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A sensitive virus yield assay for evaluation of Antivirals against Zika Virus

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A B S T R A C T

Despite the rapid spread of Zika virus (ZIKV) infection and associated neurological complications in the America's, prophylactic or therapeutic countermeasures are not currently available. This is mostly due to the fact that until recently there was no presumed need for medical intervention since there was no association between ZIKV infection and significant human morbidity. Consequently, there are currently no tools due mostly to the lack of sensitive cell based assays amenable for identification of ZIKV inhibitors. To address this unmet need we have developed a cell based virus yield assay suitable for testing antivirals against Zika virus.

Using bioinformatics, several isolates of ZIKV from the Americas, Africa, and Asia were analyzed for sequence similarity. The alignment data were then used to design primers targeting a ZIKV genomic region that was highly conserved among all the ZIKV isolates. Subsequently, primers were used in a sensitive, quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay to detect ZIKV RNA. The qRT-PCR assay was found to be highly sensitive (lower limit of detection between-10-100 copies) and reproducible. Evaluation of the primers and probes used for ZIKV against another flavivirus (Dengue virus) demonstrated specificity of detection. To evaluate potential of qRT-PCR assay as an antiviral screening tool against ZIKV, Vero cells pretreated with Type I Interferons (IFN α) were infected with virus, followed by measurement of ZIKV RNA found in the cell culture supernatants using qRT-PCR assay. Dose-dependent antiviral activity of Type I Interferons and mycophenolic acid (MPA) against Zika virus in this cell culture system was confirmed using qRT-PCR. Due to reproducible assay performance, qPCR associated higher sensitivity and short duration of the assay time, this novel cell based assay will be very useful for confirming the activity of antivirals against ZIKV.

1. Introduction

Zika virus (ZIKV), a mosquito-borne flavivirus, is responsible for dengue like illness (fever, rash, joint pain), neurological symptoms (Guillain-Barre syndrome) and microcephaly in newborn babies. This ongoing outbreak has now spread to more than 50 countries around the world. Since the outbreak was first recognized, approximately 3132 travel-associated ZIKV cases, 43 locally acquired mosquito-borne cases and 731 cases of pregnant women with laboratory evidence of ZIKV infection have also been identified in the United States (www.CDC.gov). ZIKV is primarily transmitted by mosquitoes; however, sexual and perinatal transmission also have been demonstrated in a few cases(Hamel et al., 2016). The mosquito species that transmits ZIKV belong to the genus *Aedes*, and are widely distributed around the world including much of the Southern United States (www.cdc.gov). Epidemiological studies in Brazil have provided evidence of a strong causal link between ZIKV infection in pregnant women and serious neural developmental disorders such as microcephaly in fetuses and newborns (Nunes et al., 2016). Microcephaly is a neural developmental dis-

Abbreviations: ZIKV, Zika Virus; qRT-PCR, Quantitative Reverse Transcriptase-polymerase Chain reaction; CP assay, Cytoprotection assay; CPE, Cytopathic effect; IFA, Immunofluorescence Assay.

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order characterized by smaller than average head circumference in the affected babies. The underlying cause of microcephaly is malformation of cortical neurons leading to a significant diminution of the fetal cerebral cortex. Depending on the severity, the babies born with microcephaly can have severe and permanent impairments such as intellectual disability, developmental delay, motor skills defects, vision problems, and hearing loss. In addition to causing microcephaly in developing fetuses, ZIKV infection in adults has been linked to Guillain-Barré syndrome, an autoimmunue disorder that primarily affects the peripheral nervous system, and can be fatal (Lucchese and Kanduc, 2016). Due to the widespread nature of the ongoing outbreak, extensive geographic distribution of the mosquito vectors, and potentially grave outcome of infection, especially in the unborn fetus of pregnant women, the World Health Organization (WHO) has declared ZIKV to be a 'Public Health Threat of International Concern'.

Since its first isolation in 1947 from a sentinel Rhesus monkey in the Zika forest of Uganda, sporadic human infections of ZIKV have been recorded in several African and Asian countries (Weaver et al., 2016). However, it was not until 2007 when an outbreak occurred in the Pacific Island of Yap that the true epidemic potential of this virus was realized. It was estimated that 73% of Yap residents $\geq$ 3 years of age were infected with ZIKV during the outbreak. Subsequently, in 2013, a ZIKV outbreak was reported in French Polynesia with an estimated 19,000 cases (Cao-Lormeau et al., 2014). In May 2015, the WHO reported local transmission of ZIKV in Brazil, and in December, the Brazilian Ministry of Health estimated that 440,000–1,300,000 suspected cases of ZIKV associated disease had occurred in Brazil in 2015 (Hennessey et al., 2016). Phylogenetic analyses performed on isolates collected from various time periods and geographic locations indicated two distinct ZIKV lineages, the African lineage and the Asian lineage; the African lineage was further subdivided into East African and West African clades (Faye et al., 2014). The isolates within the Asian lineage share >99% nucleotide sequence identity, while the nucleotide sequence identity between members of Asian and African lineages is 89% (Lanciotti et al., 2016). The ZIKV strain responsible for the ongoing outbreak in the Americas belongs to the Asian lineage.

ZIKV is a member of the genus *Flavivirus*, family *Flaviviridae*, and is related to other human pathogenic flaviviruses such as dengue, St. Louis encephalitis and yellow fever viruses (Kuno et al., 1998). Like other flaviviruses, the ZIKV has an icosahedral nucleocapsid, which is surrounded by a host-cell derived lipid envelope. Embedded within the envelope are virally-encoded envelope glycoprotein spikes, which serve to bind virions to the host cell receptor(s). The ZIKV genome consists of an 11 kb single-stranded, positive-sense RNA, which contains a single open reading frame of 10,794 nucleotides flanked by two non-coding regions. The ORF is translated by host cell machinery shortly after nucleocapsid uncoating in the cytoplasm thus producing a precursor polyprotein. This polyprotein is co- and post-translationally cleaved into individual viral proteins that include three structural proteins (capsid, premembrane, and envelope) and seven nonstructural proteins (NS1, NS2, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Hamel et al., 2016).

Currently, no vaccine or specific therapy is available against ZIKV. In view of the global threat posed by ZIKV, effective therapies and a vaccine are urgently needed. This warrants accelerated efforts towards rapid discovery and confirmation of antiviral compounds that can readily be 'fed' into the drug development process A key requirement for the implementation of an efficient drug discovery and developmental program is the availability of an assay that allows detection and quantification of the intended action of the chemical compound(s) against the virus. Importantly, the assay should produce a robust signal, have a high sensitivity and specificity. Additionally, a cell-based confirmatory assay is preferred as they provide a better understanding of pharmacological interaction between the virus and the compound in a physiological environment. Moreover, with regards to evaluation of potential drug candidates, a cell-based system provides opportunity to eliminate compounds that are cell impermeable, cytotoxic, or rapidly degraded within cells; thus, allowing selection of scaffolds that are amenable to downstream applications in the drug development process.

Here, we describe the development and optimization of a cell-based Zika virus yield assay for assessing the antiviral activity of compounds against ZIKV. The assay is based on qRT-PCR detection and quantitation of ZIKV yield from infected cells treated with a candidate compound. To design the assay, we first aligned nucleotide sequences of several ZIKV isolates from the Americas, Asia, and Africa. The aligned sequences were subjected to bioinformatics analyses to identify regions that display high sequence identity among all ZIKV isolates, but not other flaviviruses. Specific primers and probe were then designed based on these conserved regions, and qRT-PCR assay was performed using the RNA from various ZIKV isolates or dengue virus-1/4 (DENV1/4) as a specificity control. The assay was found to be specific (signal detected in all ZIKV-infected, but not DENV1/4-infected cells), and highly sensitive (lower limit of detection- between 10 and 100 copies of ZIKV RNA). Furthermore, the assay has a high dynamic range (10^1–10^6 gene copies) and Z' factor >0.5, indicating that the assay is sufficiently robust for testing compounds. To provide proof-of-concept, we analyzed the dose response of antiviral activity of Type I Interferons (IFN α) and MPA against ZIKV, and confirmed that this assay could detect antivirals against ZIKV.

2. Materials and methods

2.1. Sequence analysis

Comparative ZIKV genomic sequence analysis and alignments were performed using the NCBI BLAST Suite (http://www.ncbi.nlm.nih.gov/pubmed). The "Megablastn" and "Primer-blast" programs were used for the genomic comparisons and sequence specific primer homology analysis, respectively.

2.2. Viruses, Cell Culture, and Antiviral compounds (IFN-α, MPA and Cyclosporine A)

Vero cells (CCL-81, ATCC, Manassa, VA) were grown in Dulbecco's Minimal Essential Medium (DMEM, Lonza, Walkersville, MD), supplemented with 10% Fetal Bovine Serum (FBS), NEAA and L-Glutamine according to standard culture conditions. A commercially available mixture of protease (Pronase, Roche Life sciences, Indianapolis, IN) was used to release viral RNA for qRT-PCR. IFN α was obtained from ProSpec East Brunswick, NJ. Mycophenolic acid (MPA) (Cat#S2487) and Cycloproine A (Cat# S2286) were purchased from Selleckchem (Houston, TX). ZIKV isolates were obtained from the following sources: DAK MR766 and IbH 30656 (BEI Resources, Manassas, VA), PRVABC59 (CDC, Atlanta, GA), FSS13025 (UTMB Arbovirus reference collection, Galveston, TX), and ZIKV East African Lineage (Zeptometrix, Buffalo, NY). In addition, the following Zika virus isolates were used for sequence analysis in this study: Brazil ZIKV2015 (Genbank Accession #KU497555.1) (Calvet et al., 2016), Brazilian isolate Natal RGN, Bahia, Brazil (Seq. Accession# KU527068.1) (Calvet et al., 2016) (Mlakar et al., 2016). Viral stocks of different isolates were amplified in Vero cells (ATCC, Manassas, VA) and quantitated according to the standard plaque assay methodology. Strain of virus used for each experiment is mentioned in the figure legend as appropriate.

Table 1
ZIKV qRT-PCR Primers and probes.

qRT-PCR Primers and Probe	Sequence	Strain Specificity	Position
Zika-Dual For	5′-ATATCGGACATGGCTTCGGA-3′	MR766	1069–1085
		FSS13025	1063–1082
		IBH30656	1063–1082
Zika-Dual Rev	5′-GTTCTTTTGCAGACATATTGAGTG-3′	MR766	1260–1237
		FSS13025	1154–1131
		PRVABC59	1260–1237
Zika-Dual Probe	5′ FAM –TGCCCAACA/ZEN/C-AAGGTGAAGCCTACCT-IAbkBkFQ	MR766	1196–1221
		FSS13025	1090–1115
		PRVABC59	1196–1221
		IBH30656	1090–1115
Zika-PRV/BRA-F	5′-TGAGGCATCAATATCAGACATG-3′	PRVABC59	1159–1180
Zika-IBH-Rev	5GTTCTTTTACAGACATATTGAGTGTC-3′	IBH30656	1154–1129

Table 2
Comparison of different conditions for inoculum preparation.

Treatment		Time (Mins)	CT Values (Mean ± SD)
Heating	95 °C	0	18.6 ± 0.1
		5	19.3 ± 0.2
		15	19.2 ± −0.1
		30	19.4 ± −0.1
Pronase	0.8 mg/mL	30	19.2 ± −0.1
		60	20.2 ± −0.1
		90	20.4 ± −0.1
	0.4 mg/mL	30	19.9 ± −0.1
		60	20.8 ± −0.1
		90	21.9 ± −0.3
	0.08 mg/mL	30	18.7 ± −0.1
		60	19.4 ± −0.1
		90	20.6 ± −0.1

2.3. Design of primers and probes

PCR primers were designed to bind the NS1 region of the ZIKV genome. All primers were subjected to sequence analysis including; specific ZIKV strain homology- and hybridization-potential across different clades. Primer and probe sequences also were characterized for compatible melting temperatures (Tm), and self-dimer and hairpin potential using the Integrated DNA Technologies (IDT, Coralville, Iowa) "Primer Analysis software" tool. All the primers and probes were synthesized at IDT Technologies. The probe has a 5′6-FAM reporter and an internal (9th position) ZEN quencher and 3′ IBFQ Iowa black quencher as shown in Table 1.

2.4. RNA positive control for qRT-PCR standard curve preparation

PCR primers with T7 DNA dependent RNA polymerase promoter sequences flanking the target region were designed and synthesized as mentioned above. The forward primer 5′ATCTCACCA*TAATACGA-CTCACTATAGGG*AAAGTCATATACTTGGTCATGA TAC-3′ (T7 promoter region is italicized and underlined) and reverse primers 5′TCTGAAAAGTCAAGGCCTGTC CT3′ were subjected to single step RT-PCR (Applied Biosystems, Fast Virus 4x Master mix, Cat # 4444436, Life Technologies Corporation Carlsbad, CA) using purified viral RNA template (QIAmp Viral RNA Mini kit, Cat. 52906, Qiagen, Germantown, MD). Products of RT-PCR were column purified using QIAquick gel extraction kit (Cat # 28706, Qiagen). RNA positive controls were prepared using an *in vitro* transcription assay kit (mMessage Machine T7 kit, Cat # AM1344, Life Technologies Corporation, Carlsbad, CA) using purified PCR fragments as templates. A standard curve using RNA positive control was used for absolute quantitation of genome copies from test samples.

2.5. Virus infection for antiviral activity measurement by qRT-PCR

Vero cells were seeded at a density of 1×10^4 cells/well in 96 well plates (Cat # 3595, Corning clear flat bottom 96 well plates, Fisher Scientific, Rockville, MD). After pretreatment with compounds (30 min to overnight as indicated), cells were infected with ZIKV at an MOI of 0.01. Cell culture supernatants were collected at 72 h post-infection and either used immediately or stored at −70 °C before being subjected to qRT-PCR. Compound cytotoxicity were assessed by MTS (CellTiter®96 Reagent, Promega, Madison WI) dye reduction. Antiviral activity was calculated by 4-parameter curve fitting to determine EC_{50} (compound concentration inhibiting virus replication by 50%). Effect of compounds on cell viability was measured by CC_{50} (compound concentration resulting in 50% reduction in cell viability). Selectivity Index was calculated as the ratio between CC_{50} and EC_{50}. Z-factor determinations were made according to the published protocol (Zhang et al., 1999).

2.6. Quantitative RT-PCR (qRT-PCR)

Five μL of cell culture supernatants (diluted 100-fold in calcium- and magnesium-free PBS, Cat. #17-512F, Lonza Walkersville, MD), were used as inoculum in a 20 μL qRT-PCR with 500 nM of forward and reverse primers and 200 nM probe using the Applied Biosystems Fast Virus 4 x Master Mix (Cat# 4444436 Life Technologies Corporation Carlsbad, CA). qRT-PCR cycling (Applied Biosystems Inc HT 7900) conditions included an initial reverse transcription (RT) for 5 min at 53 °C, followed by 1 min at 95 °C (enzyme activation) and 45 cycles of 2 step cycling at 95 °C for 5 s, then 60 °C for 50 s. A standard curve using positive control RNA as template over a dynamic range of 6-logs (10^6 to 10^1) was used in triplicate for relative quantitation of test samples.

2.7. Dengue virus qRT-PCR

The same qRT-PCR conditions as noted above were used for two different serotypes of dengue virus (DENV1 and DENV4). The templates used for absolute quantitation of DENV were prepared from a cloned fragment overlapping the RT-PCR target (DENV1) and synthetic RNA (DENV4). Forward (DEN-1F: CAAAAG GAAGTCGTGCAATA) and reverse (DEN-1 R: CTGAGTGAATTCTCTCTACTGAACC) primers with probe (DEN–1 P: FAM-CATGTGGTTGGGAGCACGC-BHQ1) were used for detection of DENV1. Similarly, forward (DEN–4 F: TTGTCCTAATGATG CTGGTCG) and reverse (DEN-4 R: TCCACCTGAGACTCCTTCCA) primers with probe (DEN–4 P: FAM-TTCCTACTCCTACGCATCGCATTCC G-BHQ1a-Q) were used for the detection of DENV4.

Table 3
Specificity of qRT-PCR assay.

RNA TemplateAt approx. 1E4 viral copies	qRT-PCR based Detection (C_T Values)		
	ZIKV Specific Primers/probe	DENV1 Specific Primers/probe	DENV4 Specific Primers and probe
ZIKV strain FSS13025	27.0	UND	UND
DENV1	UND	24.6	UND
DENV4	UND	UND	24.9

Table 4
Evaluation of compounds in the Zika virus yield assay.

Compounds	Class	Zika Virus (Vero Cells) (uM)			Literature
		ZIKV Yield assay (EC_{50})	Cytotoxicity (CC_{50})	SI CC_{50}/ZIKV Yield assay EC_{50}	
IFNa	Immune modifier	7.7 IU/mL	>1000 IU/mL	>129	Contreras and Arumugaswami, 2016
Cyclosporine A	Cyclophilin Inhibitor	6.26	7.37	1.2	Barrows et al., 2016
MPA	IMPDH Inhibitor	<0.32	>100	>313	Barrows et al., 2016

2.8. Immunofluorescence analysis

Vero cells were seeded at a density of 1×10^4 cells/well in 96-well plates followed by pretreatment with IFN-α and infection with ZIKV isolates (MOI of 0.01). At the indicated time points post-treatment, the cells were gently fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100, and blocked with 2% BSA for 30 min followed by staining with Anti-Flavivirus antibody (MAB10216, EMD Millipore, Billerica, MA) for 1 h. After washing three times with PBS containing 0.05% Tween-20, the cells were stained with donkey anti-mouse antibodies labeled with Alexa Fluor 488 (Thermo Fisher Scientific) for 1 h. The cells were washed three times with PBS containing 0.05% Tween-20, and the nuclei were stained with 1 μg/mL of Hoechst 33342. Staining was detected by fluorescence microscopy. The number of positive cells was counted using a Spectramax MiniMax300 (Molecular Devices). Proportions of positive cells in compound-treated wells are reported as percentage of virus infected cells without any compound treatment.

3. Results and discussion

Prophylactic or therapeutic strategies are urgently needed to control the rapid spread of Zika virus infections. Though a prophylactic strategy for ZIKV (*i.e.,* vaccination) seems more practical based upon the delicate target population (pregnant women and babies), therapeutic strategies might be useful in subsets of target populations such as travelers, immunosuppressed patients, and the elderly individuals. This patient population may be unable (or might not have sufficient time) to develop protective immunity in response to a prophylactic vaccine. In addition, ZIKV infection is associated with an autoimmune disease (Guillaine Barre Syndrome), and emerging evidence suggests an antibody-mediated enhancement of cross infection between ZIKV and Dengue virus, both of which often co-circulate in South/Central America. These disease complications, might enhance possible risks of vaccine-associated adverse events, highlighting the need for effective antivirals against Zika virus. The current work is focused on the development of a sensitive qRT-PCR-based virus yield assay to enable the discovery of effective antivirals against Zika. Assay development was performed methodically using sequence analysis to guide PCR primer and probe selection followed by optimization of the ZIKV virus yield assay.

3.1. ZIKV genome sequence analysis

To identify the conserved regions in the genome of ZIKV, pairwise comparison was performed. ZIKV isolates of African (MR-766, Uganda), American (PRVABC59), and Asian (FSS13025, Cambodian) lineage, and a clinically relevant strain isolated from amniotic fluid of fetus with microcephaly (Brazil ZIKV2015) (Calvet et al., 2016) were used for this purpose. Initial analysis revealed that, the FSS13025 strain (Asian sequence lineage isolated in Cambodia) and PRVABC59 (American sequence lineage isolated in Puerto Rico) are 98–99% homologous to the currently circulating Zika virus isolates at the nucleotide level (Brazil ZIKV2015, and Brazilian isolate Natal RGN (Calvet et al., 2016) (Mlakar et al., 2016) (Data not shown). Comparison of the African and Asian lineages of ZIKV suggested less than 90% sequence identity at the nucleotide level. Heterogeneity changes to ZIKV genomic sequences and codon bias might be suggestive of viral adaptation as documented in the literature (Plotkin and Kudla, 2011) (Longdon et al., 2014). Higher similarity between ZIKV isolates of Asian and American origin is consistent with the previous observations (Zhu et al., 2016).

Different groups have targeted ZIKV NS5 (Faye et al., 2013) (Faye et al., 2008) and NS1 (Cao-Lormeau et al., 2014; Gourinat et al., 2015; Lanciotti et al., 2008, 2016; Musso et al., 2015) for qRT-PCR assay development. ZIKV NS1 region sequence analysis suggested a high degree of homology between different ZIKV isolates, but not with NS1 from Hepacivirus and Pestivirus genus constituents (Song et al., 2016). Though NS1 is conserved, four changes in NS1 (E842D, K859R, A984V and V1026I) between the African and Asian lineages are reported (Zhu et al., 2016) in the literature. However, the significance of these changes remains unknown. Lanciotti et al. has previously reported a sensitive and specific qRT-PCR based assay to detect ZIKV by targeting the NS1 region (Lanciotti et al., 2008). Based upon this literature and to reduce potential cross-reactivity with other Flaviviruses, ZIKV NS1 was selected in this work as a target for qRT-PCR primer and probe development. Primers and PCR probes proximal to those mentioned in Lanciotti et al. (Lanciotti et al., 2008), with modified target sequences, were used for assay development in our experiments. While, both isolates of MR766 (Africa) and FSS13025 (Asian) could be detected using the same set of primer/probe combinations, detection of ZIKV PRVABC59 and IBH30656 required a slightly different combination (usage of Zika-PRV/BRA-F as forward and Zika-IBH-Rev as reverse primers, respectively) (Table 1). Additionally, based upon sequence similarity observed in GenBank database, primer-probe combinations described in this work (Table 1) also are expected to detect several other ZIKV isolates from Brazil.

3.2. Comparison of different test sample preparation conditions for qRT-PCR

Methods to release RNA from virus particles such as Thermal and protease digestions are described previously (Al-Siyabi et al.,

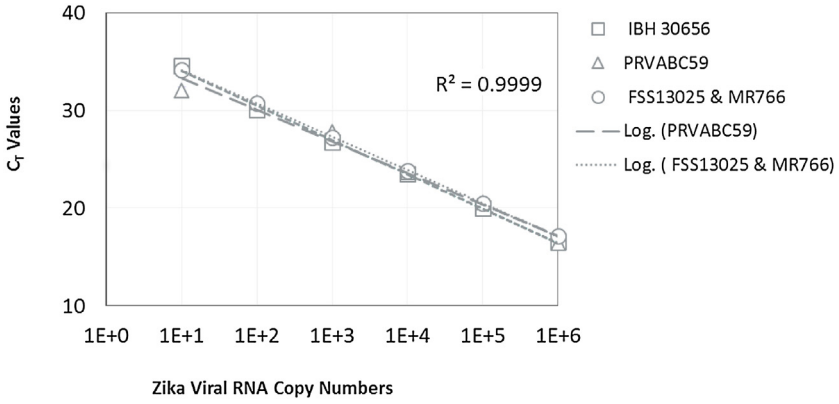

Fig. 1. Sensitivity of qRT-PCR assay.
Serial dilutions of RNA from different ZIKV isolates (IBH 30656, PRVABC59, FSS3025 and MR766) were subjected to qRT-PCR as described in the methods section. CT values are shown on the Y-axis.

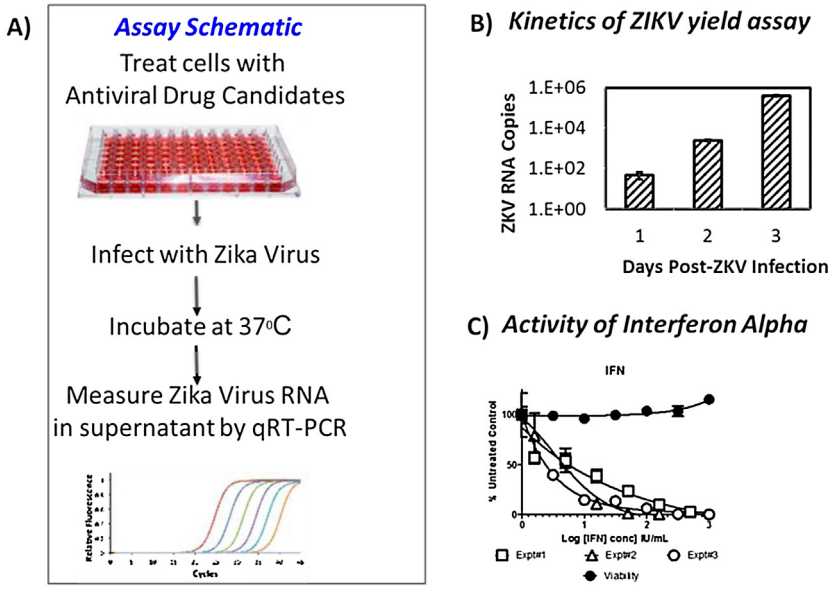

Fig. 2. Zika Virus Yield Assay.
A) Assay Schematic: Illustration of Zika virus yield assay. B) Kinetics of ZIKV assay: Zika virus infected Vero cell culture supernatants (MOI of 0.01) were subjected to virus yield assay as measured by qRT-PCR at different time points post-infection (day 1 to 3). ZIKV RNA copies (pattern filled bars) are shown on the left and right axis respectively. C) Activity of IFNa in the Zika virus yield assay: Different concentrations of IFNa were used in the Zika virus yield assay as illustrated in the Assay schematic in (A). Virus yield from the IFNa treated samples were normalized to virus infected controls. Data from three independent experiments (Mean ± SEM) is shown in the graph.

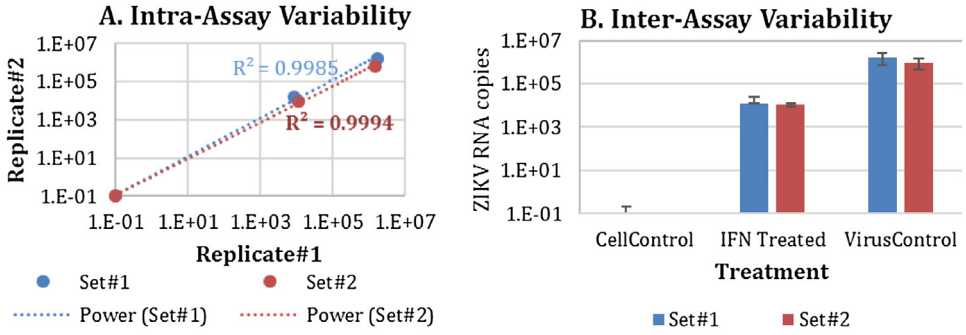

Fig. 3. Intra and Inter assay variability.
A) Intra-assay Variability: Twenty four (24 wells) in 2 x 96-well plates (Replicate#1 and 2) on two different days (Set#1 and Set#2) were treated with either 500 IU/mL of IFNa or no compound, followed by with or without virus infection as indicated in materials and methods. ZIKV virus yield assay was performed 3 days post-infection. B) Virus RNA copies for each treatment group (Virus control, IFNa treated control, cell control) (Mean ± SD) from each replicate were compared with each other as shown in the figure, followed by calculation of regression coefficient for each line. Virus RNA copies for each treatment group (Virus control, IFNa treated control, cell control) from two independent experiments (Set#1 and Set#2) performed on two different days (as mentioned above) are shown in the figure (Mean ± SD).

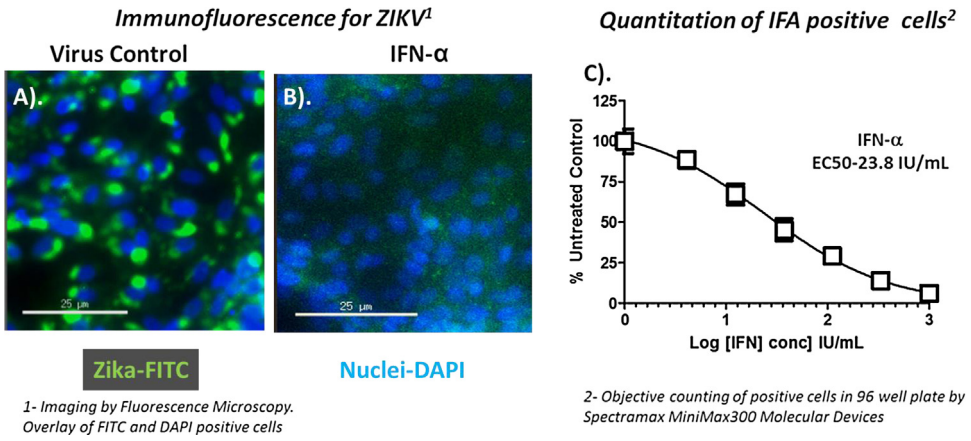

Fig. 4. ZIKV Immunofluorescence assay.
A) Immunofluorescence assay for Zika virus: Vero cells (African Green Monkey Kidney origin) with and without Zika virus infection were stained with anti-Flaviviral antibody (Millipore) and subjected to immunofluorescence microscopy. Specificity of the assay is demonstrated by detection of FITC positive cells only in Zika virus infected but not in the mock infected cell controls and lower staining in Virus infected but IFN treated cells. B) Antiviral activity of IFN in Zika virus assay: Vero cells (African Green Monkey Kidney origin) infected with Zika virus and treated with increasing concentrations of Interferon alpha (0 to 1000 IU/mL) were subjected to IFA based quantitation. ZIKV positive cells were quantitated by Spectramax MiniMax300 (Molecular Devices). Proportion of Zika positive cells relative to untreated controls are plotted on the graph.IFN demonstrates an EC50 of 28.3 IU/mL in this assay.

2013) (Klebe et al., 1996) (Thomson and Dietzgen, 1995). To optimize the detection of viral RNA found in the infected cell culture supernatants, various methods such as heat and pronase (a mixture of proteases) treatments (Table 2) were tested. Different incubation times (ranging from 5 to 90 min) with pronase and thermal treatments suggested that additional treatment failed to increase the amount of detectable viral RNA in the inoculum. The lower Ct value (18.6 ± 0.1), indicative of the best sensitivity, was observed with the non-heated sample added directly into the reaction mixture. These results suggest that neither the thermal nor the pronase pretreatment was necessary for optimal ZIKV RNA detection. The heating associated with the reverse transcription step (5 min at 53 °C) in the qRT-PCR process might be sufficient for the release of the virus-associated RNA or ZIKV RNA may be released into the cell culture supernatant due to Virus CPE mediated cell lysis. Similar observations were made for Chikungunya virus (Pastorino et al., 2005). The low level of cytopathic effect (CPE) observed, even at a low multiplicity of infection (MOI) of ZIKV (data now shown) at day 3, and lack of increase in the Ct values, even with heat treatment, supports this hypothesis. Considering optimal detection of ZIKV RNA in the infected cell culture supernatants was achieved without any additional treatment, subsequent experiments were conducted without this step. Exclusion of additional time-consuming RNA releasing methods from the assay protocol might be advantageous as it decreases the possibility of PCR contamination and the overall assay time.

3.3. Specificity of qRT-PCR assay

One of the challenges associated with ZIKV research and diagnosis is the possible serological cross reactivity between Zika and other flaviviruses (http://www.cdc.gov/zika/pdfs/denvchikvzikv-testing-algorithm.pdf). Due to the significant epidemiological coincidence associated with Dengue and Zika virus infections (Roth et al., 2014), Dengue virus was used as a prototypical flavivirus to rule out cross reactivity and confirm ZIKV specificity of qRT-PCR conditions. ZIKV primer-probe-sets were used to detect Dengue virus in supernatants of cells infected with Dengue virus and vice versa (Table 3). The ZIKV primer and probe sets could only detect ZIKV and not DENV1 and DENV4 suggesting a high degree of specificity for ZIKV and no cross reactivity. Sequence similarity searches of ZIKV primers and probes with additional Flaviviruses using the software (NCBI Primer-blast) (YFV and others) ruled out any potential cross reactivity with other flaviviruses.

3.4. Sensitivity and reproducibility

Serial dilutions of ZIKV RNA (from four ZIKV isolates) generated by *in vitro* transcription reaction were tested in the ZIKV qRT-PCR assay to determine sensitivity of detection. Our results suggest linear amplification over 6-logs with an R^2 value of more than 0.99 and a lower limit of detection of 10–100 copies per reaction (Fig. 1). This sensitivity is in the same range as previously published reports (Faye et al., 2013). Importantly, this enhanced assay sensitivity provides the ability to distinguish between samples with virus titers in close range.

3.5. Cell-based virus yield assay for ZIKV

There are few reports in the literature regarding detection of RNA in the culture supernatants without RNA extraction (Klebe et al., 1996) (Thomson and Dietzgen, 1995). Taqman-based assays have been used for other viruses (such as Sindbis, Chikungunya virus) (Adelman et al., 2012) (Zhang and Zhu, 2015) (Pastorino et al., 2005). To detect the virus yield from Zika virus-infected Vero cells with (MOI of 0.01), we evaluated the cell culture supernatant by ZIKV qRT-PCR (Schematically illustrated in Fig. 2A).

To determine the optimal Kinetics of ZIKV RNA levels in infected Vero cells ZIKV virus yield assay was performed over 3-days (Fig. 2B). A Linear increase in ZIKV RNA (from 45.7 ± 17.6 to $396{,}453.3 \pm 44{,}072.2$ copies) was observed from day 1 to day 3, suggesting that the dynamic range (10^6) and assay window of qRT-PCR based ZIKV yield assay was highest at day 3 resulting in a large signal difference between positive and negative controls and a robust Z' factor of >0.5.

IFN-α used as a positive control in our assays, with concurrent measurement of viability (Fig. 2C), showed an EC_{50} (Mean ± SEM) of 7.7 ± 4.5 IU/mL from three independent experiments with a selectivity index of >129 (Table 4). To test the reproducibility of the assay, we tested the highest concentration (500 IU/mL) of interferon in multiple wells (24 wells) on two different plates (intra-assay variability) over two different days (Inter-assay variability) along with cell and virus controls. Comparison of the data from both the replicate plates tested on the same day suggested a robust correlation

($R^2 = 0.99$) (Fig. 3A) of the ZIKV RNA copies. Similarly, the evaluation of the ZIKV virus yield assay from two independent experiments (Set#1 and 2) performed on two different days suggested similar results (Fig. 3B). To further evaluate the antiviral activity of compounds published in the literature (Barrows et al., 2016) (Contreras and Arumugaswami, 2016), MPA and Cyclosporine A (CsA) were tested in the ZIKV yield assay (Table 4). MPA showed potent activity (EC_{50} of <0.32 uM and SI of >313), whereas Cyclosporine A (CsA) showed activity in the virus yield assay but exhibited cytotoxicity resulting in narrow selectivity index. Minimal intra and inter-assay variability (Fig. 3A and B), consistent activity of IFN-α (Fig. 2C EC_{50} 7.7 ± 4.5 IU/mL), and confirmation of the antiviral activity of published compounds (Table 4) (IFN-α, MPA) are additional characteristics of the ZIKV yield assay suitable for antiviral evaluation.

3.6. IFA-based antiviral assay

The antiviral activity of IFN-α observed in the qRT-PCR based ZIKV yield assay was further confirmed by IFA. Fewer ZIKV-positive cells were detected in the cells treated with IFN-α compared to untreated virus controls (Fig. 4A and B). Subcellular analysis indicated that ZIKV proteins are primarily localized to the cytoplasm. These results further confirm the antiviral activity of IFN-α observed using the ZIKV yield assay.

3.7. Advantages of the qRT-PCR based virus yield assay

Zika virus antiviral assays have focused on traditional endpoints such as CP. In these assays, higher amounts of viral inoculum and longer duration (3–5 days) of exposure to antivirals are required to observe a quantifiable CPE– masking the antiviral effect of weaker compounds. Although the CP assay is cost-effective and easier to set-up, its use is restricted to testing against cytopathic viruses, potentially failing to detect weak antivirals and prone to identify non-specific cell death inhibitors (false positives). Additionally, CPE suffers the disadvantage of a small dynamic range which fails to sufficiently differentiate antivirals with different efficacies for reducing viral load.

CPE reduction assay was used to show the in vitro antiviral activity of nucleoside analogs against ZIKV (Eyer et al., 2016) (Zmurko et al., 2016). Recently, Barrows et al. used a high content screening (HCS) method to investigate the anti-ZIKV activity of 774 FDA approved compounds (Barrows et al., 2016). However, the ZIKV virus yield assay described in the current report, seems to be a more comprehensive assay capturing different steps from virus infection to virus yield (with the measurement of virus yield in the infected culture supernatants) as opposed to the detection of intracellular ZIKV proteins produced after infection by high content screening described in Barrows et al. Our results suggest that the novel ZIKV yield assay described in this report is specific to ZIKV, reproducible, utilizes the sensitivity of PCR based detection and is comparable to other assays published in literature (Barrows et al., 2016) (Contreras and Arumugaswami, 2016). The high dynamic range of this assay (10^1–10^6 genome copies) combined with direct detection and quantification of viral genomes offer additional benefits for using this assay to confirm antiviral activity of primary hits identified in a drug screening program. ZIKV yield assay might provide higher resolution of antiviral activities between similar chemical series which could aid in better structure activity relationship determinations. In summary, ZIKV yield assay described herein will be a valuable tool to advance ZIKV drug discovery efforts to combat this global public health concern.

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