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Research Paper

# A Rapid Zika Diagnostic Assay to Measure Neutralizing Antibodies in Patients

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ABSTRACT

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. To overcome this limitation, we have developed a high-throughput assay for ZIKV and dengue virus (DENV) diagnosis that can attain the "gold standard" of the current PRNT assay. The new assay is homogeneous and utilizes luciferase viruses to quantify the neutralizing antibody titers in a 96-well format. Using 91 human specimens, we showed that the reporter diagnostic assay 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, our results suggest that, along with the viral RT-PCR assay, the reporter virus-based serologic assay could be potentially used as the first-line test for clinical diagnosis of ZIKV infection as well as for vaccine clinical trials.

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## 1. Introduction

Many flaviviruses cause significant human morbidity and mortality, including the four serotypes of dengue virus (DENV-1 to -4), Zika virus (ZIKV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV). These viruses belong to the genus *Flavivirus* within the family Flaviviridae, and are predominantly transmitted by mosquitoes or ticks. Besides insect vectors, flaviviruses could also be transmitted through other routes. In the case of ZIKV, the virus was found to be transmitted by the *Aedes* spp. mosquitoes as well as through maternofetal route, sexual intercourse, blood transfusion, and organ transplantation (Musso and Gubler, 2016; Shan et al., 2016a). The genome of flavivirus is a single-strand, positive-sense RNA of approximately 11,000 nucleotides. It consists of a 5′ untranslated region (UTR), a single open-reading frame (ORF), and a 3′ UTR. The ORF encodes three structural proteins [capsid (C), precursor membrane (prM), and envelope (E)] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form virus particles, and are responsible for attachment and entry into host cells. The nonstructural proteins function in viral replication, virion assembly, and evasion of host immune response (Lindenbach et al., 2013).

The diagnosis to differentiate different flavivirus infections has been challenging. Three factors could contribute to this challenge. (i) Many flavivirus infections are asymptomatic and, even in patients exhibiting symptoms, infections with different flaviviruses produce similar disease syndromes, making it difficult to clinically differentiate them. Some flaviviruses, such as DENV, ZIKV, and YFV as well as some non-flavi-arboviruses (e.g., Chikungunya virus), often co-circulate in the same geographic regions. (ii) The viremic phase is short during flavivirus infection. When patients with symptoms present to clinics, their viremia is often at low or undetectable levels, imposing a narrow diagnostic window for detection of viral components. Virus and viral components can be detected by a number of assays, including RT-PCR, ELISA, other immunoassays, and virus isolation, among which RT-PCR is the most popular assay because of its sensitivity and specificity

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(Lanciotti et al., 2008). (iii) Due to the short duration of flavivirus viremic phase, host response-based serologic assays play an important role in patient diagnosis, among which IgM-capture ELISA is the most commonly used assay. The IgM ELISA-positive specimens are recommended for confirmation using a Plaque Reduction Neutralization Test (PRNT). For ELISA-based ZIKV serologic diagnosis, besides the IgM-capture ELISA developed by CDC (Lanciotti et al., 2008), a number of viral E- and NS1-based tests have been developed, including the InBios' E-based IgM-capture ELISA [received Emergency Use Authorization (EUA) approval from FDA], EuroImmun's NS1-based indirect ELISA (approved for clinical use in Europe), and NovaTec's NS1-based IgM-capture ELISA (for investigational research use). A multiplex microsphere immunoassay using ZIKV NS1 and NS5 antigens (in addition to E protein) was recently reported to improve the assay specificity (Wong et al., 2017), supporting the previous notion that antibody responses to flavivirus nonstructural proteins could be more virus-type specific than those to the structural proteins (Garcia et al., 1997; Shu et al., 2000; Stettler et al., 2016; Wong et al., 2003).

Since PRNT remains the "gold standard" for arbovirus serology, specimens with positive IgM-capture ELISA results are recommended for confirmation in the PRNT assay. However, PRNT assay is time consuming (with a turnaround time of more than a week), labor intensive, and low throughput. These constraints place PRNT as a rate-limiting step in patient diagnosis. The delay of PRNT results could lead to compromised patient care. Therefore, there is an urgent need to develop a rapid PRNT assay with an improved throughput and turnaround time. In this communication, we report a homogeneous high-throughput neutralization assay using a reporter ZIKV and DENV-2. Using 91 human sera, we demonstrated that the reporter virus assay generated diagnostic results equivalent to those obtained with the traditional plaque assay. Importantly, the reporter virus test has shortened the turnaround time to <48 h, increased the assay dynamic range by approximately 2.5 folds, and enabled a 96-well high-throughput format.

## 2. Materials and Methods

### 2.1. 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, UT) and 1% penicillin/streptomycin at 37 °C with 5% $CO_2$. For the traditional PRNT assay, we 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., 2016b; Zou et al., 2011). 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., 2016b). For both ZIKV and DENV-2, the luciferase reporter gene was engineered at the beginning of the open-reading-frame of the viral genome, as detailed in previous publications (Shan et al., 2016b; Zou et al., 2011). The cDNA clones for reporter ZIKV and DENV-2 are available for research use upon request.

### 2.2. 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 (specimens 1–10 in Table 1) from University of Texas Medical Branch (UTMB) that were submitted for routine screening for agents other than Zika virus, and 81 samples (specimens 11–91 in Table 1) 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)]. The UTMB samples were carefully selected from the patients with least possibility of exposure to ZIKV and DENV infection; these samples served as negative specimens with neither ZIKV nor DENV infections (numbers 1–10 in Table 1). As described recently (Wong et al., 2017), the sera from New York State Department of Health (specimens 11–91 in Table 1) 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.

### 2.3. 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 ($1.5 \times 10^4$ 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, TX) containing 2% FBS and 1% penicillin/streptomycin, followed by 2-fold serial dilution ($2^1$–$2^9$). Thirty microliters of each serum dilution were mixed thoroughly with 30 μl reporter ZIKV or DENV-2 and incubated at 37 °C for 1 h 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 h. The intracellular luciferase signals were measured using ViviRen substrates (Promega, Madison, WI) 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 ($NT_{90}$). Raw data of the reporter assay are available up request.

### 2.4. Plaque Reduction Neutralization Test (PRNT)

A standard double-layer plaque assay (Shi et al., 2002) was performed to determine the PRNTs of each patient serum. We 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 1/2 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 h, 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 h, 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

**Table 1**
Comparison of neutralization titers from plaque assay ($PRNT_{90}$) and reporter virus assay ($NT_{90}$).[a]

| Group number | Specimen number | Plaque assay | | Luciferase assay | |
|---|---|---|---|---|---|
| | | ZIKV | DENV | ZIKV | DENV |
| Group I | 1–10[b] | <10 | <10 | <10 | <10 |
| Group II | 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 |
| Group III | 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 |
| Group IV | 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 | 17,346 |
| | 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 | 24,147 |
| | 80 | 1280 | 5120 | 2937 | 3174 |
| | 81 | 1280 | 20,480 | 7729 | 31,361 |
| | 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 | 20,480 | 2555 | 18,316 |
| | 89 | 5120 | 5120 | 934 | 2353 |
| | 90 | 5120 | 1280 | 6352 | 1237 |
| | 91 | 5120 | 5120 | 12,068 | 8925 |

[a] The $PRNT_{90}$ and $NT_{90}$ values were derived from two to three replicate experiments.
[b] The results for specimens 1–10 were identical and, therefore, are combined in this section.

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 ($PRNT_{90}$).

## 3. Results

### 3.1. Assay Design

We 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, we 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. We 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. 1). We 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. 1). Fig. 2 summarizes the optimal assay protocol. Specifically, Vero cells ($1.5 \times 10^4$ 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 DENV that had been pre-incubated with serially diluted patient sera at 37 °C for 60 min. At 24 h p.i., 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 <48 h.

### 3.2. 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 were 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 1, group I specimens (n = 10; specimen numbers 1–10) were negative in neutralizing ZIKV and DENV. Group II specimens (n = 6; specimen numbers 11–16) were negative or marginally positive in neutralizing ZIKV, but positive in neutralizing DENV. Group III specimens (n = 23; specimen numbers 17–39) were positive in neutralizing ZIKV, but negative or marginally positive in neutralizing DENV. Group IV specimens (n = 52 patients; specimen numbers 40–91) were positive in neutralizing both ZIKV and DENV. It is worth pointing out that, due to possible cross-neutralization of antibodies between ZIKV and DENV, patients from group IV

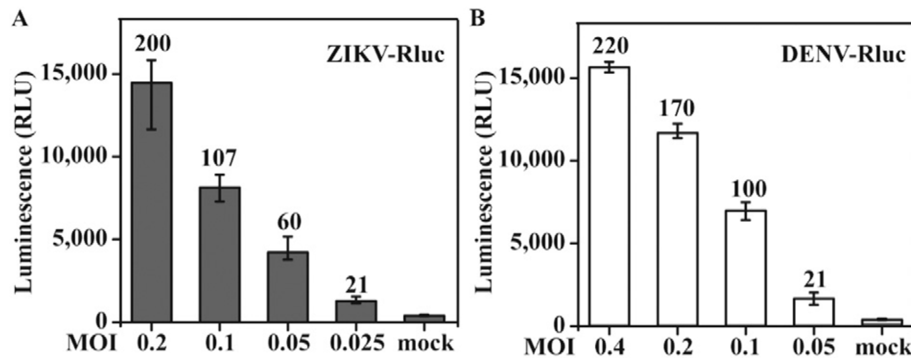


**Fig. 1.** Optimization of the inoculums of *Renilla* luciferase (Rluc) ZIKV (A) and DENV-2 (B) for the neutralization assay. The experimental protocol is detailed in Materials and Methods. Different MOIs of virus inoculum and their luciferase activities at 24 h post-infection are presented. Ratios 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.

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. The complex interpretation of PRNT or other neutralization test results have been well documented in flavivirus literature (Kuno et al., 1993; Midgley et al., 2011).

### 3.3. Comparison of Traditional PRNT and Reporter Virus Assays

All 91 patient samples were subjected to the reporter ZIKV and DENV assay. Table 1 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. 3 shows the scatter plot of 90% neutralization titers derived from the two assay formats for ZIKV and DENV, with $R^2$ correlation coefficients of 0.41 and 0.63, respectively, suggesting a general concordance between the reporter and plaque assays. (ii) Specimens from groups II and III exhibited virus type-specific neutralizating activities against DENV and ZIKV, respectively, when tested with both plaque

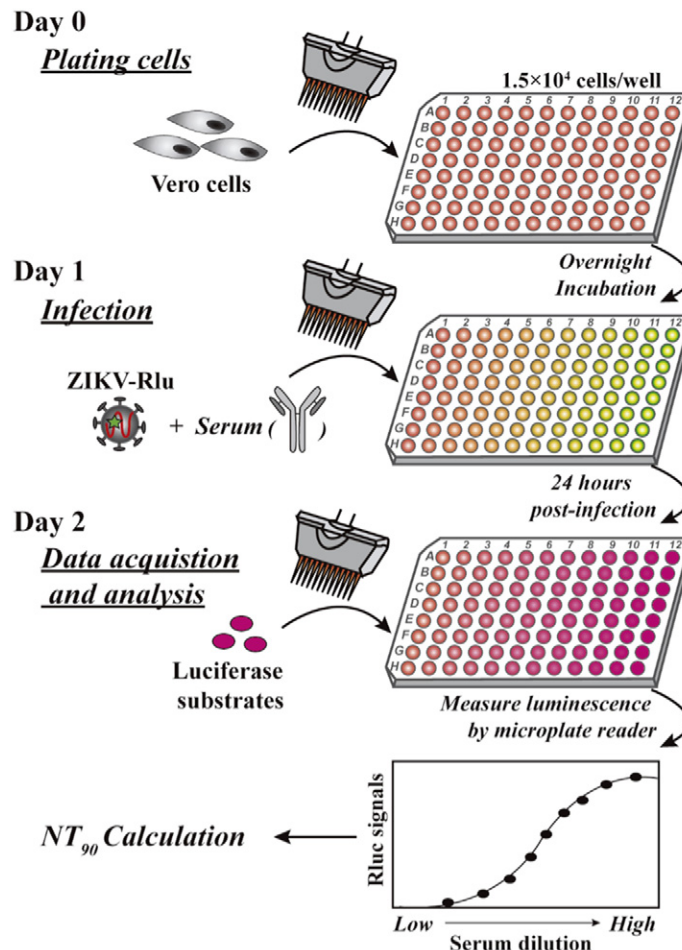


**Fig. 2.** Experimental scheme of reporter virus-based infection assay to measure neutralization titers of specimens. See text for details.

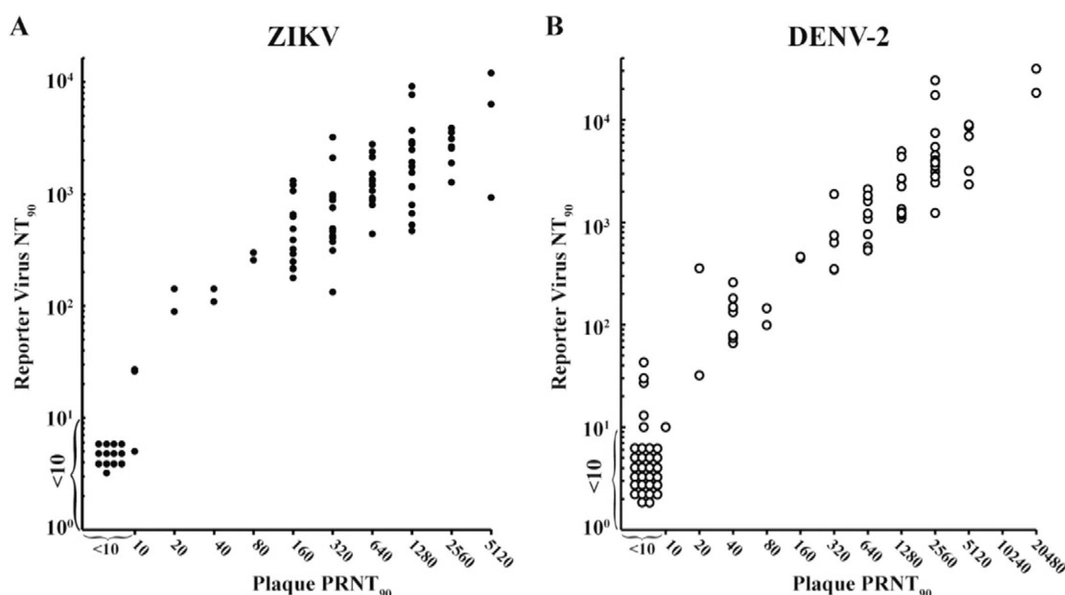


**Fig. 3.** Scatter plots of plaque assay-derived $PRNT_{90}$ and reporter assay-derived $NT_{90}$ values for ZIKV and DENV.

and reporter virus assays (Table 1). 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). 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 maintains the relative specificity of the traditional plaque assay.

## 4. Discussion

The current recommendation for diagnosis of ZIKV infection includes three main assays (Musso and Gubler, 2016; Staples et al., 2016). (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). (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 non-structural 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; Shu et al., 2002; Stettler et al., 2016; Wong et al., 2003). 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). 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.

The goal of this study was to develop a rapid assay to replace the traditional plaque-based PRNT assay. We took advantage of our 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 h. 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 may 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. In addition, the reporter assay could be used to test the difference in neutralization titers between the acute and convalescent serum samples from individual patients; a greater than four-fold rise in antibody titers between acute and convalescent phase of illness indicates a recent infection for diagnostic confirmation. 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. Despite the above improvements, it should be noted that the reporter neutralization assay still relies upon both virus-specific and cross-reactive epitopes of E protein. Therefore, the new assay does not overcome the issue of cross reactivity discussed above. It should also be noted that the current study used a set of well characterized archive samples to develop the reporter assay. A validation study is needed to further develop the assay using prospectively enrolled patients presenting with acute febrile illness.

The current reporter assay used luciferase ZIKV and DENV-2. It is ideal to expand the reporter viruses to DENV-1, -3, and -4. The reporter virus-based neutralization assay could be further expanded to other flaviviruses (Deas et al., 2005; Shustov et al., 2007; Zhang et al., 2016) 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 cell culture (Samsa et al., 2009) and in small animal models (Schoggins et al., 2012), as well as for siRNA/CRISPR library screening or antiviral drug discovery (Puig-Basagoiti et al., 2005). For serologic diagnosis, the reporter viruses are superior to *trans* packaged virus-like particles using reporter replicons (Hanna et al., 2005; Harvey et al., 2004; Khromykh et al., 1998) because once stable reporter viruses have been established, they could be produced in large quantities. Besides PRNT, the reporter virus-based neutralization assay is also more quantitative and higher throughput than other neutralization assays, such as micro-neutralization test and hemagglutination inhibition test (Taketa-Graham et al., 2010).

In summary, we have developed a reporter ZIKV assay that could 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 <48 h, it could potentially be used as the first-line of diagnostic test without prior IgM ELISA test. The reporter ZIKV assay could 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, could deliver a rapid diagnosis of ZIKV infection.

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## Conflict of Interest Statement

The authors (C.S. and P.Y.S.) have filed a patent related to the technology presented in this paper.

## Author Contributions

C.S., X.X., P.R., Y.Y., A.F., and A.P.D. performed experiments and data analysis. C.S., X.X., P.R. M.J.L., A.D.P., L.D.K., S.J.W., and P.Y.S. interpreted the results. C.S., X.X., P.R., M.J.L., S.J.W., and P.Y.S. wrote the manuscript.

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## References

Deas, T.S., Binduga-Gajewska, I., Tilgner, M., Ren, P., Stein, D.A., Moulton, H.M., Iversen, P.L., Kauffman, E.B., Kramer, L.D., Shi, P.-Y., 2005. Inhibition of flavivirus infections by antisense oligomers specifically suppressing viral translation and RNA replication. J. Virol. 79, 4599–4609.

Dowd, K.A., Ko, S.Y., Morabito, K.M., Yang, E.S., Pelc, R.S., DeMaso, C.R., Castilho, L.R., Abbink, P., Boyd, M., Nityanandam, R., et al., 2016. Rapid development of a DNA vaccine for Zika virus. Science 354, 237–240.

Garcia, G., Vaughn, D.W., Del Angel, R.M., 1997. Recognition of synthetic oligopeptides from nonstructural proteins NS1 and NS3 of dengue-4 virus by sera from dengue virus-infected children. Am. J. Trop. Med. Hyg. 56, 466–470.

Hanna, S.L., Pierson, T.C., Sanchez, M.D., Ahmed, A.A., Murtadha, M.M., Doms, R.W., 2005. N-linked glycosylation of West Nile virus envelope proteins influences particle assembly and infectivity. J. Virol. 79, 13262–13274.

Harvey, T., Liu, W., Wang, X., Linedale, R., Jacobs, M., Davidson, A., Le, T., Anraku, I., Suhrbier, A., Shi, P., et al., 2004. Tetracycline-inducible packaging cell line for production of flavivirus replicon particles. J. Virol. 78, 531–538.

Khromykh, A.A., Varnavski, A.N., Westaway, E.G., 1998. Encapsidation of the flavivirus Kunjin replicon RNA by using a complementation system providing Kunjin virus structural proteins in trans. J. Virol. 72, 5967–5977.

Kuno, G., Gubler, D.J., Oliver, A., 1993. Use of 'original antigenic sin' theory to determine the serotypes of previous dengue infections. Trans. R. Soc. Trop. Med. Hyg. 87, 103–105.

Lanciotti, R.S., Kosoy, O.L., Laven, J.J., Velez, J.O., Lambert, A.J., Johnson, A.J., Stanfield, S.M., Duffy, M.R., 2008. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg. Infect. Dis. 14, 1232–1239.

Lindenbach, B.D., Murray, C.L., Thiel, H.J., Rice, C.M., 2013. Flaviviridae. In: Knipe, D.M., Howley, P.M. (Eds.), sixth ed. Fields Virology vol. 1. Lippincott William & Wilkins, Philadelphia, pp. 712–746.

Midgley, C.M., Bajwa-Joseph, M., Vasanawathana, S., Limpitikul, W., Wills, B., Flanagan, A., Waiyaiya, E., Tran, H.B., Cowper, A.E., Chotiyarnwong, P., et al., 2011. An in-depth analysis of original antigenic sin in dengue virus infection. J. Virol. 85, 410–421.

Musso, D., Gubler, D.J., 2016. Zika virus. Clin. Microbiol. Rev. 29, 487–524.

Puig-Basagoiti, F., Deas, T.S., Ren, P., Tilgner, M., Ferguson, D.M., Shi, P.-Y., 2005. High-throughput assays using luciferase-expressing replicon, virus-like particle, and full-length virus for West Nile virus drug discovery. Antimicrob. Agents Chemother. 49, 4980–4988.

Samsa, M.M., Mondotte, J.A., Iglesias, N.G., Assuncao-Miranda, I., Barbosa-Lima, G., Da Poian, A.T., Bozza, P.T., Gamarnik, A.V., 2009. Dengue virus capsid protein usurps lipid droplets for viral particle formation. PLoS Pathog. 5, e1000632.

Schoggins, J.W., Dorner, M., Feulner, M., Imanaka, N., Murphy, M.Y., Ploss, A., Rice, C.M., 2012. Dengue reporter viruses reveal viral dynamics in interferon receptor-deficient mice and sensitivity to interferon effectors in vitro. Proc. Natl. Acad. Sci. U. S. A. 109, 14610–14615.

Shan, C., Xie, X., Barrett, A.D.T., Garcia-Blanco, M.A., Tesh, R.B., Vasconcelos, P.F.C., Vasilakis, N., Weaver, S.C., Shi, P.Y., 2016a. Zika virus: diagnosis, therapeutics, and vaccine. ACS Infect. Dis. 2, 170–172.

Shan, C., Xie, X., Muruato, A.E., Rossi, S.L., Roundy, C.M., Azar, S.R., Yang, Y., Tesh, R.B., Bourne, N., Barrett, A.D., et al., 2016b. An infectious cDNA clone of Zika virus to study viral virulence, mosquito transmission, and antiviral inhibitors. Cell Host Microbe 19, 891–900.

Shi, P.Y., Tilgner, M., Lo, M.K., Kent, K.A., Bernard, K.A., 2002. Infectious cDNA clone of the epidemic West Nile virus from New York City. J. Virol. 76, 5847–5856.

Shu, P.Y., Chen, L.K., Chang, S.F., Yueh, Y.Y., Chow, L., Chien, L.J., Chin, C., Lin, T.H., Huang, J.H., 2000. Dengue NS1-specific antibody responses: isotype distribution and serotyping in patients with dengue fever and dengue hemorrhagic fever. J. Med. Virol. 62, 224–232.

Shu, P.Y., Chen, L.K., Chang, S.F., Yueh, Y.Y., Chow, L., Chien, L.J., Chin, C., Yang, H.H., Lin, T.H., Huang, J.H., 2002. Potential application of nonstructural protein NS1 serotype-specific immunoglobulin G enzyme-linked immunosorbent assay in the seroepidemiologic study of dengue virus infection: correlation of results with those of the plaque reduction neutralization test. J. Clin. Microbiol. 40, 1840–1844.

Shustov, A., Mason, P., Frolov, I., 2007. Production of pseudoinfectious yellow fever virus with a two-component genome. J. Virol. 81, 11737–11748.

Staples, J.E., Dziuban, E.J., Fischer, M., Cragan, J.D., Rasmussen, S.A., Cannon, M.J., Frey, M.T., Renquist, C.M., Lanciotti, R.S., Munoz, J.L., 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.

Stettler, K., Beltramello, M., Espinosa, D.A., Graham, V., Cassotta, A., Bianchi, S., Vanzetta, F., Minola, A., Jaconi, S., Mele, F., et al., 2016. Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. Science 353, 823–826.

Taketa-Graham, M., Powell Pereira, J.L., Baylis, E., Cossen, C., Oceguera, L., Patiris, P., Chiles, R., Hanson, C.V., Forghani, B., 2010. High throughput quantitative colorimetric microneutralization assay for the confirmation and differentiation of West Nile virus and St. Louis encephalitis virus. Am. J. Trop. Med. Hyg. 82, 501–504.

Wong, S.J., Boyle, R.H., Demarest, V.L., Woodmansee, A.N., Kramer, L.D., Li, H., Drebot, M., Koski, R.A., Fikrig, E., Martin, D.A., et al., 2003. An immunoassay targeting nonstructural protein 5 to differentiate West Nile virus infection from dengue and St. Louis encephalitis virus infections, and form flavivirus vaccination. J. Clin. Microbiol. 41, 4217–4223.

Wong, S.J., Furuya, A., Zou, J., Xie, X., Dupuis 2nd, A.P., Kramer, L.D., Shi, P.Y., 2017. A multiplex microsphere immunoassay for Zika virus diagnosis. EBioMedicine 16 (2017), 136–140.

Zhang, P.T., Shan, C., Li, X.D., Liu, S.Q., Deng, C.L., Ye, H.Q., Shang, B.D., Shi, P.Y., Lv, M., Shen, B.F., et al., 2016. Generation of a recombinant West Nile virus stably expressing the Gaussia luciferase for neutralization assay. Virus Res. 211, 17–24.

Zou, G., Xu, H.Y., Qing, M., Wang, Q.Y., Shi, P.Y., 2011. Development and characterization of a stable luciferase dengue virus for high-throughput screening. Antivir. Res. 91, 11–19.