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TITLE:

Isolation and Quantification of Zika Virus from Multiple Organs in a Mouse

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SUMMARY:

The goal of the protocol is to demonstrate the techniques used to investigate viral disease by isolating and quantifying Zika virus, from multiple organs in a mouse following infection.

ABSTRACT:

The methods being presented demonstrate laboratory procedures for the isolation of organs from Zika virus infected animals and the quantification of viral load. The purpose of the procedure is to quantify viral titers in peripheral and CNS areas of the mouse at different time points post infection or under different experimental conditions to identify virologic and immunological factors that regulate Zika virus infection. The organ isolation procedures demonstrated allow for both focus forming assay quantification and quantitative PCR assessment of viral titers. The rapid organ isolation techniques are designed for the preservation of virus titer. Viral titer quantification by focus forming assay allows for the rapid throughput assessment of Zika virus. The benefit of the focus forming assay is the assessment of infectious virus, the limitation of this assay is the potential for organ toxicity reducing the limit of detection. Viral titer assessment is combined with quantitative PCR, and using a recombinant RNA copy control viral genome copy number within the organ is assessed with low limit of detection. Overall these techniques provide an accurate rapid high throughput method for the analysis of Zika viral titers in the periphery and

CNS of Zika virus infected animals and can be applied to the assessment of viral titers in the organs of animals infected with most pathogens, including Dengue virus.

INTRODUCTION:

Zika virus (ZIKV) is an arbovirus that belongs to the flaviviridae family, which includes important neuroinvasive human pathogens such as Powassan virus (POWV), Japanese encephalitis virus (JEV), and West Nile virus (WNV)[1]. Following its isolation and identification, there have been periodic reports of human ZIKV infections in Africa and Asia[2-5], and epidemics within Central and South America (reviewed in[6]). However, it was not until recently that ZIKV was thought to cause severe disease[7]. Now there are thousands of cases of neurological disease and birth defects linked to ZIKV infections. The rapid emergence of ZIKV has prompted many questions relating to: why there is an increase in disease severity, what is the immunological response to ZIKV infection and are there viral and/or immune mediated pathologies linked to the increase in neurological manifestations and birth defects. There is now a rush to understand the central nervous system (CNS) related disease associated with ZIKV as well as the need to rapidly test the efficacy of the antivirals and vaccines against ZIKV. It is against this backdrop that we have developed methods for the rapid analysis of ZIKV titers in both the periphery and CNS using a ZIKV-specific focus forming assays (FFA).

Small animal models are important for understanding disease progression and for the early evaluation of vaccines, therapeutics, and anti-virals. We have established small animal models for the study of arbovirus disease by using various mouse strains to model human infection and protection against viral pathogens[8-22]. Using this prior experience, we began to modify techniques used for the assessment of WNV and Dengue virus, a related flavivirus for the assessment of ZIKV titer in both peripheral organs as well as the CNS[21,23,24]. The advantages of these methods over other assays are: 1) that they combine the ability to harvest both peripheral and CNS organs for the analysis; 2) the methods are adaptable for flow cytometry, for measurements of innate and adaptive immune responses, along with viral titers on the same animal in the same organ; 3) the harvest technique is adaptable for histological analysis; 4) the ZIKV FFA is a rapid high throughput method for viral titer analysis; and 5) these methods can be applied to the assessment of viral titers in the organs of animals infected with most pathogens[25].

PROTOCOL:

All procedures of the present study are in accordance with the guidelines set by the St. Louis University Animal Care and Use Committee. SLU is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

1. Organ isolation

NOTE: The virus is not stable at room temperature (RT) so the number of animals harvested at one time must be planned carefully to preserve viral titers.

1.1. Infect mice using the chosen dose and route, based upon the phenotype required. For this protocol, infect 8-10 week old male and female type I interferon receptor deficient (Ifnar1$^{-/-}$) C57BL/6 mice.

1.1.1. Anesthetize Ifnar1$^{-/-}$ mice with a cocktail of Ketamine (90 mg/kg) and Xylazine (10 mg/kg). Test pedal reflex by a firm toe pinch to confirm anesthesia. Administer 1 x 10^5 focus forming units (FFU) of ZIKV in 50 µL subcutaneously (SC) via the footpad.

1.2. Prepare all the materials needed for harvest the day before: 2 scissors, forceps and 20 mL of 70% ethanol (EtOH) in a 50 mL conical for disinfection of tools.

1.2.1. Prepare syringes and needles: a 20 mL syringe for perfusion, 23-gauge needles (approximately 1 per cage) and 1 mL syringe with 25-gauge needle (1 per 3-5 mouse). Prior to weighing tubes, add steel beads (in hood) to 1.5 mL O-ring screw cap tubes (one for each organ). The tubes need to be appropriate for homogenization.

1.3. Prepare *the day of* the harvest the materials needed for the perfusion and organ freezing.

1.3.1. Fill an ice bucket with dry ice and 70% EtOH to make an ice bath. Prepare sterile phosphate buffered saline (PBS) assuming that 25-30 mL per mouse of PBS for each perfusion is needed 5-10 mL for the spinal cord). Obtain anesthesia, a cocktail of Ketamine and Xylazine, and assume 300 µL per mouse. Place all the materials and any additional tubes required for flow cytometry, etc., in a secondary container to transport to the animal facility.

1.4. Follow all necessary procedures for entry including donning and doffing personal protection equipment during entry and exit into the animal facility.

CAUTION: All the procedures are to be carried out in a certified biosafety cabinet within a Biosafety level 2 laboratory (BSL-2) facility.

1.5. Administer anesthesia, 200-500 µL, intraperitoneally depending on size and weight of the animal. If working alone, administer anesthesia to one animal at a time to avoid killing the animals before organ harvest.

1.5.1. Confirm anesthesia dose by pinching toe with forceps to evaluate the pedal reflex. Do not continue unless completely nonresponsive. Use pins to secure the mouse to a wax board. At this point, douse the mouse in 70% ethanol to avoid hair contamination in the organ harvest procedure

1.6. Terminally bleed by heart puncture.

1.6.1. Using the scissors and forceps, open the animal through the chest cavity to expose the heart. Collect blood via heart puncture (~800 µL), this can be used for multiple assays including

clinical chemistry, hematology, flow cytometry to detect antigen specific responses, and real time quantitative PCR (qRT-PCR) for the analysis of viral copy number.

1.6.2. For viral RNA analysis by qRT-PCR, collect blood in an EDTA tube if extracting from whole blood or in a microcentrifuge tube if extracting from serum. (For serum, spin tube at maximum speed in centrifuge, room temp, for 20 minutes and remove serum to a separate tube). Add a linear polyacrylamide as a carrier to the blood sample after finishing. RNA can then be analyzed or stored at -80 °C to further process at a later date.

1.7. Fill the 20 mL syringe with PBS and perfuse mice by inserting the butterfly needle into the left ventricle. Puncture the right atrium to allow blood and PBS to exit. Slowly administer the PBS while checking the color of the liver to confirm that the animal is completely perfused. Liver should change from deep red to pink salmon color.

1.7.1. *If* preparing the organs for histology, leave butterfly needle in place and then perfuse with 20 mL of 4% paraformaldehyde. If so, it is convenient to have the syringe connected to a 3-way stopcock with both syringes attached to it, turning the valve ON and OFF as one alternates between PBS and PFA.

1.8. Harvest organs into labeled, weighed tubes. For peripheral organs, follow an established order for harvest: liver, spleen, kidney and lungs.

1.8.1. Take only one lobe from the liver; it does not matter which one, but for all experiments always try to take the same lobe with the same size piece. Similarly, for kidneys and lungs, take the same kidney and lung. If flow cytometry is also to be completed, any organ can be cut in half. Store the half to be used for cytometry in Roswell Park Memorial Institute medium (RPMI) at room temperature until the harvest is complete.

1.8.2. Immediately after harvest, put each organ in a labeled tube and place in the dry ice bath. Virus titer reduces over time at room temperature, so the amount of time it takes to harvest organs is extremely important. There must be consistency between mice, so after safety, the next priority is speed.

NOTE: If harvesting organs for viral titers, it is very helpful to have a second individual harvest the brain at this point, to preserve viral titers.

1.9. Remove the remaining organs from the mouse body cavity to gain access to the spine and skull.

1.9.1. Remove the mouse from the board and remove the pelt, followed by the removal of the arms and legs.

Remove the head of the mouse with a decapitation, blunt or surgical scissors to harvest the brain by cutting the skull with a serrated LaGrange scissors through the *foramen magnum*. Then, peel off the skull with forceps and scoop out the brain with a spatula.

1.9.2. Using strong, blunted scissors, remove the ribs and other bones surrounding the spine. Then, cut across the pelvic bone, exposing the *vertebral foramen* at the lumbar level. The small tip of the spinal cord should be visible at this point.

1.9.3. Use a 10 mL syringe filled with PBS and a 18-gauge needle to expel the spinal cord by "flushing" the cord from lumbar to cervical spine over a Petri dish.

1.9.4. Carefully, place the beveled tip of the needle inside the vertebral foramen, avoiding excessive pressure to prevent the needle to trespassing the vertebral body. Hold strongly to exert pressure on the vertebral body and the needle and press the syringe plunger to expel the cord. Immediately transfer the spinal cord in the labeled tube and place in the dry ice bath.

1.10. Repeat the procedure with all the animals until the harvest is completed. Place the harvest tools in the 70% ethanol between animals. Focus on consistency and speed. The length of time between each organ harvest should remain consistent so as to not bias viral titer results.

1.11. When finished, disinfect the biosafety cabinet and all material prior to removing it from the animal facility.

1.12. Remove each tube from the ice bath and weigh tubes to determine organs weight. To determine the organs weight, subtract the weight of the empty tube from the weight of the organ containing tube.

NOTE: At this point organs can be frozen at -80 °C to further process at a later date or organs can be homogenized immediately before freezing individual aliquots. Freeze thawing samples multiple times decreases viral titer. Therefore, it is important to do same procedure for all experiments within a single project.

2. Organ homogenization

2.1. Prepare 3 labeled, 1.5 mL snap-capped tubes for each organ to be homogenized.

2.2. If samples are not being homogenized immediately after harvest, remove tubes from -80 °C. The samples do not need to be thawed to homogenize.

2.3. Put the samples on ice to keep them cold to minimize viral titer loss. Then, add 1 mL of cold DMEM containing 5% FBS to each organ containing tube.

2.4. Immediately beat tubes in bead beater according to manufacturer's instructions. Homogenize all organs with steel beads in a bead-homogenizer instrument. Check to ensure each organ has been completely homogenized.

2.5. Spin down organ debris in microfuge at 12,000 x *g* for 5 min in a microfuge that has been chilled to 8 °C. Then return the tubes to the ice bucket. In the biosafety cabinet, aliquot samples into tubes for necessary assays. Then return the tubes to the ice bucket.

2.6. For focus forming assay, aliquot 500 µL into a labeled tube. Then place tubes in the rack on an ice bucket. Aliquot 50 µL into a tube for RNA for fluorogenic quantitative RT-PCR (qRT-PCR) to measure viral genome copy number.

2.7. Isolate total RNA from the organs of the infected animals using a commercial RNA isolation kit. Determine flavivirus viral RNA using the primer probe sets specific for ZIKV, which recognizes unique sequences in each flavivirus genome. Determine viral copy number using a copy control plasmid containing a defined positive single-stranded RNA generated in vitro using T7 polymerase containing the ZIKV target sequences.

2.7.1. Aliquot the remaining sample, which is approximately 300 µL, into the third tube and store at -80 °C if needed.

NOTE: If samples were not previously frozen, freeze at -80 °C. If samples had been previously frozen, continue onto the focus forming assay and/or RNA isolation before stopping.

3. **Zika virus focus forming assay** [26]

NOTE: It is important to include a no virus control and a positive control. The positive control is a dilution series of a virus stock with a known concentration. Not all controls need to be on the same plate, but as the assay becomes larger than 5 plates, more controls should be added, and spread out among plates. Take care not to scratch the monolayer with either the pipet tips or by vigorous washing. Multiple organs can be titered on the same day or on different days. But an individual organ should not be titered over multiple days because different assay conditions can impact viral titer. It is strongly recommended to run an individual organ on a single day.

3.1. Prior to the day of the assay, prepare the cells and reagents needed for the focus forming assay.

3.1.1. Prepare *growth media* containing 500 mL of DMEM with 5 mL of HEPES and 25 mL of FBS. Have Vero-World Health Organization (WHO) cells growing in growth media at 37 °C, 5% CO_2 prior to the start of the assay. The Vero cells should not be a high passage or ever grown over 100% confluency prior to the start of the assay.

3.1.2. Prepare 500 mL of a 2% methylcellulose solution by autoclaving a 1 L glass media bottle with 10 g of methylcellulose and a large stir bar and a separate 1L glass media bottle with 500 mL of H_2O. If the autoclaved water has cooled reheat in microwave until bottle is hot to the touch, but not boiling.

3.1.3. Gently pour warm/hot water into bottle of methylcellulose while in the tissue culture hood. Partially cap bottle and stir on the hotplate until methylcellulose is in solution (1-4 h). Aliquot the 2% methylcellulose solution into sterile 50 mL conical tube. 2% Methylcellulose can be stored at 4 °C until needed.

3.1.4. Prepare a 5% Paraformaldehyde solution in PBS for fixing the plates and stored at 4 °C until needed. Prepare a 1x focus forming assay wash buffer by adding 0.05% Triton X-100 to PBS and stored at RT. Prepare a 1x FFA staining buffer by adding 1 mg/mL saponin to PBS and stored at 4 °C until needed. These can be prepared one- two weeks in advance.

3.2. Calculate the number of flat-bottom 96 well plates needed for the assay such that each organ is plated in triplicate. Include enough additional wells for positive and negative controls.

3.2.1. Grow up enough Vero cells in growth media to complete the assay designed. Trypsinize the Vero cells and count them resuspending them at 1.5 x 10^5 cells per mL in growth media. Plate Vero cells in the 96 well flat-bottom plates at 3.0 x 10^4 Vero-WHO cells/well in growth media by adding 200 µL per well.

3.2.2. Incubate plates at 37°C at 5% CO_2 overnight, make sure the plates are level in the incubator so the cells are equally distributed within the well.

NOTE: Each laboratory grows Vero cells slightly differently; the target is a 90-95% confluent monolayer in each well on the day of the assay for Zika virus.

3.3. Dilute Zika virus samples the day of the assay. If the organ samples had been previously homogenized, remove samples from the -80 °C freezer and allow them to thaw before placing the samples on ice for the assay.

3.3.1. On ice, prepare a round bottom 96 well plate by adding 180 µL of cold growth media to rows B through H leaving row A empty. Add 150 µL of each homogenized organ sample to row A of the round bottom plate.

3.3.2. Prepare serial 10-fold dilutions of each sample, using a multi-channel pipette. Dilute samples in a round bottom 96 well plate by adding 20 µL of sample into 180 µL of growth media, changing pipette tips between each dilution.

3.4. Prepare the focus forming plate by removing the media from the flat-bottom 96 well plate covering Vero cells. Do this immediately before adding the virus samples to prevent the monolayer from drying out.

3.4.1. Add 100 µL of the virus dilution to each well in the Vero plate. Add sample using the same set of tips by going from the lowest to the highest concentration. Rock plates side-to-side 2-4 times being careful not to swirl.

3.4.2. Incubate at 37 °C, 5% CO_2 for 1-2 h. Make sure the plates are level within the incubator. During the incubation warm up 2% methylcellulose to RT.

3.4.3. Dilute 2% methylcellulose solution in growth media. The dilution should be at a ratio of approximately 2:1 of 2% methylcellulose to growth media. Keep at room temp until it is time to use it. Add 1-2 drops/well (set the pipet to 125 µL) of the methylcellulose: growth media to each well of the 96 well plate.

3.4.4. Incubate 32-40 h at 37 °C, 5% CO_2. As everyone's Vero cells grow slightly differently and factors including cell confluency and strain of Zika virus can change the incubation time.

3.5. Fix the Vero cells using the prepared 5% paraformaldehyde solution.

3.5.1. Add 50 µL of 5% paraformaldehyde to each well over the top of the methylcellulose layer in the biosafety cabinet. Incubate for 60 min at RT. The fixing can go overnight at 4 °C but cover the plate with parafilm to reduce evaporation.

3.5.1. Dump overlay and media off cells into a disposal container inside the biosafety cabinet. Wash gently with PBS, 150 µL/well. Remove PBS from the plates and remove the plate from the BSL-2.

3.5.2. Repeat the PBS wash 2x adding 150 µL/well. Then remove the PBS. Add 150 µL/well 1x FFA wash buffer and let sit for 5-10 min at RT to permeabilize the fixed cells.

3.6. Detect infection using primary Zika antibody. Prepare the primary antibody 4G2 (D1-4G2-4-15) at a concentration of 1 µg/mL in *FFA staining buffer.* Prepare enough antibody for the whole assay.

NOTE: Stay away from lab diapers or other high-lint absorbent material as the fibers will negatively impact the imaging of the foci.

3.6.1. Remove FFA wash buffer from the plates. Add 50 µL/well of the primary antibody in the FFA staining buffer. Seal the plates with parafilm and incubate overnight at 4°C on a rocking platform. The assay can be done with an incubation for 2 h at RT.

3.7. Visualize the infection by the addition of a secondary HRP conjugated antibody. Prepare the secondary Goat anti-mouse HRP-labeled antibody at a concentration of 1:5000 in *FFA staining buffer.* Prepare enough antibody for the whole assay.

3.7.1. Wash cells 3x with FFA wash buffer, removing the wash buffer by flicking into the sink each time. Stain the cells with the secondary antibody in FFA staining buffer at 50 µL/well. Incubate 1-2 h at RT.

3.7.2. Wash cells 3x with FFA wash buffer, removing the wash buffer by flicking into the sink each time. Add 50 µL/well of the Trueblue Substrate.

3.7.3. Watch the plates carefully, waiting 2-15 min until spots are fully defined and minimal background. After the spots are visible, wash gently with water, using a hand to shield the monolayer from the force of the water running. Tap dry on paper towels (NOT DIAPERS) and image as soon as possible.

3.7.4. Spots may be counted manually or using an automated spot counter. If counted manually, a dissecting scope can be used to aid in visualization.

3.7.5. For each sample, select a dilution with easily distinguished foci (e.g., 20 to 200 per well) and calculate titer in focus-forming units per mL (FFU/ml), using the average of duplicate wells: FFU/mL = (mean foci/well) × (dilution factor) ÷ (mL inoculum).

REPRESENTATIVE RESULTS:

To evaluate ZIKV titers using the protocol described above *Ifnar1*$^{-/-}$ mice were infected with ZIKV (PRVABC59) via subcutaneous (SC) injection to the footpad. Here, the administration of 1x10^5 FFU of ZIKV to 8-12 week old *Ifnar1*$^{-/-}$ mice SC is not lethal but the virus can replicate in both the periphery and CNS. This dose and route are used to study host pathogen immune responses and pathogenicity. Administration of 1 x 10^5 FFU of ZIKV to a 8-12 week old *Ifnar1* $^{-/-}$ mouse intravenous (IV) injection is between 80 to 100% lethal, with the animal succumbing to infection between 8 to 14 days post virus injection. We routinely use this administration route to determine efficacy of antivirals and therapeutics, as well as preclinical vaccine candidate testing.

Four 10-12 week-old *Ifnar1*$^{-/-}$ mice were infected with 1 x 10^5 FFU of ZIKV SC and spleens, livers, kidneys, spinal cords and brains were harvested four days post infection by the methods detailed above (**Figure 1**). The amount of ZIKV in the tissues was assayed by focus forming assay (FFA) using Vero cells in a 96 well format as described above. Using the FFA, tissue viral load is expressed as focus forming units (FFU) per g of tissue. Similar to what was observed in a previous study of ZIKV infection of *Ifnar1*$^{-/-}$ [26], we saw viremia following a sampling of viral titers in different organs four days post ZIKV infection. These results indicate that the methods used for organ harvest and tittering by focus forming assay can be used to detect titer in both peripheral organs and the CNS within the same animal. Interestingly, we did not expect to see high viral titers in both the periphery and the CNS four days post infection in the *Ifnar1*$^{-/-}$ mice because all of the *Ifnar1*$^{-/-}$ survive ZIKV infection with this dose and route. We are continuing to explore this observation to understand how ZIKV can continue to replicate in the CNS of *Ifnar1*$^{-/-}$ without causing lethality.

When performing the focus forming assay (FFA), there are multiple technical mistakes an investigator can make which will result in suboptimal FFA results. The most common mistakes are: 1) organ toxicity; 2) vigorous pipetting; 3) fiber contamination; and 4) incorrect cell plating density. We discuss each of these issues below and illustrate the outcome in **Figure 2**. One of the more common issues that occurs with both the FFA and plaque assay is organ toxicity (**Figure 2A**

red arrow). We believe organ toxicity is driven by the high concentration of intracellular components released during organ homogenization. Organ toxicity varies based upon the organ and is seen in organs harvested from uninfected animals, with the liver being the most toxic and the spleen the least. Toxicity is reduced as the organ is serially diluted on the FFA plate. However, toxicity alters the sensitivity of the assay resulting in a change in the limit of detection. As shown in **Figure 2A** if the viral titer in the organ is lower than the toxicity the FFA will not be able to accurately record the viral titers. **Figure 2B** illustrates toxicity in wells a1-4, but the viral titer is sufficiently high to overcome organ toxicity as seen in wells b3 and b4. To overcome this limitation in the FFA, we also perform quantitative real-time PCR on organ titer samples. In **Figure 2C,** we illustrate several common technical errors. Vigorous pipetting or washing can remove the monolayer (**Figure 2C** *), if this occurs in wells with foci that data will be lost leading to inaccurate reporting of titer results. Fibers or hairs, that are present in lab bench absorbent paper can contaminate individual wells (**Figure 2C** $) this can cause significant errors if using an automated counting program. While most automated counting programs have fiber exclusion options, we have not found it to be highly effective at excluding fibers from the analysis. The solution to this is to manually count the wells, which can be very time consuming and is not practical for the analysis of large assays. Cell density is another issue which can dramatically impact the success of a focus forming assay **(Figure 2D)**. If cells are not at the right density at the start of the assay the number and size of the spots will be impacted. As shown in **Figure 2D**, columns 1-3, cells at approximately 60% confluency at the start of the assay compared to cells plated at 90% confluency columns 4-6 will dramatically impact the focus forming assay. To overcome this obstacle small pilot assays should be run to optimize cell density and fixation times as individual laboratory conditions will impact the success for the assay.

For studies when different groups of infected animals are compared, the statistical analysis that is performed is dependent on the distribution of data. Either, parametric or nonparametric tests are used to assess statistical significance. For parametric tests, ANOVA is utilized to detect overall effect, and individual treatment groups will be compared using Dunn's test. In case the distribution of data does not satisfy requirements for parametric analysis, nonparametric tests are employed. The Kruskal–Wallis test is used to detect the overall treatment effect, and the Mann–Whitney U test is used to perform pair-wise comparisons. For the results present here we did not compare the animals with a second data set harvested at this time point so we did not perform statistical analysis on the data set shown.

FIGURE AND TABLE LEGENDS:

Figure 1: Viral replication in the periphery and CNS. Viral burden in the peripheral and CNS tissues after *IFNAR1*$^{-/-}$ mice are given 1x10^5 FFU of ZIKV SC. On day 4 (n=4 per group) post-infection organs were harvested, snap frozen, weighed, and homogenized. Levels of virus were quantified by focus forming assay. The limit of detection is 100-500 FFU/gram based upon organ. Data is shown as Log_{10} focus-forming unit per gram of tissue.

Figure 2: Common Difficulties with the Focus-forming assay. For all the focus forming assays shown viral antigen was detected with an anti-flavivirus MAb, followed by immunoperoxidase

staining (purple). (**A**) Vero cells were grown to a 90% confluency and infected with a 10-fold serial dilution of supernatant from liver harvested from a 8 week old C57BL/6 mouse, IV infected with $5x10^7$ FFU of ZIKV 4 days previously. The red arrow indicates the highest or "neat" concentration of liver supernatant demonstrating the toxicity at this concentration. (**B**) Vero cells were grown to a 90% confluency and infected with a 10 fold dilution of supernatant from ZIKV infected kidney cells. In this case samples in column 1 and 2 are from a C57BL/6 mouse and col 3 and 4 are from a $Ifnar1^{-/-}$. Both mice were 8 weeks old infected with $1x10^5$ FFU of ZIKV IV and sacrificed 4 days post infection. Similar to (**A**) there is some toxicity seen at the highest concentration (row a) but the viral titers observed in column 3 and 4 overcome the limit of detection issues allowing for accurate titers to be detected. (**C**) Vero cells were plated. The selected wells show to common technical errors. The * demonstrates an area where the monolayer was removed due to vigorous pipetting. The $ is placed over a well where a fiber can be seen. (**D**) Vero cell concentration affects the sensitivity of the assay. In this plate Vero cells were plated at 1.0×10^4 cells/well in column 1-3 and 3.0×10^4 Vero-WHO cells/well in column 4-6. Then the plate was infected with 10-fold dilutions of ZIKV PRVABC59 stock. The highest viral concentration samples are in row A and diluted down, with each row representing a 10-fold dilution.

DISCUSSION:

ZIKV infection can cause a neurological disease therefore the current animal models to study pathogenesis, immune responses and protective efficacy of vaccines and antivirals need to focus on viral control within the CNS. One of the challenges in focusing on CNS disease is that it often comes at the expense of studying peripheral infection. The organ isolation methods proposed here focuses on the need to rapidly evaluate ZIKV infection in both the periphery and the CNS in order to assess CNS mediated ZIKV associated disease and establish a model for preclinical testing of antivirals, therapeutic and vaccines. An added benefit of this technique is that it also allows for a high degree of flexibility, including the combined study of immunological responses to ZIKV or histological analysis of infection. This technique, is not restricted to just ZIKV but can also be universally applied to study a range of host-pathogen interactions, including flaviviruses such as Dengue virus[21], orthopoxviruses like monkeypox and ectromelia. The considerations and drawbacks to this technique of harvesting focus mainly of the capabilities of the experimenter. As ZIKV is not stable for long periods of time at room temperature, the amount of time it takes to harvest organs after perfusion can significantly impact the quality of the results. For most experiments that we have performed, we compare viral titers from mice treated with two conditions, so we focus our efforts on consistency of time between organ harvests not on speed. In this way the same person performs the same procedure for the whole experiment to maintain consistency. The other major consideration with this procedure is safety, we have readily performed these methods with BSL-2 (ZIKV, Dengue virus) and BSL-3 (WNV, Chikungunya virus) pathogens. It is very important to perform all procedures in a clean, well maintained, certified biosafety cabinet with disinfectant.

An FFA parallels the plaque assay, except that it uses peroxidase immunostaining to identify foci of infected cells, rather than plaques. My laboratory as well as multiple other laboratories have now successfully switched to using FFAs for all our tittering experiments[11,14,15,17,26-28]. The FFA has multiple advantages over the traditional plaque assay: a) The FFA is faster, requiring a shorter

incubation compared to a plaque assay, b) it is also higher-throughput, being performed in 96-well plates. The 96 well plate format can also accommodate smaller volumes of starting material. In addition, c) the FFA is compatible with the use of an automated plate washer and automated spot counter, greatly reducing the labor and time required for the assay. The FFA has more steps after infection, but d) with the use of multichannel pipets, or even a pipetting robot, the timing for most of the assay steps after fixation are flexible and the assay can be paused overnight or for longer. Finally, e) it may be especially useful for virus strains that do not form clear plaques, such as Dengue virus. One disadvantage of the FFA is that it requires specific antibodies to detect virus-infected cells, which may be confounding when considering diverse virus strains or mutant viruses. For the FFA as with the plaque assay the density of the cell monolayer at the time of infection is critical for the success of the assay. Cells should be used at higher confluence for a FFA compared to a plaque assay, due to the shortened length of time before fixation. As the FFA is higher throughput, more cost effective and faster than the traditional plaque assay it allows my laboratory to rapidly analyze data for studying emerging infectious diseases. The FFA is more cumulatively more cost effective for several reasons. Although antibodies are more expensive than neutral red or crystal violet, we are able to analyze more samples per plate which eliminates the cost difference. In terms of labor allocation automated spot counting and easy data entry for analysis limits labor costs and the ability to have a long-term image as a record is difficult to quantify. The future of this assay may be to move to a fluorescent-based foci for a readout as opposed to HRP. Quantitating fluorescent intensity along with spot number will extend the utility of the FFA beyond what is currently studied.

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DISCLOSURES:

The authors have nothing to disclose

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Figure 1

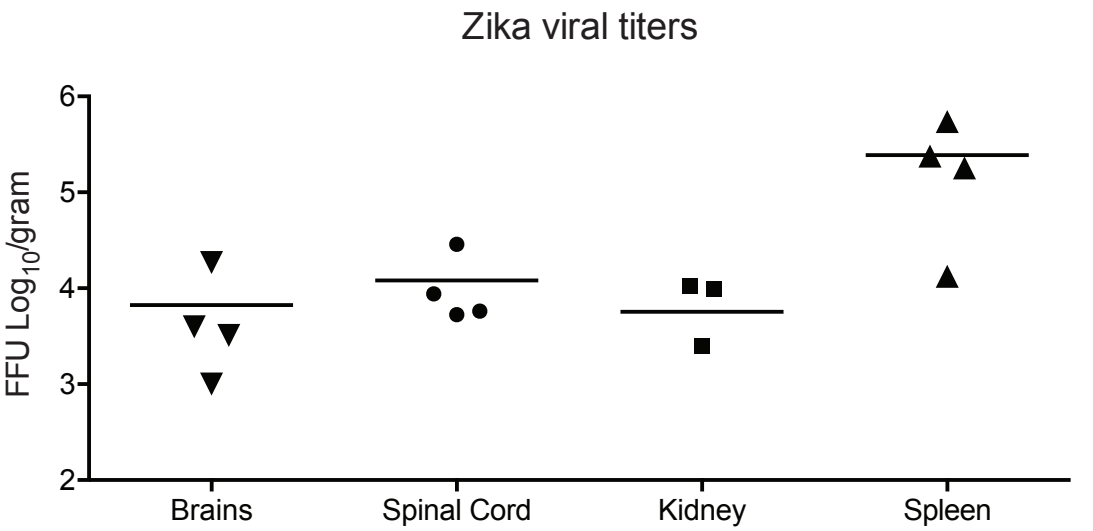
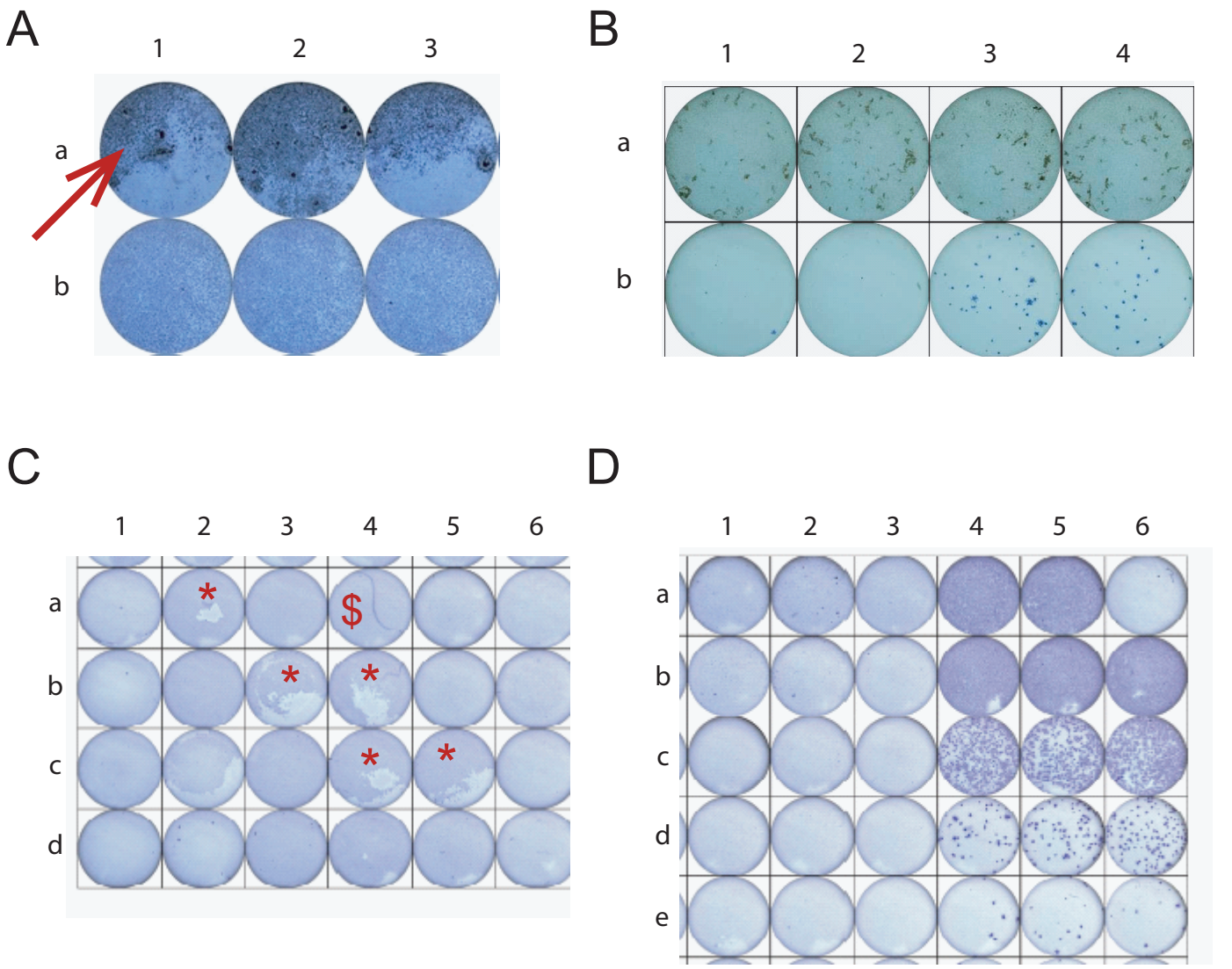


Figure 2



Name of Material/ Equipment	Company	Catalog Number
1-bromo-3-chloropropane (BCP)	MRC gene	BP151
10cc syringe	Thermo Fisher Scientific	BD 309642
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1cc TB syringe	Thermo Fisher Scientific	14-823-16H
20cc syringe	Thermo Fisher Scientific	05-561-66
24 tube beadmill	Thermo Fisher Scientific	15 340 163
3.2 mm stainless steel beads	Thermo Fisher Scientific	NC9084634
37C Tissue Culture incubator	Nuair	5800
4G2 antibody	in house	
96 well flat bottom plates	Midsci	TP92696
96well round bottom plates	Midsci	TP92697
Basix 1.5ml eppendorf tubes	Thermo Fisher Scientific	02-682-002
Concentrated Germicidal Bleach	Staples	30966CT
CTL S6 Analyzer	CTL	CTL S6 Universal An
curved cutting scissors	Fine Science Tools	14061-11
Dulbecco's Modified Eagle's Medium - high glucose With 4500 mg/L glu	MilliporeSigma	D5671
Ethanol (molecular biology-grade)	MilliporeSigma	e7023
Fetal Bovine Serum	MilliporeSigma	F0926-500ML
Forceps	Fine Science Tools	11036-20
Glacial acetic acid	MilliporeSigma	537020
Goat anti-mouse HRP-labeled antibody	MilliporeSigma	8924
HEPES 1 M	MilliporeSigma	H3537-100ML
Isopropanol (molecular biology-grade)	MilliporeSigma	I9516
Ketamine/Xylazine cocktail	Comparative Medicine	
L-glutamine	MilliporeSigma	g7513
Magmax RNA purification kit	Thermo Fisher Scientific	AM1830
Methylcellulose	MilliporeSigma	M0512
Microcentrifuge	Ependorf	5424R
MiniCollect 0.5ml EDTA tubes	Bio-one	450480

o-ring tubes	Thermo Fisher Scientific	21-403-195
one step q RT-PCR mix	Thermo Fisher Scientific	4392938
Paraformaldehyde	Thermo Fisher Scientific	EMS- 15713-S
Phosphate Buffered Saline	MilliporeSigma	d8537-500ml
Proline multichannel pipettes	Sartorius	72230/72240
Proline single channel pipettes	Sartorius	728230
RNAse free water	Thermo Fisher Scientific	10-977-023
RNAzol BD	MRC gene	RB192
Rocking Platform	Thermo Fisher Scientific	11-676-333
RPMI 1640	Fisher	MT10040CV
Saponin	MilliporeSigma	s7900
spoon/spatula	Fine Science Tools	10090-17
straight cutting scissors	Fine Science Tools	14060-11
Triton X-100	MilliporeSigma	t8787
True Blue Substrate	VWR	95059-168
Trypsin	MilliporeSigma	T3924-100ML

Comments/Description

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Dear Xiaoyan and Reviewers,

We greatly appreciate the editorial comments, they were extremely helpful. We also want to thank the reviewers for bringing up some excellent points from this study, and we thank you for inviting us to resubmit for consideration. This is a much better manuscript due to the points that were brought up by the reviewers in the previous version and addressed by the laboratory in this revised version. Below we have listed each of the reviewer's concerns and provided a point by point response in green. In addition to providing this response to the reviewer's comments, we have also provided a marked-up copy of the changes made from the previous file, as you requested.

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Changes to be made by the author(s):
1. The language in the manuscript is not publication grade. 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.
The manuscript has undergone extensive proofreading.

2. Please note that Standard Access is checked in the uploaded ALA, while in the Questionnaire Responses Open Access is selected. Please be consistent.
We have chosen to go with Open Access and have made the necessary changes.

3. Please revise lines 298-300, 322-324, 355-357, 363-365, 407-408, 411-414, and 416-422 to avoid previously published text.
These lines have been revised.

4. Please expand your Introduction to include the advantages over alternative techniques with applicable references to previous studies.
The introduction was expanded to include advantages over alternative techniques.

5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Personal pronouns have been removed.

6. 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. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
We have edit the methodology to make sure that it is written in the imperative tense. Notes are used sparingly.

7. 1.1: Please specify the age and gender of mice used.
Age and gender of the mice are added to the figure legend and to the text of the manuscript.

8. 1.1.1: Unclear sentence. Please revise. Please also mention how proper anesthetization is confirmed.
The sentence was corrected.

9. 1.6.1: What volume of blood is collected?
~800ul

10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Steps were combined.

11. 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.
Sections have been highlighted to identify the essential steps of the protocol for videography.

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Critical steps were discussed and common errors were described and illustrated within the representative results.

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Reviewer #1:

Manuscript Summary:
In the manuscript" Isolation and quantification of Zika virus from multiple organs in a mouse" the authors describe their protocol to extract various organs from a mouse and determine the viral titer. The manuscript is clearly written, includes many useful background information and for the most part the protocol is easy to follow. The authors finish by providing a comprehensive discussion of pitfalls and alternatives.

Major Concerns:
none

Minor Concerns:
Line 87: I assume this should mean anesthetized instead of athetized
Line 95: "…with steal beads added in racks to hold the samples…" gives the impression that the steel beads hold the samples. Maybe mention the steel beads in the next sentence where also homogenization is mentioned.
Line 123: "…a carrier must be added…" Like what? Can you give an example?
Line 146: to harvest the brain instead of for harvesting the brain
Line 147: Slightly confusing. Maybe break up into two sentences
Line 155: Same as above "…focus on consistency and speed. The amount of time….."
Line 185: ZIKA instead of flavivirus, since it's the only flavivirus studied here.
Line 190: and store instead of "at store at"
Line 282: Maybe list what's in the FFA buffer?
Line 382: "...represent is place over column….". Fix wording
Line 386: associated
Line 388: "... comes at the expense…" maybe say is limited to studying peripheral blood

We greatly appreciate the time and effort of the reviewer. All minor concerns were addressed in the resubmitted protocol.

Reviewer #2:

Manuscript Summary:
In this manuscript, Pinto et al. describe their highly effective methods for isolating multiple organs from ZIKV-infected mice and quantifying the viral loads via focus forming assay (FFA). This protocol is notable because the FFA allows for rapid, high throughput quantification of Zika virus from both the peripheral and CNS areas in a single mouse. While the protocol focuses on the FFA, it also describes how to harvest organs for other analyses, such as RT-qPCR, flow cytometry, and histology. To demonstrate their methods, Pinto et al. compare the FFA results under multiple conditions, specifically mouse inoculation titer and cell confluency. The advantages of FFAs over plaque assays are that FFAs take less time and starting material, and are higher throughput and better for viruses that do not form clear plaques. Although the manuscript has a number of grammar errors and should be proofread, it does make an interesting case for titering Zika virus using a FFA rather than the traditional plaque assay.

Comments:

It is not clear to me how a FFU assay is more cost effective than a PFU assay since you need to use primary antibodies and a secondary HRP antibody, followed by substrate. A plaque assay, all you need to do is add the crystal violet. The incubation time is longer (2 vers 5 days for ffu vs pfu, but then for PFU after fixing, there is only 1 step, not 4 steps. So whether its faster is also not necessarily true. It is more labor intensive than a pfu assay, but shorter in time of assay. this is not discussed, but is an important point.

* Lines 54-56: Cite ZIKV outbreaks in South America and Brazil.
A description of the increased geographic distribution of ZIKV was added to the introduction.

* Lines 72-73: What is the purpose of flow cytometry here?
We commonly use flow cytometry of peripheral blood to evaluate antigen specific T cells by tetramer or intracellular cytokine staining. This allows us to evaluate both viral load and the immune response in the same animal. This was added to the description.

* Line 88: What concentration of Xylazine?
The concentration of Xylazine was added.

* Lines 129-130: If the organs are going to be used for histology, can they be collected for other analyses?
Organs can be collected for histology and other purposes as long as the mice are not treated with paraformaldehyde intravenously.

* Lines 136-138: Preparing extra tubes for flow cytometry should be mentioned earlier in the protocol in either step 1.2 or 1.3.
The preparation of additional tubes was added to an earlier step.

* Line 167: The purpose of each set of tubes should be stated, i.e. FFA, RT-qPCR, and storage.
The purpose of each tube was added.

* Line 175: Protocol skips step 2.5.
The protocol steps were relabeled.

* Line 221, 223: Final concentrations of the wash and staining buffers are unclear.
The final concentrations of the wash and staining buffers were standardized within the protocol.

* Line 226-227: What is the number of serial dilutions?
The number of serial dilutions completed depends on the amount of virus anticipated. For wild type mice were commonly use 4 dilutions, for type I interferon deficient mice we use 8.

* Line 229: The cell concentration should be 1.5x10^5 cells/mL.
The correct cell concentration is listed.

* Lines 242-244: In what direction is the sample diluted? (e.g. A → B → etc.)
We normally dilute samples down (e.g. A → B → etc.).

* Line 258: Time range is 32-40 hours. What is the target cell confluency?
The target is a 90-95% confluent monolayer in each well on the day of the assay for Zika virus.

* Line 274: What should the concentration of primary antibody be?
The concentration of the primary antibody should be 1ug/ml.

* Line 330: What is organ toxicity?
Organ toxicity is the toxic effect of cellular debri on a vero monolayer. For example, the homogenization of the liver releases numerous enzymes that cause damage to a vero monolayer. A description of this was added to the protocol.

* Line 399-401: What are the 2 conditions?
The two conditions were described.

* Line 417: Have they tried Dengue virus? Does not plaque well.
We utilize this exact approach for DENV. There are minor variations in the length of time to allow the virus to replication that varies between serotypes. The appropriate references have been added.

Figure 1:
* Line 313: What was the route of injection?
Subcutaneous

* Lines 322-323: Why were the high viral titers surprising? Is it because of the inoculation route?
The high viral titers were surprising, because the mice have high viral loads within the CNS but survive infection. This points to a type I interferon independent mechanism of viral clearance from the CNS.

* What sex are these mice?
The mice are female.

* Why is kidney data only n = 3?
One kidney was lost. It was accidently left in the dry ice bucket and it was not noticed for several days so that sample was excluded from analysis.

*what is the limit of detection? The limit of detection varies based upon the organ.

Figure 2:

Figure 2 was redeveloped to highlight common technical errors in panels a-c. Panel d illustrates how cell density affects the sensitivity. Answers to specific questions are listed below and are added to the manuscript.

* Lines 346-348: So is 60% or 90% cell confluency better?
90% confluency is best.
* A v. B: Are the mouse strains and inoculation titers supposed to be different? Why?
The viral titer in the liver are different between the experiment shown in Fig 2A and Fig 2B. The goal is to illustrate how organ toxicity can mask low viral titers.
* A, B, C: Plate rows and columns could be annotated.

Plate rows and columns are now annotated.

* B: What do the well labels in green and red text mean? All of those unnecessary text in the figure should be removed. TNTC is not defined anywhere.

Individual specific wells are labeled according to the manuscript text. TNTC- too numerous to count, has been properly labeled.

* C: What is cell confluency in columns 1-6? What organs were plated? Why not label all wells where *, $, and & are applicable?

Panel C was separated into 2 separate panels to highlight the impact of technical errors versus cell density differences.

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March 11, 2019

Dear Dr. Nguyen,

We greatly appreciate your editorial comments on our manuscript JoVE59632R1 "Isolation and quantification of Zika virus from multiple organs in a mouse."

* Please update Dr. James Brien's email address for all communications it should be james.brien@health.slu.edu

1. Please see the attached comments in the manuscript. We have addressed all comments within the manuscript using track changes.

2. The written manuscript refers to things in Figure 2 that are not in Figure 2. Figure 2 needs more explanation to be understood. We have edited the text to align with the newest version of figure 2 and have added a greater explanation of the assay and potential pitfalls. We have also edited figure 2 to add back the arrow.

3. Please address Reviewer 2's comment comparing FFU vs PFU.

   Reviewers 2 comments:
   It is not clear to me how a FFU assay is more cost effective than a PFU assay since you need to use primary antibodies and a secondary HRP antibody, followed by substrate. A plaque assay, all you need to do is add the crystal violet. The incubation time is longer (2 vers 5 days for ffu vs pfu, but then for PFU after fixing, there is only 1 step, not 4 steps. So whether its faster is also not necessarily true. It is more labor intensive than a pfu assay, but shorter in time of assay. this is not discussed, but is an important point. Our response: In our hands the FFA is of equal cost to the plaque assay. Our antibody and development costs are approximately $0.40 per plate. Our 96 and 6 well plates are equal, but with the 96 well plate we are able to evaluate 11 additional samples with two additional dilutions, so although the antibody and development are more expensive we save money in plate costs. For a larger FFA assay we use an automated plate washer which significantly reduces labor time. In addition, with an automated imager system, there is also a reduction in labor in counting the assay and entering the data electronically. There is also the added benefit of having an image of each plate as a long term record. We have added several of these points to the discussion.

Sincerely,

Amelia K. Pinto PhD
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Saint Louis University School of Medicine