Journal of Visualized Experiments

Quantification of Antibody-dependent Enhancement of the Zika Virus in Primary Human Cells

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58691R2
Full Title:	Quantification of Antibody-dependent Enhancement of the Zika Virus in Primary Human Cells
Keywords:	Zika Virus; Dengue Virus; Antibody dependent enhancement; Pre-immunity; Flaviviruses; Human serum samples
Corresponding Author:	Sultan Asad, PhD Boston University Boston, MA UNITED STATES
Corresponding Author's Institution:	Boston University
Corresponding Author E-Mail:	sasad@bu.edu
Order of Authors:	Sultan Asad, PhD
	Fabiana Feitosa-Suntheimer
	Alexander Gold
	Berlin Londono-Renteria
	Tonya Colpitts
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	BOSTON/MA, UNITED STATES OF AMERICA

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Click here to access/download;Manuscript;58691_R2.docx

TITLE:

Quantification of Antibody-dependent Enhancement of the Zika Virus in Primary Human Cells

AUTHORS AND AFFILIATIONS:

Sultan Asad[1], Fabiana Feitosa-Suntheimer[1], Alexander Gold[1], Berlin Londono-Renteria[2], Tonya M. Colpitts[1]

[1]Department of Microbiology & National Emerging Infectious Diseases Laboratories, Boston University School of Medicine, Boston, MA

[2]Department of Entomology, Kansas State University, Manhattan, KS

Corresponding Author:

Tonya M. Colpitts (tmcol@bu.edu)

617-358-9182

E-mail Addresses of the Co-authors:

Sultan Asad (sasad@bu.edu)

Fabiana Feitosa-Suntheimer (ffeitosa@bu.edu)

Alexander Gold (asgold@bu.edu)

Berlin Londono-Renteria (blondono@ksu.edu)

KEYWORDS:

Zika virus, dengue virus, pre-immune serum, antibody-dependent enhancement, flaviviruses, viral RNA quantification

SUMMARY:

We describe a method to evaluate the effect of pre-existing immunity against dengue virus on the Zika virus infection by using human serum, primary human cells, and infection quantification by quantitative real-time polymerase chain reaction.

ABSTRACT:

The recent emergence of the flavivirus Zika and neurological complications, such as Guillain-Barré syndrome and microcephaly in infants, has brought serious public safety concerns. Among the risk factors, antibody-dependent enhancement (ADE) poses the most significant threat, as the recent re-emergence of the Zika virus (ZIKV) is primarily in areas where the population has been exposed and is in a state of pre-immunity to other closely related flaviviruses, especially dengue virus (DENV). Here, we describe a protocol for quantifying the effect of human serum antibodies against DENV on ZIKV infection in primary human cells or cell lines.

INTRODUCTION:

Among the mosquitoes-borne viral diseases, Zika infection is one of the most clinically important[1]. The infection is caused by the flavivirus ZIKV which, in most cases, uses *Aedes aegypti* as its primary vector[1,2]. However, there are studies that have reported *Aedes albopictus* as a primary vector in some ZIKV outbreaks[3]. Although the infection is asymptomatic in many cases, the most common symptoms are fever, headache, and muscle pain[2]. There is no cure or vaccine

available for ZIKV infection and the treatment available is mostly supportive. Recent outbreaks of ZIKV in South America led to severe cases of the disease and an approximately 20-fold increase in the neurodevelopmental disorder in fetuses named microcephaly[2]. As South America is an area endemic to several arboviruses such as DENV and West Nile virus, it is crucial to investigate whether prior immunity to other flavivirus(es) plays a role in the severity of ZIKV infections and disease.

Throughout the ages, viruses have evolved different strategies to increase their chance of infectivity in order to take over the host cell machinery and suppress the antiviral response. One of the most fascinating of all is the use of host pre-immune antibodies by viruses to enhance their replication with the phenomenon ADE[4]. ADE across all four serotypes of DENV has been well studied and demonstrated to increase viral titers and disease outcome[5-7]. In a previous *in vitro* study, we have shown significant enhancement of ZIKV replication due to pre-existing DENV immunity in primary human immune cells[8]. We also demonstrated a relevant *in vitro* method to quantify the capability of DENV pre-existing antibodies to enhance ZIKV replication in primary cells.

The protocol that we have developed uses human serum samples that are tested for DENV neutralization in TCID-50 or plaque reduction neutralization test (PRNT) assays, along with ZIKV in biologically relevant cells or cells derived from tissues that ZIKV can infect.

PROTOCOL:

Serum samples used in this study were obtained from human participants of a cohort from Columbia. Sample collection was approved by the internal review board (IRB) at Universidad de Pamplona (Columbia, South America) and Los Potios Hospital[8]. The samples were anonymously provided and investigators had no access to patient information. The serum samples were checked for the DENV serotype. The samples were further confirmed to neutralize DENV infection *in vitro.* For control, serum samples from healthy individuals (HC) from the USA were used.

NOTE: This protocol can be used to examine the ADE of ZIKV replication in any human cell type expressing the Fcγ receptor. The protocol consists of three parts (**Figure 1**).

1. Cell Seeding and Infection Setup

NOTE: For this particular study, human primary macrophages or U937 myelomonocytic cell line (ATCC-CRL-1593.2) were used. The cells were maintained in RPMI growth medium supplemented with 10% fetal bovine serum (FBS) at a 37 °C incubator with 5% carbon dioxide (CO_2). All the steps were carried out in a biosafety level 2 (BSL-2) biosafety cabinet in sterile conditions.

1.1. Seed 3 x 10^4 cells per well in a sterile flat-bottom 96-well plate along with 100 µL of medium and place them in the 37 °C incubator with 5% CO_2.

1.2. After seeding the cells, thaw serum samples at room temperature and make 10-fold serial dilutions (*i.e.*, 1/10, 1/100, 1/1,000, 1/10,000) by mixing the samples in serum-free medium. Aliquot the dilutions into a sterile 96-well plate. Use the virus without serum as an additional control.

1.3. Thaw the ZIKV stock at 37 °C in a water bath for 1 - 2 min and quickly transfer them to ice for future use.

1.4. Add a 0.1 multiplicity of infection (MOI) equivalent amount of ZIKV strain MR766 to the serum aliquots.

NOTE: Make sure that the dilution factor remains constant and the total volume is ~200 µL; that is enough for triplicates of each treatment. Keep three wells uninfected to use as negative control for downstream analysis.

1.5. Incubate the serum dilutions with the added virus in the 37 °C incubator with 5% CO_2 for 1 h in order to allow the DENV antibodies to form complexes with the Zika virions (for convenience, hereafter referred to as “immune complexes”).

16. Aspirate the media from the cells that were seeded in the flat-bottom 96-well plate. Wash the cells with sterile 1x phosphate-buffered saline (1x PBS).

1.7. Add 50 µL of the immune complexes to each well and incubate them in the 37 °C incubator with 5% CO_2 for 2 h.

1.8. After 2 h of incubation, aspirate the media containing the immune complexes from the cells, using a multichannel pipette, and wash the cells 2x with 1x PBS to completely remove the immune complexes and any unattached virions.

1.9. Add 100 µL of fresh complete media supplemented with 10% FBS to each well and incubate the cells in the 37 °C incubator with 5% CO_2 for 48 h.

2. RNA Extraction

2.1. Remove the 96-well plate from the incubator and transfer it to the biosafety cabinet.

2.2. Aspirate the media, wash the cells 2x with 1x PBS, and proceed to RNA extraction.

NOTE: RNA can be extracted by any method of choice (refer to the **Table of Materials**).

2.3. Add 250 µL of cell lysis buffer with 10% β-mercaptoethanol per well. Pipette the buffer up and down at least 5x along with scratching the cells with the pipette tip to speed up the lysis procedure.

2.4. Transfer the cell lysates to new, labeled, sterile 1.5 mL tubes.

2.5. Add an equal amount of 70% ethanol (250 µL). Pipette up and down 4x - 5x until the mixture is clear.

2.6. Transfer the mixture (~500 µL) to labeled silica-based columns in 2 mL collection tubes and centrifuge at 15,000 x *g* for 30 s. Discard the flow through and keep the columns in the same collection tubes.

2.7. Add 700 µL of wash buffer 1 to each column and centrifuge at 15,000 x *g* for 30 s. Discard the flow through and keep the columns in the same collection tubes.

2.8. Add 500 µL of wash buffer 2 to each column and centrifuge at 15,000 x *g* for 30 s. Discard the supernatant. Repeat this step 2x.

2.9. Transfer the columns to new 2 mL collection tubes and centrifuge at 15,000 x *g* for 2 min. Ensure that silica-based columns get completely dry and there is no ethanol left from wash buffer 2.

2.10. Discard the 2 mL tubes and place the columns in new, sterile, labeled, 1.5 mL recovery tubes.

2.11. Add prewarmed (42 °C) 30 µL of RNase-free water at the center of each column and centrifuge at 15,000 x *g* for 1 min.

2.12. Recover the eluted RNA and quantify the samples, using a spectrophotometer at a 260 nm wavelength.

NOTE: Ideally, determine the purity of the RNA by calculating the ratio between the absorbance values at 260 nm and 280 nm. Purified RNA's 260/280 ratio is ideally between 1.8 - 2.0.

3. Quantitative Real-time Polymerase Chain Reaction

NOTE: Quantitative real-time polymerase chain reaction (qRT-PCR) can be carried out by using any SYBR green mix which usually is composed of SYBR Green I dye, Taq DNA polymerase, deoxynucleotide triphosphates (dNTPs), and a passive dye. Any qPCR machine capable of the detection of SYBR green can be used to perform the reaction and acquire the data. For this experiment, a one-step RT-PCR kit was used which had a cocktail of SYBR Green I, ROX dye, Taq DNA polymerase, dNTPs, and an additional mixture of reverse transcriptase (refer to **Table of Materials**). The primers designed against the envelope region and used in this study to quantify ZIKV genomic levels are CCGCTGCCCAACACAAG for ZIKV-qF and CCACTAACGTTCTTTTGCAGACAT for ZIKV-qR. As a control, human β-2-microglobulin (B2M) was measured and used to normalize the expression of ZIKV (housekeeping gene). The primer sequences to quantify the B2M gene expression used in this study are CTCCGTGGCCTTAGCTGTG for B2M-qF and

TTTGGAGTACGCTGGATAGCCT for B2M-qR. Ensure to put three technical replicates for each sample for both the ZIKV and the B2M genes. The setup of the RT-PCR is briefly described below.

3.1. RT-PCR setup

3.1.1. Use 100 ng of RNA per sample.

3.1.2. Add 1 µL from 10 µM stocks of both forward and reverse primers of a particular gene for each reaction.

3.1.3. Add 0.25 µL of reverse transcriptase mix per sample.

3.1.4. For each individual reaction, add 12.5 µL of SYBR Green mix.

3.1.5. Add up to 25 µL of water. Make a master mix of all the above-mentioned reagents except RNA, aliquot it in the 96-well PCR plate, and add RNA last.

3.1.6. Seal the plate with transparent adhesive tape.

3.1.7. Centrifuge the plate at 1,000 x *g* for 1 min to mix the reagents.

3.1.8. Put the plate in the qPCR machine.

3.2. RT-PCR profile

NOTE: Use the RT-PCR profile shown in **Table 1**.

3.2.1. Incubate the samples at 50 °C for 10 min in order to ensure the synthesis of complementary DNA (cDNA).

3.2.2. After the cDNA synthesis, activate the Taq DNA polymerase at 95 °C for 5 min.

3.2.3. Perform 40 cycles of denaturation at 95 °C for 10 s and primer annealing and extension at 60 °C for 30 s.

3.2.4. Put the additional melt curve step (65 °C) in the end.

3.3. Data analysis

3.3.1. Monitor the melt curve by clicking the melt curve tab in the program used to run the qPCR machine, which, ideally, shows a single peak in all the samples for a particular gene to confirm the presence of only one amplicon and an amplification value more than 1.6 if the algorithm considers 2 as the 100% amplification value (the amplification value varies according to the

algorithm used by the qPCR machine). Make sure to use 0.5 °C temperature increments between steps and a minimum holding time of 10 s in the melt curve protocol, for optimal results.

3.3.2. After making sure there are a single amplicon and good amplification value, first click on the quantification data tab to get a quantitative cycle (Ct/Cq value) of each sample and export to Microsoft Excel.

3.3.3. Using a spreadsheet, calculate the average of each sample using the Ct value. Next, click in the formulas and select the average. The average CT values of each sample (replicate) are used for further analysis.

3.3.4. Next, calculate the ΔCt using the formula ΔCt = average Ct of the target gene – average Ct of the control gene (in this case, ΔCt = average Ct of the ZIKV gene – average Ct of the B2M gene).

3.3.5. Calculate ΔΔCt to normalize the data (in this case, ΔΔCt = ΔCt ZIKV samples – B2M samples).

3.3.6. Calculate the fold increase gene expression by typing the formula 2^-(ΔΔCt) for every single ΔΔCt normalized value. The results of the fold increase to perform statistical tests can be obtained as described in earlier studies[9-10].

REPRESENTATIVE RESULTS:

In **Figure 1**, there is a step-by-step diagrammatic illustration of all the steps involved to carry out the ADE protocol. It is a schematic diagram showing the whole procedure of ADE of ZIKV due to pre-existing immunity to DENV. **Figure 2** shows how human serum samples were categorized into three different groups: DENV infection-confirmed samples are referred to as the DENV-infected group, DENV antibody-confirmed samples are referred to as the DENV-exposed group, and healthy individuals' sera with no DENV-neutralizing antibodies or RNA are called the healthy control (HC) group. (**Figure 2**).

All the serum samples were allowed to make complexes with ZIKV and were then used to infect macrophage cells. After 48 h postinfection, RNA was extracted and subjected to qRT-PCR. The representative experiment in **Figure 3** demonstrates that most of the sera containing DENV serotype 1 to 4 antibodies were able to enhance ZIKV replication at different levels. The highest increase in ZIKV titers was found in the macrophages treated with sera containing DENV serotype 2 and 4 antibodies as compared to serotype 1 and 3, which showed a relatively less induction of ZIKV.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagrammatic illustration of the ADE protocol. A step-by-step illustration shows all the steps involved in the whole protocol.

Figure 2: Schematic of the experimental procedure. Three types of sera were incubated with Zika virus. Group I consisted of DENV-RNA positive (DENV-infected) samples. Group II consisted

of DENV-antibody positive (DENV-exposed) samples. Group III consisted of samples with no DENV RNA or antibody (healthy controls). The virus-sera mixture was added to human macrophages and the infection quantified. This figure has been modified from Londono-Renteria *et al.*[8].

Figure 3: DENV immune sera enhances ZIKV infection in primary human macrophages. Human sera containing DENV antibodies (DENV1-4 are serotype confirmed, Col are serotype unknown) or from healthy controls were diluted 1:10 to 1:10,000 and incubated with ZIKV. The sera are described in **Table 1**. Primary isolated human macrophages were infected either with ZIKV alone or with the ZIKV-sera mixtures. (**a**) DENV1 antibody-containing sera. (**b**) DENV2 antibody-containing sera. (**c**) DENV3 antibody-containing sera. (**d**) DENV4 antibody-containing sera. (**e**) DENV-antibody sera from Colombian individual 1. (**f**) DENV-antibody sera from Colombian individual 2. The infection was measured by qRT-PCR analysis at 48 h postinfection. Technical and biological replicates were done in triplicate. The data are pooled, and the error bars indicate standard deviation. Student's *t*-test and ANOVA were used for the statistical analysis. * $P < 0.001$. This figure has been modified from Londono-Renteria *et al.*[8].

Table 1: Thermal profile of qRT-PCR.

DISCUSSION:

Cross-reactivity of DENV antibodies leading to the ADE of other DENV serotypes has hindered the development of an effective vaccine[11]. ZIKV belongs to the same family, Flaviviridae, and has a considerable homology with other flaviviruses, especially DENV[12]. The main target of neutralizing antibodies for both ZIKV and DENV is the envelope protein, which shares a very high structural and quaternary sequence homology between the two viruses[13-15]. It has been demonstrated that pre-immunity to either DENV or ZIKV can enhance the infection of the other virus[8,16].

There are a number of different methods employed to quantify the viral infectivity in ADE experiments, ranging from plaque assays[17], intracellular viral antigen staining using antibodies conjugated with fluorescent dyes, and flow cytometry[18,19]. These assays are time-consuming and not easily adaptable for the high-throughput of multiple samples at the same time. Importantly, most of the studies used DENV-specific monoclonal antibodies to check their effect on ZIKV and examined the ADE in cell lines only[20,21]. In this protocol, we describe a simple yet effective method in which we used human pre-immune serum samples that have a neutralizing ability against DENV, along with primary human immune cells, to examine the effect of patient serum on ZIKV replication by employing qRT-PCR. This method is robust, relevant, and can be completed in three days. Although the representative results in this manuscript show its application for ZIKV, this protocol can be easily modified and used for other flaviviruses, like yellow fever virus, dengue virus, and West Nile virus. One important factor to consider is that this protocol has a limitation in distinguishing between mature and immature viral RNA.

During the protocol, it is critical to give careful consideration when performing the RNA extractions, ensuring that the environment is RNase-free during all the RNA handling steps. Furthermore, viral stocks should be thawed at 37 °C in a water bath for 1 - 2 min and immediately put on ice. However, the virus should not be kept on ice for long as, then, it can lose its infectivity.

Previous results have demonstrated that this protocol is highly convenient and adaptable to handle a large number of samples and can be completed in relatively less time than other methods. This protocol has the potential to be used as a useful assay for future ADE studies.

ACKNOWLEDGMENTS:

This work was generously supported by 1R21AI129881-01 (to T.M.C.), start-up funds from the National Emerging Infectious Diseases Laboratories, and the Boston University School of Medicine.

DISCLOSURES:

The authors have nothing to declare.

REFERENCES:

1. Hayes, E. B. Zika virus outside Africa. *Emerging Infectious Diseases.* **15**, 1347-1350 (2009).

2. Grard, G. *et al.* Zika virus in Gabon (Central Africa) - 2007: a new threat from Aedes albopictus? *PLoS Neglected Tropical Diseases.* **8** (2), e2681 (2014).

3. Fauci, A. S., Morens, D. M. Zika Virus in the Americas--Yet Another Arbovirus Threat. *New England Journal of Medicine.* **374** (7), 601-604 (2016).

4. Hawkes, R. A. Enhancement of the Infectivity of Arboviruses by Specific Antisera Produced in Domestic Fowls. *Australian Journal of Experimental Biology and Medical Science*. **42**, 465-482 (1964).

5. Musso, D., Gubler, D. J. Zika Virus. *Clinical Microbiology Reviews.* **29** (3), 487-524 (2016).

6. Vaughn, D. W. *et al.* Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *The Journal of Infectious Diseases.* **181** (1), 2-9 (2000).

7. Khandia, R. *et al.* Modulation of Dengue/Zika Virus Pathogenicity by Antibody-Dependent Enhancement and Strategies to Protect Against Enhancement in Zika Virus Infection. *Frontiers of Immunology*. **9**, 597 (2018).

8. Londono-Renteria, B. *et al.* A relevant *in vitro* human model for the study of Zika virus antibody-dependent enhancement. *Journal of General Virology.* **98** (7), 1702-1712 (2017).

9. Ganger, M. T., Dietz, G. D., Ewing, S. J. A common base method for analysis of qPCR data and the application of simple blocking in qPCR experiments. *BMC Bioinformatics.* **18** (1), 534 (2017).

10. Renn, L. A. *et al.* High-throughput quantitative real-time RT-PCR assay for determining expression profiles of types I and III interferon subtypes. *Journal of Visualized Experiments.* (97), e52650 (2015).

11. McArthur, M. A. *et al.* Dengue vaccines: recent developments, ongoing challenges and current candidates. *Expert Review of Vaccines*. **12** (8), 933-953 (2013).

12. Priyamvada, L. *et al.* Humoral cross-reactivity between Zika and dengue viruses: implications for protection and pathology. *Emerging Microbes and Infections.* **6** (5), e33 (2017).

13. Dai, L. *et al.* Molecular basis of antibody-mediated neutralization and protection against flavivirus. *IUBMB Life.* **68** (10), 783-791 (2016).

14. Dai, L. *et al.* Structures of the Zika Virus Envelope Protein and Its Complex with a Flavivirus Broadly Protective Antibody. *Cell Host & Microbe*. **19** (5), 696-704 (2016).

15. Sirohi, D. *et al.* The 3.8 A resolution cryo-EM structure of Zika virus. *Science*. **352** (6284), 467-470 (2016).

16. George, J. *et al.* Prior Exposure to Zika Virus Significantly Enhances Peak Dengue-2 Viremia in Rhesus Macaques. *Scientific Reports.* **7** (1), 10498 (2017).

17. Morens, D. M., Halstead, S. B. Measurement of antibody-dependent infection enhancement of four dengue virus serotypes by monoclonal and polyclonal antibodies. *Journal of General Virology*. **71** (Pt 12), 2909-2914 (1990).

18. Dejnirattisai, W. *et al.* Cross-reacting antibodies enhance dengue virus infection in humans. *Science*. **328** (5979), 745-748 (2010).

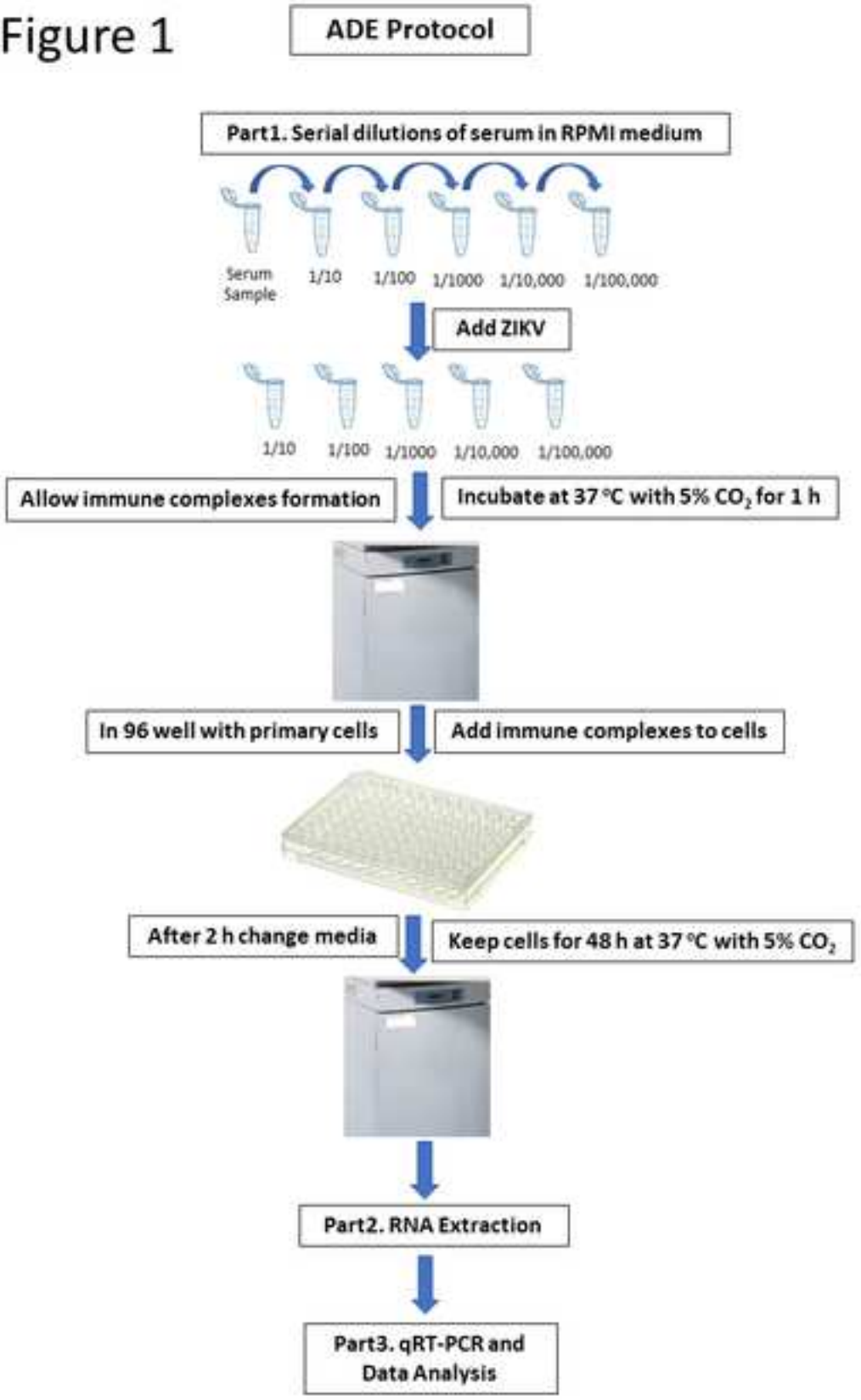
19. Priyamvada, L. *et al.* Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus. *Proceedings of the National Academy of Sciences of the United States of America.* **113** (28), 7852-7857 (2016).

20. Charles, A. S., Christofferson, R. C. Utility of a Dengue-Derived Monoclonal Antibody to Enhance Zika Infection In Vitro. *PLoS Currents*. **8** (2016).

21. Swanstrom, J. A. *et al.* Dengue Virus Envelope Dimer Epitope Monoclonal Antibodies Isolated from Dengue Patients Are Protective against Zika Virus. *MBio.* **7** (4) (2016).

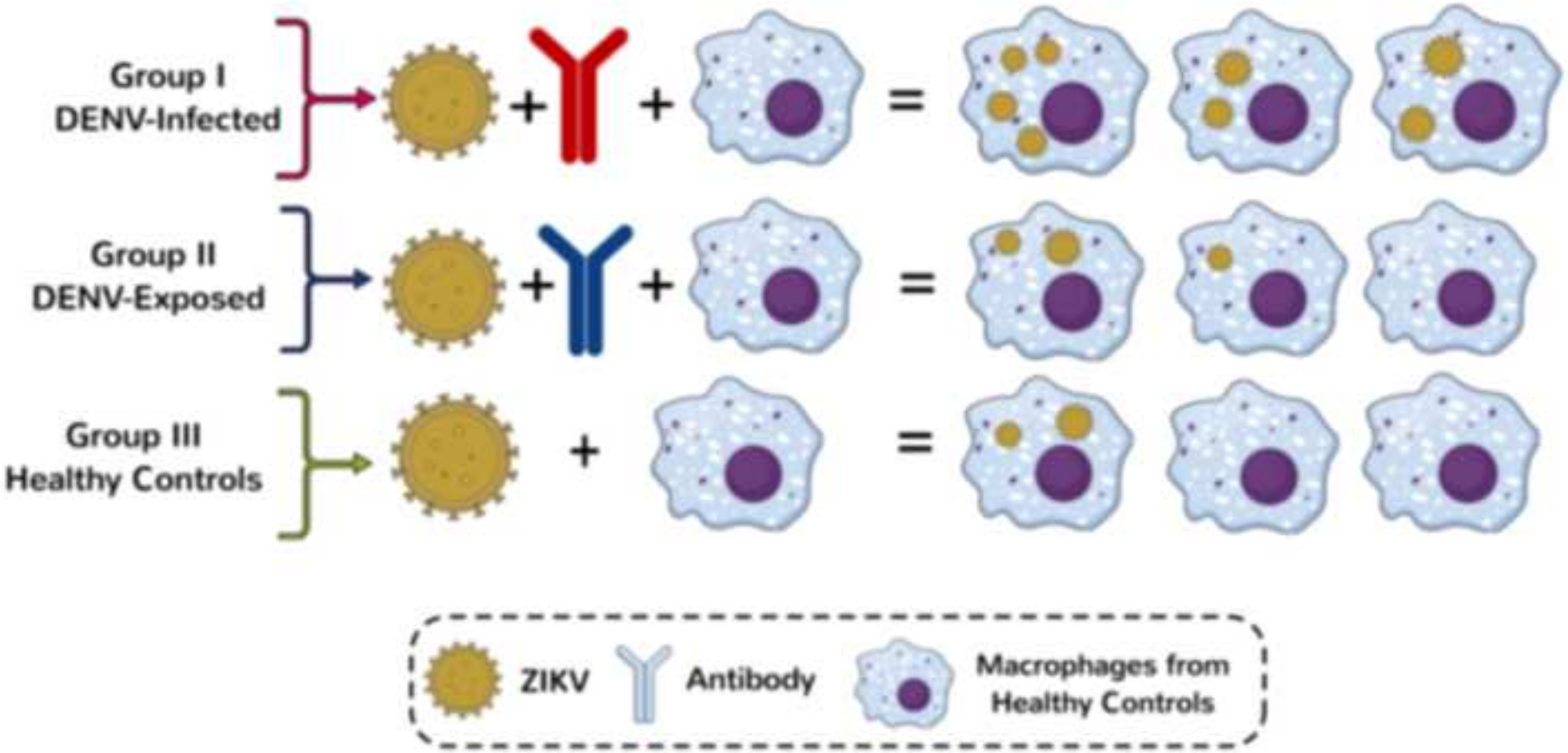
Click here to access/download;Figure;Figure1.PNG

Figure 1



Click here to access/download;Figure;Figure2.PNG

Figure 2



Click here to access/download;Figure;Figure3.PNG

Figure 3

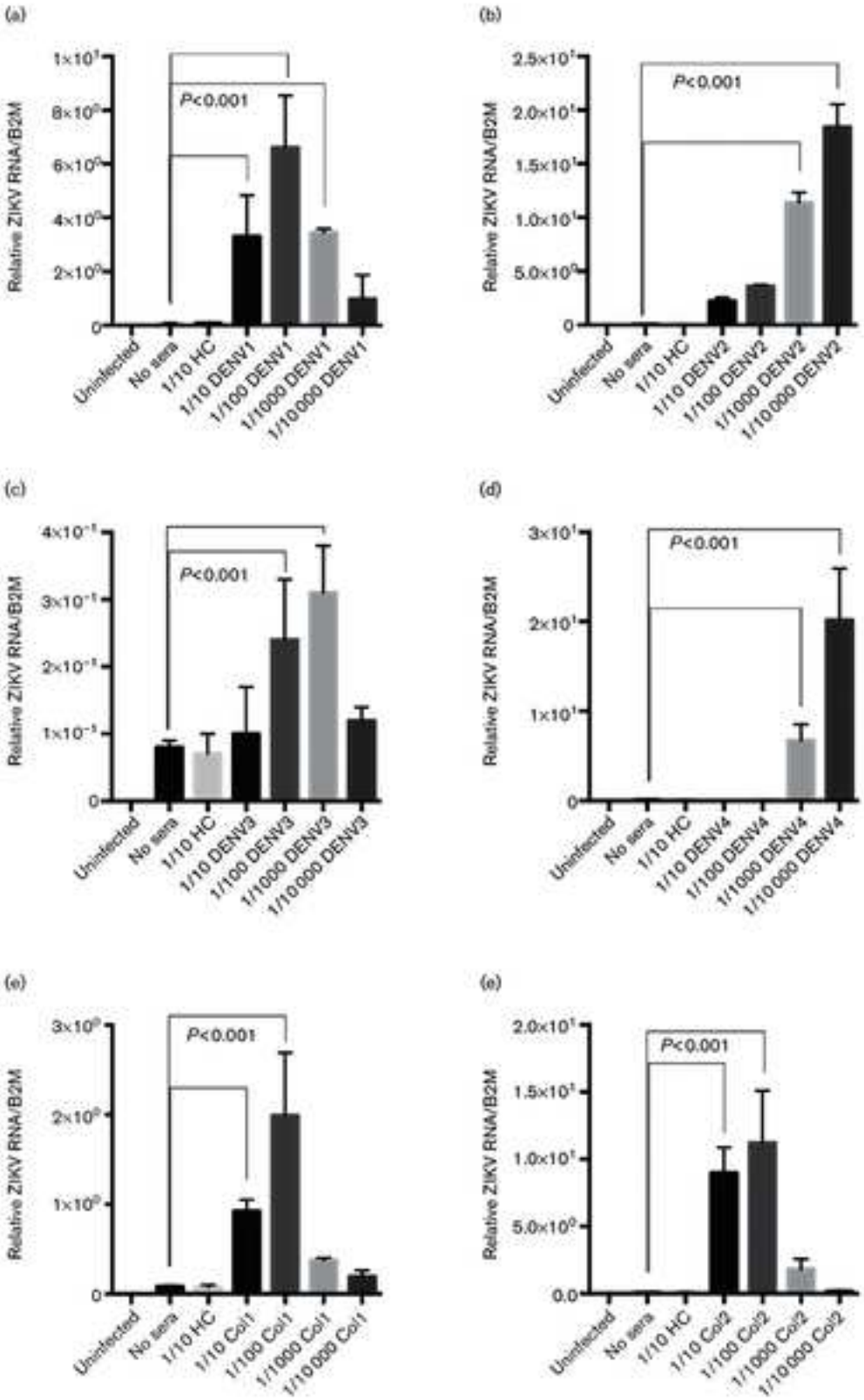


Table1

Click here to access/download;Table;JoVE-Table 1 .xlsx

S.no.	Step	Temperature
1	Reverse Transcription	50°C
2	PCR activation	95°C
3	Two Step Cycling	
	a) Denaturation	95°C
	b) Annealing and extension	60°C
4	Melt curve	65°C to 95°C

Time
10 minutes
5 minutes
10seconds 40cycles
30 seconds
In increment of 0.5 ^{o}C

40 Cycles

Click here to access/download;Table of Materials;Copy of JoVE_Materials-revised.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Fetal Bovine Serum	GEMINI	100-106	
iCycler	BioRad	785BR02188	Model No. CFX96 Optics Module
Microfuge 18 Centrifuge	Beckman Coulter	367160	
Nanodrop-1000	Thermoscientific	1072	
Quantifast SYBR-One step RT-PCR kit	Qiagen	204154	Used for 1 step RT-qPCR
RNeasy RNA Isolation Kit	Qiagen	74106	Used for RNA extraction
RPMI-medium	Gibco	11875093	

Click here to access/download;Author License Agreement (ALA);Author_License_Agreement signed.pdf

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Method for the Quantification of Antibody Dependent Enhancement of Zika Virus in Primary Human Cells
Author(s):	Sultan Asad, Fabiana Feitosa-Suntheimer, Alexander Gold, Berlin Londono-Renteria and Tonya Colpitts

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Tonya M. Colpitts
Department:	Microbiology, & National Emerging Infectious Diseases Laboratories
Institution:	Boston University **School of Medicine**
Title:	Assistant Professor
Signature:	
Date:	6-28-18

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Rebuttal Letter
Click here to access/download;Rebuttal Letter;Response to the reviewers.docx

Response to Editorial and Reviewers Comments in bold

Editorial comments:

Changes to be made by the Author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have tried our best to make this manuscript free of spelling or grammar issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Permission has been taken from Journal of general virology for the use of figure 2 and 3. Permission document has been uploaded. Figure legends have been updated as suggested.

3. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.

Each figure uploaded separately as .png

4. Figure 1: Please use SI abbreviations for all units (°C, h) and include a space between numbers and their units. Please make the number in CO2 as a subscript.

Figure 1 corrected

5. Please provide an email address for each author.

Email of each author included

6. Please spell out each abbreviation the first time it is used.

Checked the abbreviation and corrected them

7. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

All the units are changed to SI abbreviations

8. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Space included between all the numbers and their corresponding units

9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Trizol, Qiagen, Nanodrop, Bio-Rad iCycler, QuantiFast®, etc.

We have tried to make the language as generic as possible by simply assigning the name of Qiagen kit buffers according to their functions, as the original compositions of the buffers is classified.

10. Please revise the protocol to be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc. Please refrain from using bullets, dashes, or indentations.

Numbering corrected

11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Changed as suggested

12. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

Changed as suggested

13. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

We understand that protocol should be based on discrete steps. We have tried to make short and precise paragraphs.

14. Lines 161-163: Please remove the embedded table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Table removed from the manuscript and .xls file attached to the Editorial manager.

15. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. For example:
Lines 165-166: Please mention how to obtain and monitor melt curve and amplification value. What values are considered to be healthy?

Information related to the amplification values and melt cure has been added to manuscript

Line 168: How to calculate the ΔΔCt values?

Formula has been added to the manuscript

16. Please reference figures showing the experimental set-up in the Protocol.

We made this figure to give a visual demonstration of step involved in this protocol specifically for this manuscript and has not been published before.

17. Please include single-line spaces between all paragraphs, headings, steps, etc.

Revised as suggested

18. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Protocol paragraphs highlighted
19. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Done as suggested
20. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Done as suggested
21. Please describe Figure 3 in more detail in Representative Results.

More details of figure 3 has been added
22. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have highlighted all the critical steps in main protocol. And we have discussed both significance and future applications of the techniques in the discussion.

23. References: Please do not abbreviate journal titles.

Changed as suggested

24. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available.

Revised as suggested

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes a simple but effective, RT-QPCR based protocol to assess the dengue serum caused ADE on Zika infection in human macrophages or primary cell lines. This protocol is significant and timely because ZIka virus is causing world-wide human health concerns. There are a lot of efforts in developing antivirals against Zika infection and this protocol may facilitate the process of testing the potential ADE of antibody-based anti-Zika vaccines. This protocol will also be interested especially to the related new investigators who recently started to study Zika and dengue related research topics.

Major Concerns:
None.

Minor Concerns:
The authors may need to state the strain name of Zika virus they used, and which Zika gene they amplified with their qPCR primers.

Information about strain and gene used for qPCR primers has been included in the manuscript

Reviewer #2:

Manuscript Summary:
A good manuscript worth for JOVE, however minor corrections are required.

Major Concerns:
1. Line 31 it appears that (ZIKV), which uses Aedes aegypti and Aedes albopictus as its primary and secondary transmission vector, where As per Grard et al., 2007, Aedes albopictus is primary vector for Zika virus involved in the Gabonese outbreaks.

The information along with reference regarding *Aedes Albopictus* has been added to the manuscript.

2. Line 51 write full form of abbreviation PRNT.

The full form of PRNT has been added to the manuscript.

3. A little-bit brushing in language correction is required.

We have tried our best to improve the language of the manuscript.

Minor Concerns:

Add few latest references on ADE e.g. Khandia et al. (2018) Modulation of Dengue / Zika Virus Pathogenicity by Antibody-Dependent Enhancement and Strategies to Protect against Enhancement in Zika Virus Infection. Front. Immunol. doi: 10.3389/fimmu.2018.00597.

The abovesaid reference has been added

Rebuttal Letter
Click here to access/download;Rebuttal Letter;Response to editorial comments_09-27-2018.docx

Response to the editorial comments in bold

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have gone through the whole manuscript to look for the spelling and grammar issue to the best of our knowledge.

2. Please provide at least 6 keywords or phrases (there are only 3 in the manuscript).

Added key words to the total of 6.

3. Please ensure that the references appear as the following:

Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).

For more than 6 authors, list only the first author then et al.

All the references are formatted according to the editor's instructions.

4. Please do not highlight notes for filming.

Notes not highlighted as suggested.

5. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc., and h, min, s for time units.

All the units are changed to SI symbols and prefixes.

6. JoVE cannot publish manuscripts containing commercial language. This includes company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include Ominiscript, Sensiscript, etc.

All the commercial language has been removed from the manuscript.

7. Please define all abbreviations before use.

Done as suggested

8. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done as suggested

9. Line 87-88: Is this a step or a note?

This is a step. Corrected and renumbered the steps.

10. Line 172-183: Is this a step or a note? If it's not a protocol step, please do not highlight. IF it's a step, please ensure that all text is written in imperative tense.

This is a brief introduction of genes and primers used for setting up the reactions and the chemistry used to detect the signals. It will be important to let audience know which gene primers and chemistry we used. That's why we think it will be important to include it in the video.

11. Step 3.1: Please write this step in complete sentences and in imperative tense.

Done as suggested

12. Step 3.2: Please write this step in imperative tense.

Done as suggested

13. 3.3.1: How to monitor?

Information added

14. 3.3.1-3.3.3: For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

Information and steps used of software added to the manuscript

15. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

Discussion has been revised.

a) Critical steps within the protocol

Mentioned in lines 304-308

b) Any modifications and troubleshooting of the technique

Added lines 299-301

c) Any limitations of the technique

Mentioned in lines 301-303

d) The significance with respect to existing methods

Mentioned in lines 309-310

e) Any future applications of the technique

Added line 311

Permission to use figures

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Licence document.docx

Copyright Clearance Center

Note: Copyright.com supplies permissions but not the copyrighted content itself.

1 PAYMENT 2 REVIEW **3 CONFIRMATION**

Step 3: Order Confirmation

Thank you for your order! A confirmation for your order will be sent to your account email address. If you have questions about your order, you can call us 24 hrs/day, M-F at +1.855.239.3415 Toll Free, or write to us at info@copyright.com. This is not an invoice.

Confirmation Number: 11726979
Order Date: 06/28/2018

If you paid by credit card, your order will be finalized and your card will be charged within 24 hours. If you choose to be invoiced, you can change or cancel your order until the invoice is generated.

Payment Information

Sultan Asad
Boston University
sasad@bu.edu
+1 (617) 358-9560
Payment Method: CC ending in 5108

Order Details

The Journal of general virology

Order detail ID:	71267856
Order License Id:	4377661427609
ISSN:	0022-1317
Publication Type:	Journal
Volume:	
Issue:	
Start page:	
Publisher:	Microbiology Society
Author/Editor:	FEDERATION OF EUROPEAN MICROBIOLOGICAL SOCIETIES ; SOCIETY FOR GENERAL MICROBIOLOGY

Permission Status: Granted

Permission type: Republish or display content
Type of use: Republish in other published product

Hide details

Requestor type	Publisher, not-for-profit
Format	Electronic
Portion	chart/graph/table/figure
Number of charts/graphs/tables/figures	2
The requesting person/organization	Sultan Asad/Boston University
Title or numeric reference of the portion(s)	Figure 2 and figure S1
Title of the article or chapter the portion is from	A relevant in vitro human model for the study of Zika virus antibody-dependent enhancement
Editor of portion(s)	NA
Author of portion(s)	NA
Volume of serial or monograph	98: 1702-1712
Page range of portion	NA
Publication date of portion	October 2018
Rights for	Main product
Duration of use	Current edition and up to 10 years
Creation of copies for the disabled	no

privileges	
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no
Lifetime unit quantity of new product	Up to 499
Title	Method for the Quantification of Antibody Dependent Enhancement of Zika Virus in Primary Human Cells
Author	Sultan Asad, Fabiana Feitosa-Suntheimer, Alexander Gold, Berlin Londono-Renteria and Tonya Colpitts
Produced by	Department of Microbiology & National Emerging Infectious Diseases Laboratories, Boston University School of Medicine, Boston, MA and Department of Entomology, Kansas State University, Manhattan, Kansas.
Expected publication date	Oct 2018

Note: This item will be invoiced or charged separately through CCC's **RightsLink** service. More info **$ 3.50**

This is not an invoice.

Total order items: 1

Order Total: 3.50 USD