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WASHINGTON, DC

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Standard Operating Procedure Worksheet (Template)

A written standard operating procedure is required that explains how all hazardous materials are handled safely in each step of the process and addressing the items listed below.

- Procurement and storage of agents
- Protective equipment
- Biosafety cabinet use
- How solid and liquid waste is handled
- Training
- Other equipment used (i.e.: centrifuge, sonication)
- Sharps handling

Principal Investigator: **OLS**
Proposal Title: **Systems serology of ZIKV virus**
Protocol: **Plaque Reduction Neutralizing Testing (PRNT) for Measuring Neutralizing Antibodies Against Flavivirus**

PURPOSE

This protocol describes how to measure neutralizing antibodies against Dengue and Zika virus by quantifying plaque formation in a Vero cell monolayer. This protocol is based on “Guidelines for plaque reduction neutralization testing of human antibodies to dengue viruses” produced by the Initiative for Vaccine Research of the Department of Immunization, Vaccines and Biologicals at the WHO.

SCOPE

This procedural format is proposed to be utilized by all members of the OLS’s laboratory. Personnel will be trained and supervised by Dr. Maria Smith and Dr. John Doe. There will be **Mandatory Information Training on working with Zika from CDC guidelines.**
All the experimental steps will be performed in tissue culture room 800C, located at Ross Hall, 6th floor, or 5th floor room 911, which are BSL-2-enhanced lab space.
Area where ZIKV work will be conducted will be clearly identified and all personal working with ZIKV or ZIKV-infected samples will be explained the risks associated with ZIKV infection
All infectious materials will be stored will be clearly labeled with the universal biohazard symbol and in locked rooms at 4C, -20C or -80C in leak proof container. Samples will be handled according to recommended NIH-CDC procedures and the accepted standards for “universal precautions”. All personnel will wear non-absorbent disposable gowns and double gloves.
All decontamination steps will be performed using 10% freshly prepared bleach. Double-gloves will be worn at all times. All generated waste and contaminated materials will be considered biohazardous and will be placed in leak-proof, properly designated containers for subsequent collection, decontamination and disposal in accordance with the University regulations. Universal Precautions will be followed at all times.
In brief:
- Liquid biohazardous waste will be decontaminated by mixing one volume of undiluted bleach with nine volumes of liquid biohazardous waste (final dilution of 1:10) for 30 minutes. This will be drain-disposed.
- Solid biohazardous waste will be disposed in red biohazardous waste bags, which must be placed inside a medical waste container with a tight-fitting lid at all times.
No glassware will be used.
No food or drink is allowed in the lab. After every experiment, surfaces will be disinfected with 10% freshly prepared bleach followed by 70% ethanol. After performing experiments, all personnel will remove their gloves and wash their hands at the sink near the exit.

SAFETY NOTES:

- There will be **Mandatory Information Training on working with Zika from CDC guidelines.**
- Infectious agents are used in this procedure.
- All personnel upon entering to the Tissue Culture Room 616C, must put on proper Personal Protective Equipment (PPE): disposable gown and double gloves. Handling infectious material requires wearing double layer of gloves and safety goggles if handled outside the BSC.
- Handling samples and reagents will be done inside of the Biosafety Cabinet class II(BSC). No open tubes allowed outside of the BSC.
- BSC will contain the following safety measures:
  - Bleach waste bucket: Autoclavable, polypropylene, filled 1/8 with undiluted Bleach. This amount guarantees that the final concentration of bleach is always more than 10%.This bucket is used to discard small amounts of liquid (infectious or not) and to soak serological pipettes that have contacted infectious material for at least 30, before placing into the biohazard bag lined container.
  - Vacuum bottles, and HEPA filters inserted between the vacuum bottles and the central line. The bottle is filled 1/8 full with 100%, undiluted bleach, prepared daily. This amount guarantees, that the final concentration of bleach is always more than 10%. Liquid (infectious or not) can be aspirated into the primary vacuum bottle. Liquid disposal happens according to the GWU's safety regulations.
  - Biohazard bag lined container for solid waste and infectious material containing closed/ capped tubes.
- Within reach, a Spray bottle with 70% EtOH and squirt bottle with freshly prepared 10% Bleach.
- All procedures are performed using sterile laboratory supplies. All supplies (serological pipettes, barrier tips, screw cap tubes, different type of tissue culture dishes) should be within reaching distance to minimize traffic and potential contamination. Used supplies should be discarded and placed in the biohazard bag lined container (primary biohazard containment) inside of the BSC. When 2/3 full, the closed biohazard bag should be discarded into the Biological Medical Waste Box, provided by the GWU.
- All incubations are performed in a humidified 37^{o}C, 5% CO_2 incubator unless otherwise specified.
- When centrifuging tubes containing infectious material, safety caps must be used.
- After all experiments BSC should be disinfected with with 10% freshly prepared bleach followed by 70% ethanol.
- Before exiting the room, all the PPE should be disposed properly into the Biological Medical Waste Box lined with red biohazard bag.
- Personnel should wash her/his hands before exiting the room.
- Accidents and all spills must be reported immediately to the Principal Investigator and/or lab supervisor. Injured personnel must report for immediate medical evaluation, treatment, and post exposure follow-up to the Employee Health Office at GWU Hospital (900 23rd St., NW, Suite G-1090, Phone: **202-715-4275**). After hours treatment can be received at the GWU hospital emergency room. The PI must submit an Accident Report Form to the Office of Risk Management and to the Office Laboratory Safety. Call OLS for assistance at **4-8258**.

INTRODUCTION

This assay measures neutralization of Flaviviruses as a function of a reduction in plaque formation in a Vero cell monolayer. Vero cells may be obtained from ATCC® (CCL-81™) and are a kidney epithelial cell line derived from an African green monkey. Vero cells are a mammalian cell line permissive to Zika and Dengue virus infection and Vero cells have been produced in a fashion that have allowed them to be used for production of live-attenuated vaccines and are therefore, recommended for use in the PRNT.

Material	**Hazards**	**Precautions**
DAY 1. 1. **Seed Vero cells at 4x10^{5} cells/well in 24-well tissue culture plates.**	Aerosol exposures. Blood borne pathogens. Spills	Use Biosafety Cabinet class II (BSC), nitrile gloves, disposable long sleeve gown, safety goggles. Centrifugation is done in sealed buckets. Use proper pipetting device for the volume of liquid

Incubate for 2 days at 37℃, 5% CO_2 (should be 95% confluent for Day 3 infection)		used. Only barrier tips will be used. Transfer plates to the incubators in a secondary container. All solid waste is decontaminated immediately for 30 minutes with freshly prepared 10% bleach solution in the BSC and disposed in the biohazardous waste container. Liquid biohazardous waste will be decontaminated by mixing one volume of undiluted bleach with nine volumes of liquid biohazardous waste (final dilution of 1:10) for 30 minutes. This will be drain-disposed. The BSC is disinfected with freshly prepared 10% bleach, wiped with water followed by 70% ethanol, and wash hands after removal of gloves. Aspirated liquids are treated with 10% bleach for at least 30 minutes. **In case of injury or exposure** Flush eyes, nose, or mucous membranes with water for at least 15 minutes. Flush abraded or cut skin with soap and water for 15 minutes. If someone else is present in the area, get him or her to assist you. Accidents and all spills must be reported immediately to the Principal Investigator and/or lab supervisor. Injured personnel must report for immediate medical evaluation, treatment, and post exposure follow-up to the Employee Health Office at GWU Hospital (900 23rd St., NW, Suite G-1090, Phone: **202-715-4275**). After hours treatment can be received at the GWU hospital emergency room. The PI must submit an Accident Report Form to the Office of Laboratory Safety. Call OLS for assistance at **4-8258**.
DAY 2. 1. **Make 4% carboxymethylcellulose (CMC) solution in ddH_2O.** For each 24-well plate to test, mix • xxg CMC • 30 ml ddH_2O *Incubate o/n at 4℃*	Aerosols.	See safety notes above. Carboxymethylcellulose may be hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, or of inhalation. Immediately flush eyes with running water for at least 15 minutes, or wash skin with plenty of water and

DAY 3. 1. **Make overlay solution.** Autoclave 4% CMC at liquid cycle (20min at 121°C). Place in water bath to cool to 40°C Mix • 25 ml 4% CMC • 500 µl FBS (1%) 500 µl pen/strep (1%) 24 ml 2x DMEM *Final solution = DMEM w/ 2% CMC & 1%FBS*	Heat Hazard Melting Plastic	Only autoclave polypropylene and polycarbonate containers! Ask the PI when in doubt Do not autoclave polystyrene (PS), polyvinyl chloride (PVC), nylon, acrylic, low-density polyethylene (LDPE), and high-density polyethylene (HDPE)! These will melt. Wear Heat resistant gloves when placing and removing material from the autoclave.
2. **Heat inactivate sera** to be assayed in a 56°C water bath for 30 min.	Heat hazard Splash	See safety notes above. Handling samples and reagents will be done inside of the Biosafety Cabinet (BSC). No open tubes allowed outside of the BSC.
3. **Prepare antibody dilutions** in a deep well 96-well plate as illustrated in *Template A*. a) Place 240 µl of cDMEM in all wells of rows A-H in all columns being used. b) Add another 240 µl cDMEM to column1, row A-D (no virus control) *c)* Add 60 µl of each serum sample to row A from column 2 on (a 1:5 dilution for a final dilution of 1:10). ○ *In every assay, positive/negative controls should be tested in columns 1 of the first plate.* ○ *Test samples are added to subsequent wells in row A of columns 3 on, including at least one neg sample.*	Aerosols.	See safety notes above.
4. Using a multichannel pipettor, perform **serial five-fold dilutions**: • take 60 µl of sample from row A and transfer to row B. • Mix samples, then transfer 60 µl from row B to row C. • Repeat the transfer and dilution of samples through row H. After the final transfer, discard 60 µl from the last tube in the dilution series so that each tube of the dilution series contains 240 µl	Aerosols	See safety notes above.
5. **Prepare virus** by rapid thawing the required number of vials of virus by placing in an 37°C water bath and quickly putting in cooler.	Aerosols Splash	▪ Make virus dilution calculations a day ahead ▪ Check Cryovials used for virus storage for physical

• When completely thawed, dilute the virus in cDMEM to achieve a dilution that will result in 80 pfu/100 μl based on plaque titer for that specific virus lot. Final dilution of virus will be 80 pfu/ 200 μl. Mix virus dilution by inverting the tightly capped tube, and transfer virus containing medium into a sterile reservoir.		appearance (cracks, closed cap) ▪ Correct name of virus lot is recognized on the vial ▪ Cap of the vial should not get under water in the water bath ▪ Limit the thawing time up to 5 min ▪ After removing samples from the water bath, wipe Cryovials with 70% EtOH. ▪ See safety notes about handling virus containing liquid above
6. Using a multichannel pipette, dispense equal volume of virus (240 μl) to all wells EXCEPT column 1, rows A-D (no virus). Cover plates and gently tap plate to mix.	Aerosols	See safety notes above Use proper pipetting device for the volume of liquid used. Use barrier tips!
7. **Incubate** for 60 minutes at 37°C, 5% CO_2.	Spills	Use a secondary container to transfer plates to the incubator.
8. Take out experiment plate with seeded Vero cells from the incubator and check in the microscope that cells look confluent. Label each plate for appropriate samples	Spills	See safety notes above
9. One plate at a time, remove cell culture media. • Using an adjustable multichannel pipettor, transfer 200 μl of sample in duplicate **from 96 well antibody dilution plate** (ADP) to **24 well experiment plate** (EP). Evenly distribute the inoculum by rocking the plate, with a cover on it, back and forth and from side to side.	Aerosols	See safety notes above