science*

354:237-40). The larger dynamic range of the reporter virus assay suggests a higher sensitivity than the plaque assay in differentiating the neutralization titers of patient specimens. Collectively, the results demonstrate that the reporter virus assay has a more dynamic diagnostic range and maintains the relative specificity of the traditional plaque assay.

Materials and Methods

Cells and Viruses.

Vero and BHK-21 cells were purchased from the American Type Culture Collection (ATCC, Bethesda, Md.), and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, South Logan, Utah) and 1% penicillin/streptomycin at 37° C. with 5% CO_2. For the traditional PRNT assay, the inventors used ZIKV Puerto Rico strain PRVABC59 and DENV-2 New Guinea (NGC) strain. *Renilla* luciferase ZIKV (strain FSS13025) and DENV-2 (strain NGC) were prepared from the previously constructed infectious cDNA clones (Shan et al., 2016 *ACS Infectious Diseases* 2:170-72; Zou et al., 2011 *Antiviral Res* 91:11-19). Briefly, the cDNA plasmids were used to in vitro transcribe genomic RNAs. The luciferase ZIKV and DENV RNA transcripts were transfected into Vero and BHK-21 cells, respectively. The transfected cells were cultured in DMEM without phenol red (to eliminate its interference with luciferase signal measurement). On day 10 and 6 post-transfection (when cytopathic effects started to appear in the ZIKV and DENV-2 RNA-transfected cells, respectively), culture fluids were collected and quantified for viral titers using an immuno-staining focus assay and plaque assay, respectively, as previously reported (Shan et al., 2016 *Cell Host Microbe* 19:891-900).

Serum Specimens.

A total of 91 sera from de-identified clinical specimens were used in the study. The specimens came from two sources: 10 samples from University of Texas Medical Branch (UTMB) that were submitted for routine screening for agents other than Zika virus, and 81 samples from New York State Department of Health that were submitted for ZIKV IgM-capture ELISA and Arbovirus MIA testing [a WNV E protein-based microsphere immunoassay as reported previously (Wong et al., 2003 *J Clin Microbiol* 41:4217-23)]. The UTMB samples were carefully selected from the patients with least possibility of exposure to ZIKV and DENV infection. As described recently (Wong et al., 2017 *E Bio Medicine*), the sera from New York State Department of Health were almost all collected from New York State residents who returned from travels to ZIKV epidemic areas (including the Caribbean and Central and South America) from the end of 2015 to October of 2016. Most sera were collected within two months after travel with possible exposure to ZIKV. In some instances, patients requested diagnostic tests at later time points. Since many individuals were asymptomatic, the dates of disease onset were not known. The demographic profile of this population is approximately 19% Hispanic and 6% Non-Hispanic Asian and Pacific Islander. Based on this demographic profile, it is not surprising that many of these individuals may have *flavivirus* immunity, primarily to DENV and other flaviviruses as well as YF vaccines. The information about patient history with respect to vaccination and previous *flavivirus* infections is not available.

Reporter Virus-Based Neutralization Assay.

Reporter ZIKV and DENV-2 containing a *Renilla* luciferase gene was used to measure the neutralization titers of patient sera against ZIKV or DENV-2 in a 96-well plate format. Briefly, Vero cells (1.5×10^4 cells per well) were seeded into a 96-well white opaque plate (Corning Costar, St. Louis, Mo.) one day prior to infection. Patient sera were initially diluted as 10-fold in a phenol red-free DMEM medium (ThermoFisher Scientific, Sugar Land, Tex.) containing 2% FBS and 1% penicillin/streptomycin, followed by 2-fold serial dilution (2^1 to 2^9). Thirty microliters of each serum dilution were mixed thoroughly with 30 μl reporter ZIKV or DENV-2 and incubated at 37° C. for 1 hour to form antibody-virus complexes. Afterwards, 50 μl serum-virus mixtures were inoculated onto the Vero cell monolayer (containing 50 μl phenol red-free DMEM medium with 2% FBS and 1% penicillin/streptomycin). The plate was incubated at 37° C. for 24 hour. The intracellular luciferase signals were measured using ViviRen substrates (Promega, Madison, Wis.) on Cytation 5 Cell Imaging Multi-Mode Reader (Biotek, Winooski, Vt.) according to the manufacturer's instructions. Medium containing the same amounts of reporter ZIKV or DENV-2 but without specimen serum was used as non-treatment controls. Luciferase signals from the non-treatment controls were set at 100%. Luciferase signals from each diluted serum-treated samples were normalized to those from the non-treatment controls. A four-parameter sigmoidal (logistic) model in the software GraphPad Prism 7 was used to calculate the neutralization titers that suppressed 90% of the luciferase signals of the non-treatment control (NT_{90}).

Plaque Reduction Neutralization Test (PRNT).

A standard double-layer plaque assay (Shi et al., 2002 J Virol 76:5847-56) was performed to determine the PRNTs of each patient serum. The inventors used ZIKV Puerto Rico strain PRVABC59 and DENV-2 New Guinea strain in the PRNT assay. Specifically, serial dilutions of serum samples (1/10 for the first dilution followed by serial ½ dilutions) were mixed with an equal amount of virus suspension containing 200 plaque-forming units (PFU) in 0.1 ml. After incubating the mixtures at 37° C. for 1 hour, each virus-diluted serum sample (0.1 ml) was inoculated onto one well of a 6-well tissue culture plate containing a confluent monolayer of Vero cells. After incubating the plate at 37° C. for 1 hour, an agar overlay was added to the infected cell monolayer, and the plate was further incubated at 37° C. When virus plaques became visible, a second overlay containing neutral red was added, and plaques were counted. The antibody titer was determined as the serum dilution that inhibited 90% of the tested virus inoculum ($PRNT_{90}$).

SEQUENCE LISTING

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                                                  Met Lys Asn
                                                  1

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Pro Lys Lys Lys Ser Gly Gly Phe Arg Ile Val Asn Met Leu Lys Arg
    5                  10                  15

gga gta gcc cgt gtg agc ccc ttt ggg ggc ttg aag agg ctg cca gcc      212
Gly Val Ala Arg Val Ser Pro Phe Gly Gly Leu Lys Arg Leu Pro Ala
20                  25                  30                  35

gga ctt ctg ctg ggt cat ggg ccc atc agg atg gtc ttg gcg att cta      260
Gly Leu Leu Leu Gly His Gly Pro Ile Arg Met Val Leu Ala Ile Leu
                40                  45                  50

gcc ttt ttg aga ttc acg gca atc aag cca tca ctg ggt ctc atc aat      308
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            55                  60                  65

aga tgg ggt tca gtg ggg aaa aaa gag gct atg gaa ata ata aag aag      356
Arg Trp Gly Ser Val Gly Lys Lys Glu Ala Met Glu Ile Ile Lys Lys
        70                  75                  80

ttt aag aaa gat ctg gct gcc atg ctg aga ata atc aat gct agg aag      404
Phe Lys Lys Asp Leu Ala Ala Met Leu Arg Ile Ile Asn Ala Arg Lys
    85                  90                  95

gag aag aag aga cga ggc aca gat act agt gtc gga att gtt ggc ctc      452
Glu Lys Lys Arg Arg Gly Thr Asp Thr Ser Val Gly Ile Val Gly Leu
100                 105                 110                 115

ctg ctg acc aca gcc atg gca gtg gag gtc act aga cgt ggg aat gca      500
Leu Leu Thr Thr Ala Met Ala Val Glu Val Thr Arg Arg Gly Asn Ala
                120                 125                 130

tac tat atg tac ttg gac aga agc gat gct ggg gag gcc ata tct ttt      548
Tyr Tyr Met Tyr Leu Asp Arg Ser Asp Ala Gly Glu Ala Ile Ser Phe
            135                 140                 145

cca acc aca atg ggg atg aat aag tgt tat ata cag atc atg gat ctt      596
Pro Thr Thr Met Gly Met Asn Lys Cys Tyr Ile Gln Ile Met Asp Leu
        150                 155                 160

gga cac atg tgt gat gcc acc atg agc tat gaa tgc cct atg ctg gat      644
Gly His Met Cys Asp Ala Thr Met Ser Tyr Glu Cys Pro Met Leu Asp
    165                 170                 175

gag ggg gta gaa cca gat gac gtc gat tgt tgg tgc aac acg acg tca      692
Glu Gly Val Glu Pro Asp Asp Val Asp Cys Trp Cys Asn Thr Thr Ser
180                 185                 190                 195

act tgg gtt gtg tac gga acc tgc cac cac aaa aaa ggt gaa gca cgg      740
Thr Trp Val Val Tyr Gly Thr Cys His His Lys Lys Gly Glu Ala Arg
                200                 205                 210

aga tct aga aga gct gtg acg ctc ccc tcc cat tcc act agg aag ctg      788
Arg Ser Arg Arg Ala Val Thr Leu Pro Ser His Ser Thr Arg Lys Leu
            215                 220                 225

caa acg cgg tcg cag acc tgg ttg gaa tca aga gaa tac aca aag cac      836
Gln Thr Arg Ser Gln Thr Trp Leu Glu Ser Arg Glu Tyr Thr Lys His
        230                 235                 240

ctg att aga gtc gaa aat tgg ata ttc agg aac cct ggc ttc gcg tta      884
Leu Ile Arg Val Glu Asn Trp Ile Phe Arg Asn Pro Gly Phe Ala Leu
    245                 250                 255

gca gca gct gcc atc gct tgg ctt ttg gga agc tca acg agc caa aaa      932
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agg tgc ata gga gtc agc aat agg gac ttt gtg gaa ggt atg tca ggt     1028
Arg Cys Ile Gly Val Ser Asn Arg Asp Phe Val Glu Gly Met Ser Gly
            295                 300                 305

ggg act tgg gtt gat gtt gtc ttg gaa cat gga ggt tgt gtt acc gta     1076
Gly Thr Trp Val Asp Val Val Leu Glu His Gly Gly Cys Val Thr Val
        310                 315                 320

atg gca cag gac aaa ccg act gtc gac ata gag ctg gtt aca aca aca     1124
Met Ala Gln Asp Lys Pro Thr Val Asp Ile Glu Leu Val Thr Thr Thr
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tcg gac atg gct tcg gac agc cgc tgc cca aca caa ggt gaa gcc tac     1220
Ser Asp Met Ala Ser Asp Ser Arg Cys Pro Thr Gln Gly Glu Ala Tyr
                360                 365                 370

ctt gac aag caa tca gac act caa tat gtc tgc aaa aga acg tta gtg     1268
Leu Asp Lys Gln Ser Asp Thr Gln Tyr Val Cys Lys Arg Thr Leu Val
            375                 380                 385

gac aga ggc tgg gga aat gga tgt gga ctt ttt ggc aaa ggg agc ctg     1316
Asp Arg Gly Trp Gly Asn Gly Cys Gly Leu Phe Gly Lys Gly Ser Leu
        390                 395                 400

gtg aca tgc gct aag ttt gct tgc tct aag aaa atg acc ggg aag agc     1364
Val Thr Cys Ala Lys Phe Ala Cys Ser Lys Lys Met Thr Gly Lys Ser
    405                 410                 415

atc cag cca gag aat ctg gag tac cgg ata atg ctg tca gtt cat ggc     1412
Ile Gln Pro Glu Asn Leu Glu Tyr Arg Ile Met Leu Ser Val His Gly
420                 425                 430                 435

tcc cag cac agt ggg atg atc gtt aat gat aca gga cat gaa act gat     1460
Ser Gln His Ser Gly Met Ile Val Asn Asp Thr Gly His Glu Thr Asp
                440                 445                 450

gag aat aga gcg aag gtt gag ata acg ccc aat tca cca aga gcc gaa     1508
Glu Asn Arg Ala Lys Val Glu Ile Thr Pro Asn Ser Pro Arg Ala Glu
            455                 460                 465

gcc acc ctg ggg ggt ttt gga agc cta gga ctt gat tgt gaa ccg agg     1556
Ala Thr Leu Gly Gly Phe Gly Ser Leu Gly Leu Asp Cys Glu Pro Arg
        470                 475                 480

aca ggc ctt gac ttt tca gat ttg tat tac ttg act atg aat aac aag     1604
Thr Gly Leu Asp Phe Ser Asp Leu Tyr Tyr Leu Thr Met Asn Asn Lys
    485                 490                 495

cac tgg ttg gtt cac aag gag tgg ttc cac gac att cca tta cct tgg     1652
His Trp Leu Val His Lys Glu Trp Phe His Asp Ile Pro Leu Pro Trp
500                 505                 510                 515

cac gct ggg gca gac acc gga act cca cac tgg aac aac aaa gaa gca     1700
His Ala Gly Ala Asp Thr Gly Thr Pro His Trp Asn Asn Lys Glu Ala
                520                 525                 530

ctg gta gag ttc aag gac gca cat gcc aaa agg cag act gtc gtg gtt     1748
Leu Val Glu Phe Lys Asp Ala His Ala Lys Arg Gln Thr Val Val Val
            535                 540                 545

cta ggg agt caa gaa gga gca gtt cac acg gcc ctt gct gga gct ctg     1796
Leu Gly Ser Gln Glu Gly Ala Val His Thr Ala Leu Ala Gly Ala Leu
        550                 555                 560

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    565                 570                 575

aaa tgt cgc ctg aaa atg gac aaa ctt aga ttg aag ggc gtg tca tac     1892
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ctg cac ggg aca gtc aca gtg gag gta cag tac gca ggg aca gat gga     1988
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            615                 620                 625

cct tgc aag gtt cca gct cag atg gcg gtg gac atg caa act ctg acc     2036
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Glu Asn Ser Lys Met Met Leu Glu Leu Asp Pro Pro Phe Gly Asp Ser
660                 665                 670                 675

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                680                 685                 690

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            695                 700                 705

gcc aag aga atg gca gtc ttg gga gac aca gcc tgg gac ttt gga tca     2276
Ala Lys Arg Met Ala Val Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser
        710                 715                 720

gtt ggg ggt gct ctc aac tca ctg ggc aag ggc atc cat caa att ttt     2324
Val Gly Gly Ala Leu Asn Ser Leu Gly Lys Gly Ile His Gln Ile Phe
    725                 730                 735

gga gca gct ttc aaa tca ttg ttt gga gga atg tcc tgg ttc tca caa     2372
Gly Ala Ala Phe Lys Ser Leu Phe Gly Gly Met Ser Trp Phe Ser Gln
740                 745                 750                 755

att ctc att gga acg ttg ctg gtg tgg ttg ggt ctg aat aca aag aat     2420
Ile Leu Ile Gly Thr Leu Leu Val Trp Leu Gly Leu Asn Thr Lys Asn
                760                 765                 770

gga tct att tcc ctt atg tgc ttg gcc tta ggg gga gtg ttg atc ttc     2468
Gly Ser Ile Ser Leu Met Cys Leu Ala Leu Gly Gly Val Leu Ile Phe
            775                 780                 785

tta tcc aca gcc gtc tct gct gat gtg ggg tgc tcg gtg gac ttc tca     2516
Leu Ser Thr Ala Val Ser Ala Asp Val Gly Cys Ser Val Asp Phe Ser
        790                 795                 800

aag aag gaa acg aga tgc ggt aca ggg gtg ttc gtc tat aac gac gtt     2564
Lys Lys Glu Thr Arg Cys Gly Thr Gly Val Phe Val Tyr Asn Asp Val
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Glu Ala Trp Arg Asp Arg Tyr Lys Tyr His Pro Asp Ser Pro Arg Arg
820                 825                 830                 835

ttg gca gca gca gtc aag caa gcc tgg gaa gat ggg atc tgt ggg atc     2660
Leu Ala Ala Ala Val Lys Gln Ala Trp Glu Asp Gly Ile Cys Gly Ile
                840                 845                 850

tcc tct gtt tca aga atg gaa aac atc atg tgg aga tca gta gaa ggg     2708
Ser Ser Val Ser Arg Met Glu Asn Ile Met Trp Arg Ser Val Glu Gly
            855                 860                 865

gag ctc aac gca atc ctg gaa gag aat gga gtt caa ctg acg gtc gtt     2756
Glu Leu Asn Ala Ile Leu Glu Glu Asn Gly Val Gln Leu Thr Val Val
        870                 875                 880

gtg gga tct gta aaa aac ccc atg tgg aga ggt cca cag aga ttg ccc     2804
Val Gly Ser Val Lys Asn Pro Met Trp Arg Gly Pro Gln Arg Leu Pro
    885                 890                 895

gtg cct gtg aac gag ctg ccc cat ggc tgg aag gct tgg ggg aaa tcg     2852
Val Pro Val Asn Glu Leu Pro His Gly Trp Lys Ala Trp Gly Lys Ser
900                 905                 910                 915
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tac ttc gtc agg gca gca aag aca aat aac agc ttt gtc gtg gat ggt     2900
Tyr Phe Val Arg Ala Ala Lys Thr Asn Asn Ser Phe Val Val Asp Gly
                920                 925                 930

gac aca ctg aag gaa tgc cca ctc aaa cat aga gca tgg aac agc ttt     2948
Asp Thr Leu Lys Glu Cys Pro Leu Lys His Arg Ala Trp Asn Ser Phe
            935                 940                 945

ctt gtg gag gat cat ggg ttc ggg gta ttt cac act agt gtc tgg ctc     2996
Leu Val Glu Asp His Gly Phe Gly Val Phe His Thr Ser Val Trp Leu
        950                 955                 960

aag gtt aga gaa gat tat tca tta gag tgt gat cca gcc gtc att gga     3044
Lys Val Arg Glu Asp Tyr Ser Leu Glu Cys Asp Pro Ala Val Ile Gly
    965                 970                 975

aca gcc gct aag gga aag gag gct gtg cac agt gat cta ggc tac tgg     3092
Thr Ala Ala Lys Gly Lys Glu Ala Val His Ser Asp Leu Gly Tyr Trp
980                 985                 990                 995

att gag agt gag aag  aac gac aca tgg agg  ctg aag agg gcc cac       3137
Ile Glu Ser Glu Lys  Asn Asp Thr Trp Arg  Leu Lys Arg Ala His
                1000                1005                1010

ctg atc gag atg aaa  aca tgt gaa tgg cca  aag tcc cac aca ttg       3182
Leu Ile Glu Met Lys  Thr Cys Glu Trp Pro  Lys Ser His Thr Leu
                1015                1020                1025

tgg aca gat gga ata  gaa gaa agt gat ctg  atc ata ccc aag tct       3227
Trp Thr Asp Gly Ile  Glu Glu Ser Asp Leu  Ile Ile Pro Lys Ser
                1030                1035                1040

tta gct ggg cca ctc  agc cat cac aac acc  aga gag ggc tac agg       3272
Leu Ala Gly Pro Leu  Ser His His Asn Thr  Arg Glu Gly Tyr Arg
                1045                1050                1055

acc caa atg gaa ggg  cca tgg cat agt gaa  gag ctt gaa att cgg       3317
Thr Gln Met Glu Gly  Pro Trp His Ser Glu  Glu Leu Glu Ile Arg
                1060                1065                1070

ttt gag gaa tgc cca  ggc act aag gtc cac  gtg gag gaa aca tgt       3362
Phe Glu Glu Cys Pro  Gly Thr Lys Val His  Val Glu Glu Thr Cys
                1075                1080                1085

gga aca aga gga cca  tct ctg aga tca acc  act gca agc gga agg       3407
Gly Thr Arg Gly Pro  Ser Leu Arg Ser Thr  Thr Ala Ser Gly Arg
                1090                1095                1100

gtg atc gag gaa tgg  tgc tgc agg gag tgc  aca atg ccc cca ctg       3452
Val Ile Glu Glu Trp  Cys Cys Arg Glu Cys  Thr Met Pro Pro Leu
                1105                1110                1115

tcg ttc cgg gct aaa  gat ggt tgt tgg tat  gga atg gag ata agg       3497
Ser Phe Arg Ala Lys  Asp Gly Cys Trp Tyr  Gly Met Glu Ile Arg
                1120                1125                1130

ccc agg aaa gaa cca  gaa agt aac tta gta  agg tca atg gtg act       3542
Pro Arg Lys Glu Pro  Glu Ser Asn Leu Val  Arg Ser Met Val Thr
                1135                1140                1145

gca gga tca act gat  cac atg gat cac ttc  tcc ctt gga gtg ctt       3587
Ala Gly Ser Thr Asp  His Met Asp His Phe  Ser Leu Gly Val Leu
                1150                1155                1160

gtg att ctg ctc atg  gta cag gaa ggg cta  aag aag aga atg acc       3632
Val Ile Leu Leu Met  Val Gln Glu Gly Leu  Lys Lys Arg Met Thr
                1165                1170                1175

aca aag atc atc ata  agc aca tca atg gca  gtg ctg gta gct atg       3677
Thr Lys Ile Ile Ile  Ser Thr Ser Met Ala  Val Leu Val Ala Met
                1180                1185                1190

atc ctg gga gga ttt  tca atg agt gac ctg  gct aag ctt gca att       3722
Ile Leu Gly Gly Phe  Ser Met Ser Asp Leu  Ala Lys Leu Ala Ile
                1195                1200                1205

ttg atg ggt gcc acc  ttc gcg gaa atg aac  act gga gga gat gtt       3767
Leu Met Gly Ala Thr  Phe Ala Glu Met Asn  Thr Gly Gly Asp Val
                1210                1215                1220
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gct cat ctg gcg ctg  ata gcg gca ttc aaa  gtc aga cct gcg ttg       3812
Ala His Leu Ala Leu  Ile Ala Ala Phe Lys  Val Arg Pro Ala Leu
                1225                1230                1235

ctg gta tct ttc att  ttc aga gct aat tgg  aca ccc cgt gag agc       3857
Leu Val Ser Phe Ile  Phe Arg Ala Asn Trp  Thr Pro Arg Glu Ser
                1240                1245                1250

atg ctg ctg gcc ttg  gcc tcg tgt ctt ctg  caa act gcg atc tcc       3902
Met Leu Leu Ala Leu  Ala Ser Cys Leu Leu  Gln Thr Ala Ile Ser
                1255                1260                1265

gcc ttg gaa ggc gac  ctg atg gtt ccc atc  aat ggt ttt gct ttg       3947
Ala Leu Glu Gly Asp  Leu Met Val Pro Ile  Asn Gly Phe Ala Leu
                1270                1275                1280

gcc tgg ttg gca ata  cga gcg atg gtt gtt  cca cgc act gac aac       3992
Ala Trp Leu Ala Ile  Arg Ala Met Val Val  Pro Arg Thr Asp Asn
                1285                1290                1295

atc acc ttg gca atc  ctg gct gct ctg aca  cca ctg gcc cgg ggc       4037
Ile Thr Leu Ala Ile  Leu Ala Ala Leu Thr  Pro Leu Ala Arg Gly
                1300                1305                1310

aca ctg ctt gtg gcg  tgg aga gca ggc ctt  gct act tgc ggg ggg       4082
Thr Leu Leu Val Ala  Trp Arg Ala Gly Leu  Ala Thr Cys Gly Gly
                1315                1320                1325

ttc atg ctc ctt tct  ctg aag ggg aaa ggc  agt gtg aag aag aac       4127
Phe Met Leu Leu Ser  Leu Lys Gly Lys Gly  Ser Val Lys Lys Asn
                1330                1335                1340

tta cca ttt gtc atg  gcc ctg gga cta acc  gct gtg agg ctg gtc       4172
Leu Pro Phe Val Met  Ala Leu Gly Leu Thr  Ala Val Arg Leu Val
                1345                1350                1355

gac ccc atc aac gtg  gtg gga ctg ctg ttg  ctc aca agg agt ggg       4217
Asp Pro Ile Asn Val  Val Gly Leu Leu Leu  Leu Thr Arg Ser Gly
                1360                1365                1370

aag cgg agc tgg ccc  cct agt gaa gta ctc  aca gct gtt ggc ctg       4262
Lys Arg Ser Trp Pro  Pro Ser Glu Val Leu  Thr Ala Val Gly Leu
                1375                1380                1385

ata tgc gca ttg gct  gga ggg ttc gcc aag  gcg gat ata gag atg       4307
Ile Cys Ala Leu Ala  Gly Gly Phe Ala Lys  Ala Asp Ile Glu Met
                1390                1395                1400

gct ggg ccc atg gcc  gcg gtc ggt ctg cta  att gtc agt tac gtg       4352
Ala Gly Pro Met Ala  Ala Val Gly Leu Leu  Ile Val Ser Tyr Val
                1405                1410                1415

gtc tca gga aag agt  gtg gac atg tac att  gaa aga gca ggt gac       4397
Val Ser Gly Lys Ser  Val Asp Met Tyr Ile  Glu Arg Ala Gly Asp
                1420                1425                1430

atc aca tgg gaa aaa  gat gcg gaa gtc act  gga aac agt ccc cgg       4442
Ile Thr Trp Glu Lys  Asp Ala Glu Val Thr  Gly Asn Ser Pro Arg
                1435                1440                1445

ctc gat gtg gca cta  gat gag agt ggt gat  ttc tcc cta gtg gag       4487
Leu Asp Val Ala Leu  Asp Glu Ser Gly Asp  Phe Ser Leu Val Glu
                1450                1455                1460

gat gat ggt ccc ccc  atg aga gag atc ata  ctc aaa gtg gtc ctg       4532
Asp Asp Gly Pro Pro  Met Arg Glu Ile Ile  Leu Lys Val Val Leu
                1465                1470                1475

atg gcc atc tgt ggc  atg aac cca ata gcc  ata ccc ttt gca gct       4577
Met Ala Ile Cys Gly  Met Asn Pro Ile Ala  Ile Pro Phe Ala Ala
                1480                1485                1490

gga gcg tgg tac gtg  tat gtg aag act gga  aaa agg agt ggt gct       4622
Gly Ala Trp Tyr Val  Tyr Val Lys Thr Gly  Lys Arg Ser Gly Ala
                1495                1500                1505

cta tgg gat gtg cct  gct ccc aag gaa gta  aaa aag ggg gag acc       4667
Leu Trp Asp Val Pro  Ala Pro Lys Glu Val  Lys Lys Gly Glu Thr
                1510                1515                1520
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aca gat gga gtg tac  aga gta atg act cgt  aga ctg cta ggt tca       4712
Thr Asp Gly Val Tyr  Arg Val Met Thr Arg  Arg Leu Leu Gly Ser
                1525                1530                1535

aca caa gtt gga gtg  gga gtc atg caa gag  ggg gtc ttc cac act       4757
Thr Gln Val Gly Val  Gly Val Met Gln Glu  Gly Val Phe His Thr
                1540                1545                1550

atg tgg cac gtc aca  aaa gga tcc gcg ctg  aga agc ggt gaa ggg       4802
Met Trp His Val Thr  Lys Gly Ser Ala Leu  Arg Ser Gly Glu Gly
                1555                1560                1565

aga ctt gat cca tac  tgg gga gat gtc aag  cag gat ctg gtg tca       4847
Arg Leu Asp Pro Tyr  Trp Gly Asp Val Lys  Gln Asp Leu Val Ser
                1570                1575                1580

tac tgt ggt cca tgg  aag cta gat gcc gcc  tgg gac ggg cac agc       4892
Tyr Cys Gly Pro Trp  Lys Leu Asp Ala Ala  Trp Asp Gly His Ser
                1585                1590                1595

gag gtg cag ctc ttg  gcc gtg ccc ccc gga  gag aga gcg agg aac       4937
Glu Val Gln Leu Leu  Ala Val Pro Pro Gly  Glu Arg Ala Arg Asn
                1600                1605                1610

atc cag act ctg ccc  gga ata ttt aag aca  aag gat ggg gac att       4982
Ile Gln Thr Leu Pro  Gly Ile Phe Lys Thr  Lys Asp Gly Asp Ile
                1615                1620                1625

gga gca gtt gcg ctg  gac tac cca gca gga  act tca gga tct cca       5027
Gly Ala Val Ala Leu  Asp Tyr Pro Ala Gly  Thr Ser Gly Ser Pro
                1630                1635                1640

atc cta gat aag tgt  ggg aga gtg ata gga  ctc tat ggt aat ggg       5072
Ile Leu Asp Lys Cys  Gly Arg Val Ile Gly  Leu Tyr Gly Asn Gly
                1645                1650                1655

gtc gtg atc aaa aat  ggg agt tac gtt agt  gcc atc acc caa ggg       5117
Val Val Ile Lys Asn  Gly Ser Tyr Val Ser  Ala Ile Thr Gln Gly
                1660                1665                1670

agg agg gag gaa gag  act cct gtt gag tgc  ttc gag cct tcg atg       5162
Arg Arg Glu Glu Glu  Thr Pro Val Glu Cys  Phe Glu Pro Ser Met
                1675                1680                1685

ctg aag aag aag cag  cta act gtc tta gac  ttg cat cct gga gct       5207
Leu Lys Lys Lys Gln  Leu Thr Val Leu Asp  Leu His Pro Gly Ala
                1690                1695                1700

ggg aaa acc agg aga  gtt ctt cct gaa ata  gtc cgt gaa gcc ata       5252
Gly Lys Thr Arg Arg  Val Leu Pro Glu Ile  Val Arg Glu Ala Ile
                1705                1710                1715

aaa aca aga ctc cgc  act gtg atc tta gct  cca acc agg gtt gtc       5297
Lys Thr Arg Leu Arg  Thr Val Ile Leu Ala  Pro Thr Arg Val Val
                1720                1725                1730

gct gct gaa atg gag  gaa gcc ctt aga ggg  ctt cca gtg cgt tat       5342
Ala Ala Glu Met Glu  Glu Ala Leu Arg Gly  Leu Pro Val Arg Tyr
                1735                1740                1745

atg aca aca gca gtc  aat gtc acc cat tct  ggg aca gaa atc gtt       5387
Met Thr Thr Ala Val  Asn Val Thr His Ser  Gly Thr Glu Ile Val
                1750                1755                1760

gac tta atg tgc cat  gcc acc ttc act tca  cgt cta cta cag cca       5432
Asp Leu Met Cys His  Ala Thr Phe Thr Ser  Arg Leu Leu Gln Pro
                1765                1770                1775

atc aga gtc ccc aac  tat aat ctg tat att  atg gat gag gcc cac       5477
Ile Arg Val Pro Asn  Tyr Asn Leu Tyr Ile  Met Asp Glu Ala His
                1780                1785                1790

ttc aca gat ccc tca  agt ata gca gca aga  gga tac att tca aca       5522
Phe Thr Asp Pro Ser  Ser Ile Ala Ala Arg  Gly Tyr Ile Ser Thr
                1795                1800                1805

agg gtt gag atg ggc  gag gcg gct gcc atc  ttc atg act gcc acg       5567
Arg Val Glu Met Gly  Glu Ala Ala Ala Ile  Phe Met Thr Ala Thr
                1810                1815                1820
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cca cca gga acc cgt  gac gca ttc ccg gac  tcc aac tca cca att       5612
Pro Pro Gly Thr Arg  Asp Ala Phe Pro Asp  Ser Asn Ser Pro Ile
                1825                1830                1835

atg gac acc gaa gtg  gaa gtc cca gag aga  gcc tgg agc tca ggc       5657
Met Asp Thr Glu Val  Glu Val Pro Glu Arg  Ala Trp Ser Ser Gly
                1840                1845                1850

ttt gat tgg gtg acg  gat cat tct gga aaa  aca gtt tgg ttt gtt       5702
Phe Asp Trp Val Thr  Asp His Ser Gly Lys  Thr Val Trp Phe Val
                1855                1860                1865

cca agc gtg agg aat  ggc aat gag atc gca  gct tgt ctg aca aag       5747
Pro Ser Val Arg Asn  Gly Asn Glu Ile Ala  Ala Cys Leu Thr Lys
                1870                1875                1880

gct gga aaa cgg gtc  ata cag ctc agc aga  aag act ttt gag aca       5792
Ala Gly Lys Arg Val  Ile Gln Leu Ser Arg  Lys Thr Phe Glu Thr
                1885                1890                1895

gag ttc cag aaa aca  aaa cat caa gag tgg  gac ttc gtc gtg aca       5837
Glu Phe Gln Lys Thr  Lys His Gln Glu Trp  Asp Phe Val Val Thr
                1900                1905                1910

act gac att tca gag  atg ggc gcc aac ttt  aaa gct gac cgt gtc       5882
Thr Asp Ile Ser Glu  Met Gly Ala Asn Phe  Lys Ala Asp Arg Val
                1915                1920                1925

ata gat tcc agg aga  tgc cta aag ccg gtc  ata ctt gat ggc gag       5927
Ile Asp Ser Arg Arg  Cys Leu Lys Pro Val  Ile Leu Asp Gly Glu
                1930                1935                1940

aga gtc att ctg gct  gga ccc atg cct gtc  aca cat gcc agc gct       5972
Arg Val Ile Leu Ala  Gly Pro Met Pro Val  Thr His Ala Ser Ala
                1945                1950                1955

gcc cag agg agg ggg  cgc ata ggc agg aac  ccc aac aaa cct gga       6017
Ala Gln Arg Arg Gly  Arg Ile Gly Arg Asn  Pro Asn Lys Pro Gly
                1960                1965                1970

gat gag tat ctg tat  gga ggt ggg tgc gca  gag act gat gaa gac       6062
Asp Glu Tyr Leu Tyr  Gly Gly Gly Cys Ala  Glu Thr Asp Glu Asp
                1975                1980                1985

cat gca cac tgg ctt  gaa gca aga atg ctt  ctt gac aac att tac       6107
His Ala His Trp Leu  Glu Ala Arg Met Leu  Leu Asp Asn Ile Tyr
                1990                1995                2000

ctc caa gat ggc ctc  ata gcc tcg ctc tat  cga cct gag gcc gac       6152
Leu Gln Asp Gly Leu  Ile Ala Ser Leu Tyr  Arg Pro Glu Ala Asp
                2005                2010                2015

aaa gta gca gct att  gag gga gag ttc aag  ctt agg acg gag caa       6197
Lys Val Ala Ala Ile  Glu Gly Glu Phe Lys  Leu Arg Thr Glu Gln
                2020                2025                2030

agg aag acc ttt gtg  gaa ctc atg aaa aga  gga gat ctt cct gtt       6242
Arg Lys Thr Phe Val  Glu Leu Met Lys Arg  Gly Asp Leu Pro Val
                2035                2040                2045

tgg ctg gcc tat cag  gtt gca tct gcc gga  ata acc tac aca gat       6287
Trp Leu Ala Tyr Gln  Val Ala Ser Ala Gly  Ile Thr Tyr Thr Asp
                2050                2055                2060

aga aga tgg tgc ttt  gat ggc acg acc aac  aac acc ata atg gaa       6332
Arg Arg Trp Cys Phe  Asp Gly Thr Thr Asn  Asn Thr Ile Met Glu
                2065                2070                2075

gac agt gtg ccg gca  gag gtg tgg acc aga  tac gga gag aaa aga       6377
Asp Ser Val Pro Ala  Glu Val Trp Thr Arg  Tyr Gly Glu Lys Arg
                2080                2085                2090

gtg ctc aaa ccg agg  tgg atg gac gcc aga  gtt tgt tca gat cat       6422
Val Leu Lys Pro Arg  Trp Met Asp Ala Arg  Val Cys Ser Asp His
                2095                2100                2105

gcg gcc ctg aag tca  ttc aaa gag ttt gcc  gct ggg aaa aga gga       6467
Ala Ala Leu Lys Ser  Phe Lys Glu Phe Ala  Ala Gly Lys Arg Gly
                2110                2115                2120
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gcg gcc ttt gga gtg  atg gaa gcc ctg gga  aca ctg cca gga cat       6512
Ala Ala Phe Gly Val  Met Glu Ala Leu Gly  Thr Leu Pro Gly His
                2125                2130                2135

atg aca gag aga ttc  cag gag gcc att gac  aac ctc gct gtg ctc       6557
Met Thr Glu Arg Phe  Gln Glu Ala Ile Asp  Asn Leu Ala Val Leu
                2140                2145                2150

atg cgg gca gag act  gga agc agg ccc tac  aaa gcc gcg gcg gcc       6602
Met Arg Ala Glu Thr  Gly Ser Arg Pro Tyr  Lys Ala Ala Ala Ala
                2155                2160                2165

caa tta ccg gag acc  cta gag act atc atg  ctt ttg ggg ttg ctg       6647
Gln Leu Pro Glu Thr  Leu Glu Thr Ile Met  Leu Leu Gly Leu Leu
                2170                2175                2180

gga aca gtc tcg ctg  gga atc ttt ttc gtc  ttg atg cgg aac aag       6692
Gly Thr Val Ser Leu  Gly Ile Phe Phe Val  Leu Met Arg Asn Lys
                2185                2190                2195

ggc ata ggg aag atg  ggc ttt gga atg gtg  act ctt ggg gcc agc       6737
Gly Ile Gly Lys Met  Gly Phe Gly Met Val  Thr Leu Gly Ala Ser
                2200                2205                2210

gca tgg ctt atg tgg  ctc tcg gaa att gag  cca gcc aga att gca       6782
Ala Trp Leu Met Trp  Leu Ser Glu Ile Glu  Pro Ala Arg Ile Ala
                2215                2220                2225

tgt gtc ctc att gtt  gtg ttc cta ttg ctg  gtg gtg ctc ata cct       6827
Cys Val Leu Ile Val  Val Phe Leu Leu Leu  Val Val Leu Ile Pro
                2230                2235                2240

gag cca gaa aag caa  aga tct ccc cag gac  aac caa atg gca atc       6872
Glu Pro Glu Lys Gln  Arg Ser Pro Gln Asp  Asn Gln Met Ala Ile
                2245                2250                2255

atc atc atg gta gca  gtg ggt ctt ctg ggc  ttg att acc gcc aat       6917
Ile Ile Met Val Ala  Val Gly Leu Leu Gly  Leu Ile Thr Ala Asn
                2260                2265                2270

gaa ctc gga tgg ttg  gag aga aca aag agt  gac cta agc cat cta       6962
Glu Leu Gly Trp Leu  Glu Arg Thr Lys Ser  Asp Leu Ser His Leu
                2275                2280                2285

atg gga agg aga gag  gag ggg gca act ata  gga ttc tca atg gac       7007
Met Gly Arg Arg Glu  Glu Gly Ala Thr Ile  Gly Phe Ser Met Asp
                2290                2295                2300

att gac ctg cgg cca  gcc tca gct tgg gct  atc tat gct gct ctg       7052
Ile Asp Leu Arg Pro  Ala Ser Ala Trp Ala  Ile Tyr Ala Ala Leu
                2305                2310                2315

aca act ttc att acc  cca gcc gtc caa cat  gca gtg acc act tca       7097
Thr Thr Phe Ile Thr  Pro Ala Val Gln His  Ala Val Thr Thr Ser
                2320                2325                2330

tac aac aac tac tcc  tta atg gcg atg gcc  acg caa gct gga gtg       7142
Tyr Asn Asn Tyr Ser  Leu Met Ala Met Ala  Thr Gln Ala Gly Val
                2335                2340                2345

ttg ttc ggt atg ggt  aaa ggg atg cca ttc  tat gca tgg gac ttt       7187
Leu Phe Gly Met Gly  Lys Gly Met Pro Phe  Tyr Ala Trp Asp Phe
                2350                2355                2360

gga gtc ccg ctg cta  atg ata ggt tgc tac  tca caa tta aca ccc       7232
Gly Val Pro Leu Leu  Met Ile Gly Cys Tyr  Ser Gln Leu Thr Pro
                2365                2370                2375

ctg acc cta ata gtg  gcc atc att ttg ctc  gtg gcg cac tac atg       7277
Leu Thr Leu Ile Val  Ala Ile Ile Leu Leu  Val Ala His Tyr Met
                2380                2385                2390

tac ttg atc cca ggg  ctg cag gca gca gct  gcg cgt gct gcc cag       7322
Tyr Leu Ile Pro Gly  Leu Gln Ala Ala Ala  Ala Arg Ala Ala Gln
                2395                2400                2405

aag aga acg gca gct  ggc atc atg aag aac  cct gtt gtg gat gga       7367
Lys Arg Thr Ala Ala  Gly Ile Met Lys Asn  Pro Val Val Asp Gly
                2410                2415                2420
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ata gtg gtg act gac  att gac aca atg aca  att gac ccc caa gtg       7412
Ile Val Val Thr Asp  Ile Asp Thr Met Thr  Ile Asp Pro Gln Val
                2425                2430                2435

gag aaa aag atg gga  cag gtg cta ctc ata  gca gta gct gtc tcc       7457
Glu Lys Lys Met Gly  Gln Val Leu Leu Ile  Ala Val Ala Val Ser
                2440                2445                2450

agc gcc ata ctg tcg  cgg acc gcc tgg ggg  tgg ggt gag gct ggg       7502
Ser Ala Ile Leu Ser  Arg Thr Ala Trp Gly  Trp Gly Glu Ala Gly
                2455                2460                2465

gcc ctg atc aca gct  gca act tcc act ttg  tgg gag ggc tct ccg       7547
Ala Leu Ile Thr Ala  Ala Thr Ser Thr Leu  Trp Glu Gly Ser Pro
                2470                2475                2480

aac aag tac tgg aac  tcc tcc aca gcc acc  tca ctg tgt aac att       7592
Asn Lys Tyr Trp Asn  Ser Ser Thr Ala Thr  Ser Leu Cys Asn Ile
                2485                2490                2495

ttt agg gga agc tac  ttg gct gga gct tct  cta atc tac aca gta       7637
Phe Arg Gly Ser Tyr  Leu Ala Gly Ala Ser  Leu Ile Tyr Thr Val
                2500                2505                2510

aca aga aac gct ggc  ttg gtc aag aga cgt  ggg ggt gga acg gga       7682
Thr Arg Asn Ala Gly  Leu Val Lys Arg Arg  Gly Gly Gly Thr Gly
                2515                2520                2525

gag acc ctg gga gag  aaa tgg aag gcc cgc  ctg aac cag atg tcg       7727
Glu Thr Leu Gly Glu  Lys Trp Lys Ala Arg  Leu Asn Gln Met Ser
                2530                2535                2540

gcc ctg gag ttc tac  tcc tac aaa aag tca  ggc atc acc gag gtg       7772
Ala Leu Glu Phe Tyr  Ser Tyr Lys Lys Ser  Gly Ile Thr Glu Val
                2545                2550                2555

tgc aga gaa gag gcc  cgc cgc gcc ctc aag  gac ggt gtg gca acg       7817
Cys Arg Glu Glu Ala  Arg Arg Ala Leu Lys  Asp Gly Val Ala Thr
                2560                2565                2570

gga ggc cac gct gtg  tcc cga gga agt gca  aag ctg aga tgg ttg       7862
Gly Gly His Ala Val  Ser Arg Gly Ser Ala  Lys Leu Arg Trp Leu
                2575                2580                2585

gtg gag agg gga tac  ctg cag ccc tat gga  aag gtc att gat ctt       7907
Val Glu Arg Gly Tyr  Leu Gln Pro Tyr Gly  Lys Val Ile Asp Leu
                2590                2595                2600

gga tgt ggc aga ggg  ggc tgg agt tac tat  gcc gcc acc atc cgc       7952
Gly Cys Gly Arg Gly  Gly Trp Ser Tyr Tyr  Ala Ala Thr Ile Arg
                2605                2610                2615

aaa gtt caa gaa gtg  aaa gga tac aca aaa  gga ggc cct ggt cat       7997
Lys Val Gln Glu Val  Lys Gly Tyr Thr Lys  Gly Gly Pro Gly His
                2620                2625                2630

gaa gaa ccc atg ttg  gtg caa agc tat ggg  tgg aac ata gtc cgt       8042
Glu Glu Pro Met Leu  Val Gln Ser Tyr Gly  Trp Asn Ile Val Arg
                2635                2640                2645

ctt aag agt ggg gtg  gac gtc ttt cat atg  gcg gct gag ccg tgt       8087
Leu Lys Ser Gly Val  Asp Val Phe His Met  Ala Ala Glu Pro Cys
                2650                2655                2660

gac acg ttg ctg tgt  gat ata ggt gag tca  tca tct agt cct gaa       8132
Asp Thr Leu Leu Cys  Asp Ile Gly Glu Ser  Ser Ser Ser Pro Glu
                2665                2670                2675

gtg gaa gaa gca cgg  acg ctc aga gtc ctc  tcc atg gtg ggg gat       8177
Val Glu Glu Ala Arg  Thr Leu Arg Val Leu  Ser Met Val Gly Asp
                2680                2685                2690

tgg ctt gaa aaa aga  cca gga gcc ttt tgt  ata aaa gtg ttg tgc       8222
Trp Leu Glu Lys Arg  Pro Gly Ala Phe Cys  Ile Lys Val Leu Cys
                2695                2700                2705

cca tac acc agc act  atg atg gaa acc ctg  gag cga ctg cag cgt       8267
Pro Tyr Thr Ser Thr  Met Met Glu Thr Leu  Glu Arg Leu Gln Arg
                2710                2715                2720
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agg tat ggg gga gga  ctg gtc aga gtg cca  ctc tcc cgc aac tct       8312
Arg Tyr Gly Gly Gly  Leu Val Arg Val Pro  Leu Ser Arg Asn Ser
                2725                2730                2735

aca cat gag atg tac  tgg gtc tct gga gcg  aaa agc aac acc ata       8357
Thr His Glu Met Tyr  Trp Val Ser Gly Ala  Lys Ser Asn Thr Ile
                2740                2745                2750

aaa agt gtg tcc acc  acg agc cag ctc ctt  ttg ggg cgc atg gac       8402
Lys Ser Val Ser Thr  Thr Ser Gln Leu Leu  Leu Gly Arg Met Asp
                2755                2760                2765

ggg ccc agg agg cca  gtg aaa tat gaa gag  gat gtg aat ctc ggc       8447
Gly Pro Arg Arg Pro  Val Lys Tyr Glu Glu  Asp Val Asn Leu Gly
                2770                2775                2780

tct ggc acg cgg gct  gtg gta agc tgc gct  gaa gct ccc aac atg       8492
Ser Gly Thr Arg Ala  Val Val Ser Cys Ala  Glu Ala Pro Asn Met
                2785                2790                2795

aag atc att ggt aac  cgc att gag agg atc  cgc agt gag cac gcg       8537
Lys Ile Ile Gly Asn  Arg Ile Glu Arg Ile  Arg Ser Glu His Ala
                2800                2805                2810

gaa acg tgg ttc ttt  gac gag aac cac cca  tat agg aca tgg gct       8582
Glu Thr Trp Phe Phe  Asp Glu Asn His Pro  Tyr Arg Thr Trp Ala
                2815                2820                2825

tac cat gga agc tac  gag gcc ccc aca caa  ggg tca gcg tcc tct       8627
Tyr His Gly Ser Tyr  Glu Ala Pro Thr Gln  Gly Ser Ala Ser Ser
                2830                2835                2840

cta ata aac ggg gtt  gtc agg ctc ctg tca  aaa ccc tgg gat gtg       8672
Leu Ile Asn Gly Val  Val Arg Leu Leu Ser  Lys Pro Trp Asp Val
                2845                2850                2855

gtg act gga gtc aca  gga ata gcc atg acc  gac acc aca ccg tat       8717
Val Thr Gly Val Thr  Gly Ile Ala Met Thr  Asp Thr Thr Pro Tyr
                2860                2865                2870

ggt cag caa aga gtt  ttc aag gaa aaa gtg  gac act agg gtg cca       8762
Gly Gln Gln Arg Val  Phe Lys Glu Lys Val  Asp Thr Arg Val Pro
                2875                2880                2885

gac ccc caa gaa ggc  act cgt cag gtt atg  agc atg gtc tct tcc       8807
Asp Pro Gln Glu Gly  Thr Arg Gln Val Met  Ser Met Val Ser Ser
                2890                2895                2900

tgg ttg tgg aaa gag  tta ggc aaa cac aaa  cgg cca cga gtc tgt       8852
Trp Leu Trp Lys Glu  Leu Gly Lys His Lys  Arg Pro Arg Val Cys
                2905                2910                2915

acc aaa gaa gag ttc  atc aac aag gtt cgt  agc aac gca gca tta       8897
Thr Lys Glu Glu Phe  Ile Asn Lys Val Arg  Ser Asn Ala Ala Leu
                2920                2925                2930

ggg gca ata ttt gaa  gag gaa aaa gag tgg  aag act gca gtg gaa       8942
Gly Ala Ile Phe Glu  Glu Glu Lys Glu Trp  Lys Thr Ala Val Glu
                2935                2940                2945

gct gtg aac gat cca  agg ttc tgg gct cta  gtg gac aag gaa aga       8987
Ala Val Asn Asp Pro  Arg Phe Trp Ala Leu  Val Asp Lys Glu Arg
                2950                2955                2960

gag cac cac ctg aga  gga gag tgc cag agc  tgt gtg tac aac atg       9032
Glu His His Leu Arg  Gly Glu Cys Gln Ser  Cys Val Tyr Asn Met
                2965                2970                2975

atg gga aaa aga gaa  aag aaa caa ggg gaa  ttt gga aag gcc aag       9077
Met Gly Lys Arg Glu  Lys Lys Gln Gly Glu  Phe Gly Lys Ala Lys
                2980                2985                2990

ggc agc cgc gcc atc  tgg tac atg tgg cta  ggg gct aga ttt cta       9122
Gly Ser Arg Ala Ile  Trp Tyr Met Trp Leu  Gly Ala Arg Phe Leu
                2995                3000                3005

gag ttc gaa gcc ctt  gga ttc ttg aac gag  gat cac tgg atg ggg       9167
Glu Phe Glu Ala Leu  Gly Phe Leu Asn Glu  Asp His Trp Met Gly
                3010                3015                3020
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aga gag aat tca gga  ggt ggt gtt gaa ggg  cta gga tta caa aga       9212
Arg Glu Asn Ser Gly  Gly Gly Val Glu Gly  Leu Gly Leu Gln Arg
                3025                3030                3035

ctc gga tat gtc tta  gaa gag atg agt cgc  ata cca gga gga agg       9257
Leu Gly Tyr Val Leu  Glu Glu Met Ser Arg  Ile Pro Gly Gly Arg
                3040                3045                3050

atg tat gca gat gat  act gct ggc tgg gac  acc cgc atc agc agg       9302
Met Tyr Ala Asp Asp  Thr Ala Gly Trp Asp  Thr Arg Ile Ser Arg
                3055                3060                3065

ttt gat ctg gag aat  gaa gct cta atc acc  aac caa atg gag aaa       9347
Phe Asp Leu Glu Asn  Glu Ala Leu Ile Thr  Asn Gln Met Glu Lys
                3070                3075                3080

ggg cac agg gcc ttg  gca ttg gcc ata atc  aag tac aca tac caa       9392
Gly His Arg Ala Leu  Ala Leu Ala Ile Ile  Lys Tyr Thr Tyr Gln
                3085                3090                3095

aac aaa gtg gta aag  gtc ctt aga cca gct  gaa aaa ggg aag aca       9437
Asn Lys Val Val Lys  Val Leu Arg Pro Ala  Glu Lys Gly Lys Thr
                3100                3105                3110

gtt atg gac att att  tca aga caa gac caa  agg ggg agc gga caa       9482
Val Met Asp Ile Ile  Ser Arg Gln Asp Gln  Arg Gly Ser Gly Gln
                3115                3120                3125

gtt gtc act tac gct  ctt aat aca ttt acc  aac cta gtg gtg cag       9527
Val Val Thr Tyr Ala  Leu Asn Thr Phe Thr  Asn Leu Val Val Gln
                3130                3135                3140

ctc att cgg aat atg  gag gct gag gaa gtt  cta gag atg caa gac       9572
Leu Ile Arg Asn Met  Glu Ala Glu Glu Val  Leu Glu Met Gln Asp
                3145                3150                3155

ttg tgg ctg ctg cgg  agg tca gag aaa gtg  acc aac tgg ttg cag       9617
Leu Trp Leu Leu Arg  Arg Ser Glu Lys Val  Thr Asn Trp Leu Gln
                3160                3165                3170

agc aat gga tgg gat  agg ctc aaa cga atg  gca gtc agt gga gat       9662
Ser Asn Gly Trp Asp  Arg Leu Lys Arg Met  Ala Val Ser Gly Asp
                3175                3180                3185

gat tgc gtt gtg aaa  cca att gat gat agg  ttt gca cat gct ctc       9707
Asp Cys Val Val Lys  Pro Ile Asp Asp Arg  Phe Ala His Ala Leu
                3190                3195                3200

agg ttc ttg aat gat  atg gga aaa gtt agg  aag gac aca caa gag       9752
Arg Phe Leu Asn Asp  Met Gly Lys Val Arg  Lys Asp Thr Gln Glu
                3205                3210                3215

tgg aag ccc tca act  gga tgg gac aac tgg  gaa gaa gtt ccg ttt       9797
Trp Lys Pro Ser Thr  Gly Trp Asp Asn Trp  Glu Glu Val Pro Phe
                3220                3225                3230

tgc tcc cac cac ttc  aac aag ctc cat ctc  aag gac ggg agg tcc       9842
Cys Ser His His Phe  Asn Lys Leu His Leu  Lys Asp Gly Arg Ser
                3235                3240                3245

att gtg gtt ccc tgc  cgc cac caa gat gaa  ctg att ggc cga gct       9887
Ile Val Val Pro Cys  Arg His Gln Asp Glu  Leu Ile Gly Arg Ala
                3250                3255                3260

cgc gtc tca ccg ggg  gcg gga tgg agc atc  cgg gag act gct tgc       9932
Arg Val Ser Pro Gly  Ala Gly Trp Ser Ile  Arg Glu Thr Ala Cys
                3265                3270                3275

cta gca aaa tca tat  gcg caa atg tgg cag  ctc ctt tat ttc cac       9977
Leu Ala Lys Ser Tyr  Ala Gln Met Trp Gln  Leu Leu Tyr Phe His
                3280                3285                3290

aga agg gac ctc cga  ctg atg gcc aat gcc  att tgt tca tct gtg      10022
Arg Arg Asp Leu Arg  Leu Met Ala Asn Ala  Ile Cys Ser Ser Val
                3295                3300                3305

cca gtt gac tgg gtt  cca act ggg aga act  acc tgg tca atc cat      10067
Pro Val Asp Trp Val  Pro Thr Gly Arg Thr  Thr Trp Ser Ile His
                3310                3315                3320
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gga aag gga gaa tgg  atg acc act gaa gac  atg ctt gtg gtg tgg      10112
Gly Lys Gly Glu Trp  Met Thr Thr Glu Asp  Met Leu Val Val Trp
                3325                3330                3335

aac aga gtg tgg att  gag gag aac gac cac  atg gaa gac aag acc      10157
Asn Arg Val Trp Ile  Glu Glu Asn Asp His  Met Glu Asp Lys Thr
                3340                3345                3350

cca gtt acg aaa tgg  aca gac att ccc tat  ttg gga aaa agg gaa      10202
Pro Val Thr Lys Trp  Thr Asp Ile Pro Tyr  Leu Gly Lys Arg Glu
                3355                3360                3365

gac ttg tgg tgt ggg  tct ctc ata ggg cac  aga ccg cgc acc acc      10247
Asp Leu Trp Cys Gly  Ser Leu Ile Gly His  Arg Pro Arg Thr Thr
                3370                3375                3380

tgg gct gag aac att  aaa aac aca gtc aac  atg atg cgt agg atc      10292
Trp Ala Glu Asn Ile  Lys Asn Thr Val Asn  Met Met Arg Arg Ile
                3385                3390                3395

ata ggt gat gaa gaa  aag tac gtg gac tac  cta tcc acc caa gtt      10337
Ile Gly Asp Glu Glu  Lys Tyr Val Asp Tyr  Leu Ser Thr Gln Val
                3400                3405                3410

cgc tac ttg ggc gaa  gaa ggg tcc aca cct  gga gtg cta gcaccaatct  10386
Arg Tyr Leu Gly Glu  Glu Gly Ser Thr Pro  Gly Val Leu
                3415                3420

tagtgttgtc aggcctgcta gtcagccaca gcttggggaa agctgtgcag cctgtgaccc  10446

ccccaggaga agctgggaaa ccaagcccat agtcaggccg agaacgccat ggcacggaag  10506

aagccatgct gcctgtgagc ccctcagagg acactgagtc aaaaaacccc acgcgcttgg  10566

aggcgcagga tgggaaaaga aggtggcgac cttccccacc ctttaatctg gggcctgaac  10626

tggagatcag ctgtggatct ccagaagagg gactagtggt tagaggagac cccccggaaa  10686

acgcaaaaca gcatattgac gctgggaaag accagagact ccatgagttt ccaccacgct  10746

ggccgccagg cacagatcgc cgaatagcgg cggccggtgt ggggaaatcc atgggtct    10804


<210> SEQ ID NO 2
<211> LENGTH: 3423
<212> TYPE: PRT
<213> ORGANISM: Flavivirus

<400> SEQUENCE: 2

Met Lys Asn Pro Lys Lys Lys Ser Gly Gly Phe Arg Ile Val Asn Met
1               5                  10                  15

Leu Lys Arg Gly Val Ala Arg Val Ser Pro Phe Gly Gly Leu Lys Arg
            20                  25                  30

Leu Pro Ala Gly Leu Leu Leu Gly His Gly Pro Ile Arg Met Val Leu
        35                  40                  45

Ala Ile Leu Ala Phe Leu Arg Phe Thr Ala Ile Lys Pro Ser Leu Gly
    50                  55                  60

Leu Ile Asn Arg Trp Gly Ser Val Gly Lys Lys Glu Ala Met Glu Ile
65                  70                  75                  80

Ile Lys Lys Phe Lys Lys Asp Leu Ala Ala Met Leu Arg Ile Ile Asn
                85                  90                  95

Ala Arg Lys Glu Lys Lys Arg Arg Gly Thr Asp Thr Ser Val Gly Ile
            100                 105                 110

Val Gly Leu Leu Leu Thr Thr Ala Met Ala Val Glu Val Thr Arg Arg
        115                 120                 125

Gly Asn Ala Tyr Tyr Met Tyr Leu Asp Arg Ser Asp Ala Gly Glu Ala
    130                 135                 140

Ile Ser Phe Pro Thr Thr Met Gly Met Asn Lys Cys Tyr Ile Gln Ile
145                 150                 155                 160
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Met Asp Leu Gly His Met Cys Asp Ala Thr Met Ser Tyr Glu Cys Pro
                165                 170                 175

Met Leu Asp Glu Gly Val Glu Pro Asp Asp Val Asp Cys Trp Cys Asn
            180                 185                 190

Thr Thr Ser Thr Trp Val Val Tyr Gly Thr Cys His His Lys Lys Gly
        195                 200                 205

Glu Ala Arg Arg Ser Arg Arg Ala Val Thr Leu Pro Ser His Ser Thr
    210                 215                 220

Arg Lys Leu Gln Thr Arg Ser Gln Thr Trp Leu Glu Ser Arg Glu Tyr
225                 230                 235                 240

Thr Lys His Leu Ile Arg Val Glu Asn Trp Ile Phe Arg Asn Pro Gly
                245                 250                 255

Phe Ala Leu Ala Ala Ala Ala Ile Ala Trp Leu Leu Gly Ser Ser Thr
            260                 265                 270

Ser Gln Lys Val Ile Tyr Leu Val Met Ile Leu Leu Ile Ala Pro Ala
        275                 280                 285

Tyr Ser Ile Arg Cys Ile Gly Val Ser Asn Arg Asp Phe Val Glu Gly
    290                 295                 300

Met Ser Gly Gly Thr Trp Val Asp Val Val Leu Glu His Gly Gly Cys
305                 310                 315                 320

Val Thr Val Met Ala Gln Asp Lys Pro Thr Val Asp Ile Glu Leu Val
                325                 330                 335

Thr Thr Thr Val Ser Asn Met Ala Glu Val Arg Ser Tyr Cys Tyr Glu
            340                 345                 350

Ala Ser Ile Ser Asp Met Ala Ser Asp Ser Arg Cys Pro Thr Gln Gly
        355                 360                 365

Glu Ala Tyr Leu Asp Lys Gln Ser Asp Thr Gln Tyr Val Cys Lys Arg
    370                 375                 380

Thr Leu Val Asp Arg Gly Trp Gly Asn Gly Cys Gly Leu Phe Gly Lys
385                 390                 395                 400

Gly Ser Leu Val Thr Cys Ala Lys Phe Ala Cys Ser Lys Lys Met Thr
                405                 410                 415

Gly Lys Ser Ile Gln Pro Glu Asn Leu Glu Tyr Arg Ile Met Leu Ser
            420                 425                 430

Val His Gly Ser Gln His Ser Gly Met Ile Val Asn Asp Thr Gly His
        435                 440                 445

Glu Thr Asp Glu Asn Arg Ala Lys Val Glu Ile Thr Pro Asn Ser Pro
    450                 455                 460

Arg Ala Glu Ala Thr Leu Gly Gly Phe Gly Ser Leu Gly Leu Asp Cys
465                 470                 475                 480

Glu Pro Arg Thr Gly Leu Asp Phe Ser Asp Leu Tyr Tyr Leu Thr Met
                485                 490                 495

Asn Asn Lys His Trp Leu Val His Lys Glu Trp Phe His Asp Ile Pro
            500                 505                 510

Leu Pro Trp His Ala Gly Ala Asp Thr Gly Thr Pro His Trp Asn Asn
        515                 520                 525

Lys Glu Ala Leu Val Glu Phe Lys Asp Ala His Ala Lys Arg Gln Thr
    530                 535                 540

Val Val Val Leu Gly Ser Gln Glu Gly Ala Val His Thr Ala Leu Ala
545                 550                 555                 560

Gly Ala Leu Glu Ala Glu Met Asp Gly Ala Lys Gly Arg Leu Ser Ser
                565                 570                 575
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Gly His Leu Lys Cys Arg Leu Lys Met Asp Lys Leu Arg Leu Lys Gly
            580                 585                 590

Val Ser Tyr Ser Leu Cys Thr Ala Ala Phe Thr Phe Thr Lys Ile Pro
        595                 600                 605

Ala Glu Thr Leu His Gly Thr Val Thr Val Glu Val Gln Tyr Ala Gly
    610                 615                 620

Thr Asp Gly Pro Cys Lys Val Pro Ala Gln Met Ala Val Asp Met Gln
625                 630                 635                 640

Thr Leu Thr Pro Val Gly Arg Leu Ile Thr Ala Asn Pro Val Ile Thr
                645                 650                 655

Glu Ser Thr Glu Asn Ser Lys Met Met Leu Glu Leu Asp Pro Pro Phe
            660                 665                 670

Gly Asp Ser Tyr Ile Val Ile Gly Val Gly Glu Lys Lys Ile Thr His
        675                 680                 685

His Trp His Arg Ser Gly Ser Thr Ile Gly Lys Ala Phe Glu Ala Thr
    690                 695                 700

Val Arg Gly Ala Lys Arg Met Ala Val Leu Gly Asp Thr Ala Trp Asp
705                 710                 715                 720

Phe Gly Ser Val Gly Gly Ala Leu Asn Ser Leu Gly Lys Gly Ile His
                725                 730                 735

Gln Ile Phe Gly Ala Ala Phe Lys Ser Leu Phe Gly Gly Met Ser Trp
            740                 745                 750

Phe Ser Gln Ile Leu Ile Gly Thr Leu Leu Val Trp Leu Gly Leu Asn
        755                 760                 765

Thr Lys Asn Gly Ser Ile Ser Leu Met Cys Leu Ala Leu Gly Gly Val
    770                 775                 780

Leu Ile Phe Leu Ser Thr Ala Val Ser Ala Asp Val Gly Cys Ser Val
785                 790                 795                 800

Asp Phe Ser Lys Lys Glu Thr Arg Cys Gly Thr Gly Val Phe Val Tyr
                805                 810                 815

Asn Asp Val Glu Ala Trp Arg Asp Arg Tyr Lys Tyr His Pro Asp Ser
            820                 825                 830

Pro Arg Arg Leu Ala Ala Ala Val Lys Gln Ala Trp Glu Asp Gly Ile
        835                 840                 845

Cys Gly Ile Ser Ser Val Ser Arg Met Glu Asn Ile Met Trp Arg Ser
    850                 855                 860

Val Glu Gly Glu Leu Asn Ala Ile Leu Glu Glu Asn Gly Val Gln Leu
865                 870                 875                 880

Thr Val Val Val Gly Ser Val Lys Asn Pro Met Trp Arg Gly Pro Gln
                885                 890                 895

Arg Leu Pro Val Pro Val Asn Glu Leu Pro His Gly Trp Lys Ala Trp
            900                 905                 910

Gly Lys Ser Tyr Phe Val Arg Ala Ala Lys Thr Asn Asn Ser Phe Val
        915                 920                 925

Val Asp Gly Asp Thr Leu Lys Glu Cys Pro Leu Lys His Arg Ala Trp
    930                 935                 940

Asn Ser Phe Leu Val Glu Asp His Gly Phe Gly Val Phe His Thr Ser
945                 950                 955                 960

Val Trp Leu Lys Val Arg Glu Asp Tyr Ser Leu Glu Cys Asp Pro Ala
                965                 970                 975

Val Ile Gly Thr Ala Ala Lys Gly Lys Glu Ala Val His Ser Asp Leu
            980                 985                 990
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Gly Tyr Trp Ile Glu Ser Glu Lys  Asn Asp Thr Trp Arg  Leu Lys Arg
        995                1000                1005

Ala His  Leu Ile Glu Met Lys  Thr Cys Glu Trp Pro  Lys Ser His
    1010                1015                1020

Thr Leu  Trp Thr Asp Gly Ile  Glu Glu Ser Asp Leu  Ile Ile Pro
    1025                1030                1035

Lys Ser  Leu Ala Gly Pro Leu  Ser His His Asn Thr  Arg Glu Gly
    1040                1045                1050

Tyr Arg  Thr Gln Met Glu Gly  Pro Trp His Ser Glu  Glu Leu Glu
    1055                1060                1065

Ile Arg  Phe Glu Glu Cys Pro  Gly Thr Lys Val His  Val Glu Glu
    1070                1075                1080

Thr Cys  Gly Thr Arg Gly Pro  Ser Leu Arg Ser Thr  Thr Ala Ser
    1085                1090                1095

Gly Arg  Val Ile Glu Glu Trp  Cys Cys Arg Glu Cys  Thr Met Pro
    1100                1105                1110

Pro Leu  Ser Phe Arg Ala Lys  Asp Gly Cys Trp Tyr  Gly Met Glu
    1115                1120                1125

Ile Arg  Pro Arg Lys Glu Pro  Glu Ser Asn Leu Val  Arg Ser Met
    1130                1135                1140

Val Thr  Ala Gly Ser Thr Asp  His Met Asp His Phe  Ser Leu Gly
    1145                1150                1155

Val Leu  Val Ile Leu Leu Met  Val Gln Glu Gly Leu  Lys Lys Arg
    1160                1165                1170

Met Thr  Thr Lys Ile Ile Ile  Ser Thr Ser Met Ala  Val Leu Val
    1175                1180                1185

Ala Met  Ile Leu Gly Gly Phe  Ser Met Ser Asp Leu  Ala Lys Leu
    1190                1195                1200

Ala Ile  Leu Met Gly Ala Thr  Phe Ala Glu Met Asn  Thr Gly Gly
    1205                1210                1215

Asp Val  Ala His Leu Ala Leu  Ile Ala Ala Phe Lys  Val Arg Pro
    1220                1225                1230

Ala Leu  Leu Val Ser Phe Ile  Phe Arg Ala Asn Trp  Thr Pro Arg
    1235                1240                1245

Glu Ser  Met Leu Leu Ala Leu  Ala Ser Cys Leu Leu  Gln Thr Ala
    1250                1255                1260

Ile Ser  Ala Leu Glu Gly Asp  Leu Met Val Pro Ile  Asn Gly Phe
    1265                1270                1275

Ala Leu  Ala Trp Leu Ala Ile  Arg Ala Met Val Val  Pro Arg Thr
    1280                1285                1290

Asp Asn  Ile Thr Leu Ala Ile  Leu Ala Ala Leu Thr  Pro Leu Ala
    1295                1300                1305

Arg Gly  Thr Leu Leu Val Ala  Trp Arg Ala Gly Leu  Ala Thr Cys
    1310                1315                1320

Gly Gly  Phe Met Leu Leu Ser  Leu Lys Gly Lys Gly  Ser Val Lys
    1325                1330                1335

Lys Asn  Leu Pro Phe Val Met  Ala Leu Gly Leu Thr  Ala Val Arg
    1340                1345                1350

Leu Val  Asp Pro Ile Asn Val  Val Gly Leu Leu Leu  Leu Thr Arg
    1355                1360                1365

Ser Gly  Lys Arg Ser Trp Pro  Pro Ser Glu Val Leu  Thr Ala Val
    1370                1375                1380
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Gly Leu  Ile Cys Ala Leu Ala  Gly Gly Phe Ala Lys  Ala Asp Ile
    1385                 1390                 1395

Glu Met  Ala Gly Pro Met Ala  Ala Val Gly Leu Leu  Ile Val Ser
    1400                 1405                 1410

Tyr Val  Val Ser Gly Lys Ser  Val Asp Met Tyr Ile  Glu Arg Ala
    1415                 1420                 1425

Gly Asp  Ile Thr Trp Glu Lys  Asp Ala Glu Val Thr  Gly Asn Ser
    1430                 1435                 1440

Pro Arg  Leu Asp Val Ala Leu  Asp Glu Ser Gly Asp  Phe Ser Leu
    1445                 1450                 1455

Val Glu  Asp Asp Gly Pro Pro  Met Arg Glu Ile Ile  Leu Lys Val
    1460                 1465                 1470

Val Leu  Met Ala Ile Cys Gly  Met Asn Pro Ile Ala  Ile Pro Phe
    1475                 1480                 1485

Ala Ala  Gly Ala Trp Tyr Val  Tyr Val Lys Thr Gly  Lys Arg Ser
    1490                 1495                 1500

Gly Ala  Leu Trp Asp Val Pro  Ala Pro Lys Glu Val  Lys Lys Gly
    1505                 1510                 1515

Glu Thr  Thr Asp Gly Val Tyr  Arg Val Met Thr Arg  Arg Leu Leu
    1520                 1525                 1530

Gly Ser  Thr Gln Val Gly Val  Gly Val Met Gln Glu  Gly Val Phe
    1535                 1540                 1545

His Thr  Met Trp His Val Thr  Lys Gly Ser Ala Leu  Arg Ser Gly
    1550                 1555                 1560

Glu Gly  Arg Leu Asp Pro Tyr  Trp Gly Asp Val Lys  Gln Asp Leu
    1565                 1570                 1575

Val Ser  Tyr Cys Gly Pro Trp  Lys Leu Asp Ala Ala  Trp Asp Gly
    1580                 1585                 1590

His Ser  Glu Val Gln Leu Leu  Ala Val Pro Pro Gly  Glu Arg Ala
    1595                 1600                 1605

Arg Asn  Ile Gln Thr Leu Pro  Gly Ile Phe Lys Thr  Lys Asp Gly
    1610                 1615                 1620

Asp Ile  Gly Ala Val Ala Leu  Asp Tyr Pro Ala Gly  Thr Ser Gly
    1625                 1630                 1635

Ser Pro  Ile Leu Asp Lys Cys  Gly Arg Val Ile Gly  Leu Tyr Gly
    1640                 1645                 1650

Asn Gly  Val Val Ile Lys Asn  Gly Ser Tyr Val Ser  Ala Ile Thr
    1655                 1660                 1665

Gln Gly  Arg Arg Glu Glu Glu  Thr Pro Val Glu Cys  Phe Glu Pro
    1670                 1675                 1680

Ser Met  Leu Lys Lys Lys Gln  Leu Thr Val Leu Asp  Leu His Pro
    1685                 1690                 1695

Gly Ala  Gly Lys Thr Arg Arg  Val Leu Pro Glu Ile  Val Arg Glu
    1700                 1705                 1710

Ala Ile  Lys Thr Arg Leu Arg  Thr Val Ile Leu Ala  Pro Thr Arg
    1715                 1720                 1725

Val Val  Ala Ala Glu Met Glu  Glu Ala Leu Arg Gly  Leu Pro Val
    1730                 1735                 1740

Arg Tyr  Met Thr Thr Ala Val  Asn Val Thr His Ser  Gly Thr Glu
    1745                 1750                 1755

Ile Val  Asp Leu Met Cys His  Ala Thr Phe Thr Ser  Arg Leu Leu
    1760                 1765                 1770
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Gln Pro  Ile Arg Val Pro Asn  Tyr Asn Leu Tyr Ile  Met Asp Glu
    1775                1780                1785

Ala His  Phe Thr Asp Pro Ser  Ser Ile Ala Ala Arg  Gly Tyr Ile
    1790                1795                1800

Ser Thr  Arg Val Glu Met Gly  Glu Ala Ala Ala Ile  Phe Met Thr
    1805                1810                1815

Ala Thr  Pro Pro Gly Thr Arg  Asp Ala Phe Pro Asp  Ser Asn Ser
    1820                1825                1830

Pro Ile  Met Asp Thr Glu Val  Glu Val Pro Glu Arg  Ala Trp Ser
    1835                1840                1845

Ser Gly  Phe Asp Trp Val Thr  Asp His Ser Gly Lys  Thr Val Trp
    1850                1855                1860

Phe Val  Pro Ser Val Arg Asn  Gly Asn Glu Ile Ala  Ala Cys Leu
    1865                1870                1875

Thr Lys  Ala Gly Lys Arg Val  Ile Gln Leu Ser Arg  Lys Thr Phe
    1880                1885                1890

Glu Thr  Glu Phe Gln Lys Thr  Lys His Gln Glu Trp  Asp Phe Val
    1895                1900                1905

Val Thr  Thr Asp Ile Ser Glu  Met Gly Ala Asn Phe  Lys Ala Asp
    1910                1915                1920

Arg Val  Ile Asp Ser Arg Arg  Cys Leu Lys Pro Val  Ile Leu Asp
    1925                1930                1935

Gly Glu  Arg Val Ile Leu Ala  Gly Pro Met Pro Val  Thr His Ala
    1940                1945                1950

Ser Ala  Ala Gln Arg Arg Gly  Arg Ile Gly Arg Asn  Pro Asn Lys
    1955                1960                1965

Pro Gly  Asp Glu Tyr Leu Tyr  Gly Gly Gly Cys Ala  Glu Thr Asp
    1970                1975                1980

Glu Asp  His Ala His Trp Leu  Glu Ala Arg Met Leu  Leu Asp Asn
    1985                1990                1995

Ile Tyr  Leu Gln Asp Gly Leu  Ile Ala Ser Leu Tyr  Arg Pro Glu
    2000                2005                2010

Ala Asp  Lys Val Ala Ala Ile  Glu Gly Glu Phe Lys  Leu Arg Thr
    2015                2020                2025

Glu Gln  Arg Lys Thr Phe Val  Glu Leu Met Lys Arg  Gly Asp Leu
    2030                2035                2040

Pro Val  Trp Leu Ala Tyr Gln  Val Ala Ser Ala Gly  Ile Thr Tyr
    2045                2050                2055

Thr Asp  Arg Arg Trp Cys Phe  Asp Gly Thr Thr Asn  Asn Thr Ile
    2060                2065                2070

Met Glu  Asp Ser Val Pro Ala  Glu Val Trp Thr Arg  Tyr Gly Glu
    2075                2080                2085

Lys Arg  Val Leu Lys Pro Arg  Trp Met Asp Ala Arg  Val Cys Ser
    2090                2095                2100

Asp His  Ala Ala Leu Lys Ser  Phe Lys Glu Phe Ala  Ala Gly Lys
    2105                2110                2115

Arg Gly  Ala Ala Phe Gly Val  Met Glu Ala Leu Gly  Thr Leu Pro
    2120                2125                2130

Gly His  Met Thr Glu Arg Phe  Gln Glu Ala Ile Asp  Asn Leu Ala
    2135                2140                2145

Val Leu  Met Arg Ala Glu Thr  Gly Ser Arg Pro Tyr  Lys Ala Ala
    2150                2155                2160
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Ala Ala  Gln Leu Pro Glu Thr  Leu Glu Thr Ile Met  Leu Leu Gly
    2165                 2170                 2175

Leu Leu  Gly Thr Val Ser Leu  Gly Ile Phe Phe Val  Leu Met Arg
    2180                 2185                 2190

Asn Lys  Gly Ile Gly Lys Met  Gly Phe Gly Met Val  Thr Leu Gly
    2195                 2200                 2205

Ala Ser  Ala Trp Leu Met Trp  Leu Ser Glu Ile Glu  Pro Ala Arg
    2210                 2215                 2220

Ile Ala  Cys Val Leu Ile Val  Val Phe Leu Leu Leu  Val Val Leu
    2225                 2230                 2235

Ile Pro  Glu Pro Glu Lys Gln  Arg Ser Pro Gln Asp  Asn Gln Met
    2240                 2245                 2250

Ala Ile  Ile Ile Met Val Ala  Val Gly Leu Leu Gly  Leu Ile Thr
    2255                 2260                 2265

Ala Asn  Glu Leu Gly Trp Leu  Glu Arg Thr Lys Ser  Asp Leu Ser
    2270                 2275                 2280

His Leu  Met Gly Arg Arg Glu  Glu Gly Ala Thr Ile  Gly Phe Ser
    2285                 2290                 2295

Met Asp  Ile Asp Leu Arg Pro  Ala Ser Ala Trp Ala  Ile Tyr Ala
    2300                 2305                 2310

Ala Leu  Thr Thr Phe Ile Thr  Pro Ala Val Gln His  Ala Val Thr
    2315                 2320                 2325

Thr Ser  Tyr Asn Asn Tyr Ser  Leu Met Ala Met Ala  Thr Gln Ala
    2330                 2335                 2340

Gly Val  Leu Phe Gly Met Gly  Lys Gly Met Pro Phe  Tyr Ala Trp
    2345                 2350                 2355

Asp Phe  Gly Val Pro Leu Leu  Met Ile Gly Cys Tyr  Ser Gln Leu
    2360                 2365                 2370

Thr Pro  Leu Thr Leu Ile Val  Ala Ile Ile Leu Leu  Val Ala His
    2375                 2380                 2385

Tyr Met  Tyr Leu Ile Pro Gly  Leu Gln Ala Ala Ala  Ala Arg Ala
    2390                 2395                 2400

Ala Gln  Lys Arg Thr Ala Ala  Gly Ile Met Lys Asn  Pro Val Val
    2405                 2410                 2415

Asp Gly  Ile Val Val Thr Asp  Ile Asp Thr Met Thr  Ile Asp Pro
    2420                 2425                 2430

Gln Val  Glu Lys Lys Met Gly  Gln Val Leu Leu Ile  Ala Val Ala
    2435                 2440                 2445

Val Ser  Ser Ala Ile Leu Ser  Arg Thr Ala Trp Gly  Trp Gly Glu
    2450                 2455                 2460

Ala Gly  Ala Leu Ile Thr Ala  Ala Thr Ser Thr Leu  Trp Glu Gly
    2465                 2470                 2475

Ser Pro  Asn Lys Tyr Trp Asn  Ser Ser Thr Ala Thr  Ser Leu Cys
    2480                 2485                 2490

Asn Ile  Phe Arg Gly Ser Tyr  Leu Ala Gly Ala Ser  Leu Ile Tyr
    2495                 2500                 2505

Thr Val  Thr Arg Asn Ala Gly  Leu Val Lys Arg Arg  Gly Gly Gly
    2510                 2515                 2520

Thr Gly  Glu Thr Leu Gly Glu  Lys Trp Lys Ala Arg  Leu Asn Gln
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Met Ser  Ala Leu Glu Phe Tyr  Ser Tyr Lys Lys Ser  Gly Ile Thr
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Glu Val  Cys Arg Glu Glu Ala  Arg Arg Ala Leu Lys  Asp Gly Val
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Ala Thr  Gly Gly His Ala Val  Ser Arg Gly Ser Ala  Lys Leu Arg
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Trp Leu  Val Glu Arg Gly Tyr  Leu Gln Pro Tyr Gly  Lys Val Ile
    2585                 2590                 2595

Asp Leu  Gly Cys Gly Arg Gly  Gly Trp Ser Tyr Tyr  Ala Ala Thr
    2600                 2605                 2610

Ile Arg  Lys Val Gln Glu Val  Lys Gly Tyr Thr Lys  Gly Gly Pro
    2615                 2620                 2625

Gly His  Glu Glu Pro Met Leu  Val Gln Ser Tyr Gly  Trp Asn Ile
    2630                 2635                 2640

Val Arg  Leu Lys Ser Gly Val  Asp Val Phe His Met  Ala Ala Glu
    2645                 2650                 2655

Pro Cys  Asp Thr Leu Leu Cys  Asp Ile Gly Glu Ser  Ser Ser Ser
    2660                 2665                 2670

Pro Glu  Val Glu Glu Ala Arg  Thr Leu Arg Val Leu  Ser Met Val
    2675                 2680                 2685

Gly Asp  Trp Leu Glu Lys Arg  Pro Gly Ala Phe Cys  Ile Lys Val
    2690                 2695                 2700

Leu Cys  Pro Tyr Thr Ser Thr  Met Met Glu Thr Leu  Glu Arg Leu
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Gln Arg  Arg Tyr Gly Gly Gly  Leu Val Arg Val Pro  Leu Ser Arg
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Asn Ser  Thr His Glu Met Tyr  Trp Val Ser Gly Ala  Lys Ser Asn
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Thr Ile  Lys Ser Val Ser Thr  Thr Ser Gln Leu Leu  Leu Gly Arg
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Met Asp  Gly Pro Arg Arg Pro  Val Lys Tyr Glu Glu  Asp Val Asn
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Leu Gly  Ser Gly Thr Arg Ala  Val Val Ser Cys Ala  Glu Ala Pro
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Asn Met  Lys Ile Ile Gly Asn  Arg Ile Glu Arg Ile  Arg Ser Glu
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His Ala  Glu Thr Trp Phe Phe  Asp Glu Asn His Pro  Tyr Arg Thr
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Trp Ala  Tyr His Gly Ser Tyr  Glu Ala Pro Thr Gln  Gly Ser Ala
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Ser Ser  Leu Ile Asn Gly Val  Val Arg Leu Leu Ser  Lys Pro Trp
    2840                 2845                 2850

Asp Val  Val Thr Gly Val Thr  Gly Ile Ala Met Thr  Asp Thr Thr
    2855                 2860                 2865

Pro Tyr  Gly Gln Gln Arg Val  Phe Lys Glu Lys Val  Asp Thr Arg
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Val Pro  Asp Pro Gln Glu Gly  Thr Arg Gln Val Met  Ser Met Val
    2885                 2890                 2895

Ser Ser  Trp Leu Trp Lys Glu  Leu Gly Lys His Lys  Arg Pro Arg
    2900                 2905                 2910

Val Cys  Thr Lys Glu Glu Phe  Ile Asn Lys Val Arg  Ser Asn Ala
    2915                 2920                 2925

Ala Leu  Gly Ala Ile Phe Glu  Glu Glu Lys Glu Trp  Lys Thr Ala
    2930                 2935                 2940
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Val Glu  Ala Val Asn Asp Pro  Arg Phe Trp Ala Leu  Val Asp Lys
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Glu Arg  Glu His His Leu Arg  Gly Glu Cys Gln Ser  Cys Val Tyr
    2960                 2965                 2970

Asn Met  Met Gly Lys Arg Glu  Lys Lys Gln Gly Glu  Phe Gly Lys
    2975                 2980                 2985

Ala Lys  Gly Ser Arg Ala Ile  Trp Tyr Met Trp Leu  Gly Ala Arg
    2990                 2995                 3000

Phe Leu  Glu Phe Glu Ala Leu  Gly Phe Leu Asn Glu  Asp His Trp
    3005                 3010                 3015

Met Gly  Arg Glu Asn Ser Gly  Gly Gly Val Glu Gly  Leu Gly Leu
    3020                 3025                 3030

Gln Arg  Leu Gly Tyr Val Leu  Glu Glu Met Ser Arg  Ile Pro Gly
    3035                 3040                 3045

Gly Arg  Met Tyr Ala Asp Asp  Thr Ala Gly Trp Asp  Thr Arg Ile
    3050                 3055                 3060

Ser Arg  Phe Asp Leu Glu Asn  Glu Ala Leu Ile Thr  Asn Gln Met
    3065                 3070                 3075

Glu Lys  Gly His Arg Ala Leu  Ala Leu Ala Ile Ile  Lys Tyr Thr
    3080                 3085                 3090

Tyr Gln  Asn Lys Val Val Lys  Val Leu Arg Pro Ala  Glu Lys Gly
    3095                 3100                 3105

Lys Thr  Val Met Asp Ile Ile  Ser Arg Gln Asp Gln  Arg Gly Ser
    3110                 3115                 3120

Gly Gln  Val Val Thr Tyr Ala  Leu Asn Thr Phe Thr  Asn Leu Val
    3125                 3130                 3135

Val Gln  Leu Ile Arg Asn Met  Glu Ala Glu Glu Val  Leu Glu Met
    3140                 3145                 3150

Gln Asp  Leu Trp Leu Leu Arg  Arg Ser Glu Lys Val  Thr Asn Trp
    3155                 3160                 3165

Leu Gln  Ser Asn Gly Trp Asp  Arg Leu Lys Arg Met  Ala Val Ser
    3170                 3175                 3180

Gly Asp  Asp Cys Val Val Lys  Pro Ile Asp Asp Arg  Phe Ala His
    3185                 3190                 3195

Ala Leu  Arg Phe Leu Asn Asp  Met Gly Lys Val Arg  Lys Asp Thr
    3200                 3205                 3210

Gln Glu  Trp Lys Pro Ser Thr  Gly Trp Asp Asn Trp  Glu Glu Val
    3215                 3220                 3225

Pro Phe  Cys Ser His His Phe  Asn Lys Leu His Leu  Lys Asp Gly
    3230                 3235                 3240

Arg Ser  Ile Val Val Pro Cys  Arg His Gln Asp Glu  Leu Ile Gly
    3245                 3250                 3255

Arg Ala  Arg Val Ser Pro Gly  Ala Gly Trp Ser Ile  Arg Glu Thr
    3260                 3265                 3270

Ala Cys  Leu Ala Lys Ser Tyr  Ala Gln Met Trp Gln  Leu Leu Tyr
    3275                 3280                 3285

Phe His  Arg Arg Asp Leu Arg  Leu Met Ala Asn Ala  Ile Cys Ser
    3290                 3295                 3300

Ser Val  Pro Val Asp Trp Val  Pro Thr Gly Arg Thr  Thr Trp Ser
    3305                 3310                 3315

Ile His  Gly Lys Gly Glu Trp  Met Thr Thr Glu Asp  Met Leu Val
    3320                 3325                 3330
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Val Trp  Asn Arg Val Trp Ile  Glu Glu Asn Asp His  Met Glu Asp
    3335                3340                3345

Lys Thr  Pro Val Thr Lys Trp  Thr Asp Ile Pro Tyr  Leu Gly Lys
    3350                3355                3360

Arg Glu  Asp Leu Trp Cys Gly  Ser Leu Ile Gly His  Arg Pro Arg
    3365                3370                3375

Thr Thr  Trp Ala Glu Asn Ile  Lys Asn Thr Val Asn  Met Met Arg
    3380                3385                3390

Arg Ile  Ile Gly Asp Glu Glu  Lys Tyr Val Asp Tyr  Leu Ser Thr
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Gln Val  Arg Tyr Leu Gly Glu  Glu Gly Ser Thr Pro  Gly Val Leu
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cttccgacca tcaagcattt tatccgtact cctgatgatg catggttact caccactgcg 180

atccccggga aaacagcatt ccaggtatta gaagaatatc ctgattcagg tgaaaatatt 240

gttgatgcgc tggcagtgtt cctgcgccgg ttgcattcga ttcctgtttg taattgtcct 300

tttaacagcg atcgcgtatt tcgtctcgct caggcgcaat cacgaatgaa taacggtttg 360

gttgatgcga gtgattttga tgacgagcgt aatggctggc ctgttgaaca agtctggaaa 420

gaaatgcata agcttttgcc attctcaccg gattcagtcg tcactcatgg tgatttctca 480

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ctcggtcgcc gcatacacta ttctcagaat gacttggttg agtactcacc agtcacagaa   1980

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ggaagcggaa tatatcctgt atcacatatt ctgcggccgc                         3400
```

The invention claimed is:

1. An assay for detecting flavivirus infection comprising:

contacting a sample from a subject suspected of having a flavivirus infection with a reporter Zika virus (rZIKV), the rZIKV configured to produce a detectable signal when expressed in viable cell, forming a reporter mixture and incubating the reporter mixture at a temperature of 35 to 40° C.;

contacting a host cell monolayer with the reporter mixture under cell growth conditions at about 37° C. forming an inoculated cell monolayer;

measuring the reporter signal produced by the inoculated cell monolayer and normalizing the measured signal to a control; and

calculating a ZIKV antibody titer of the sample using the reporter signal measurements.

2. The assay of claim **1**, wherein a serial dilution of the sample contacted with the rZIKV.

3. The assay of claim **1**, wherein a plurality of samples are assayed individually.

4. The assay of claim **1**, wherein the sample is a blood sample.

5. The assay of claim **1**, wherein the sample is from a pregnant subject.

6. The assay of claim **1**, wherein the subject is a mammalian subject.

7. The assay of claim **6**, wherein the subject is human.

8. The assay of claim **1**, wherein the rZIKV is a luciferase reporter ZIKV.

9. The assay of claim **1**, wherein the luciferase is *Renilla* luciferase.

10. The assay of claim **1**, wherein the cell monolayer is a Vero cell monolayer.

11. The assay of claim **1**, wherein the cell monolayers are assayed in a multi-well plate.

12. The assay of claim 1, wherein the inoculated cells are incubated for about 12, 24, 36, or 48 hours before measuring the reporter signal.

13. The assay of claim 1, further comprising:

contacting a sample from a subject suspected of having a flavivirus infection with a reporter dengue virus (rDENV), the rDENV configured to produce a detectable signal when infecting a viable cell, forming a reporter mixture and incubating the reporter mixture at a temperature of 35 to 40° C.;

contacting a host cell monolayer with the reporter mixture under cell growth conditions at about 37° C. forming an inoculated cell monolayer;

measuring the reporter signal produced by the inoculated cell monolayer and normalizing the measured signal to a control; and

calculating a DENV antibody titer of the sample using the reporter signal measurements.

14. The assay of claim 1, further comprising performing virus specific DNA amplification using a second sample from the subject suspected of having a flavivirus infection.

15. The assay of claim 14, wherein the DNA amplification is a viral RT-PCR assay.

16. A recombinant DNA expression cassette comprising a flavivirus nucleic acid segment that is at least 95% identical to the nucleic acid sequence of SEQ ID NO:1.

17. A recombinant flavivirus genome comprising a nucleic acid sequence SEQ ID NO: 1 and a heterologous nucleic acid segment.

18. The recombinant flavivirus of claim 17, wherein the heterologous nucleic acid segment encodes a reporter protein.

19. The recombinant flavivirus of claim 18, wherein the reporter protein is a fluorescent protein.

* * * * *