CAES
The Connecticut Agricultural Experiment Station
Putting Science to Work for Society since 1875

STANDARD OPERATING PROCEDURES (SOPs)

Mosquito-Borne Pathogen Testing

Agreement No. 101-226-000

Between
NEON, Incorporated
&
The Connecticut Agricultural Experiment Station

CAES
The Connecticut Agricultural Experiment Station
Putting Science to Work for Society since 1875

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Appendix A: SOP for preserving and archiving maintenance

Connecticut Agricultural Experiment Station **Protocol 1.4**
BSL3 Virus Laboratory

Title: Adding and removing samples to and from the freezer reference collections.
Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions for adding and removing material to and from the freezer reference collections.
Scope: This procedure applies to the addition and removal of samples to and from the freezer reference collections in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor. The select agent reference collections may only be accessed by individuals who have received security risk assessment clearance from the Select Agent Program. Keys for the freezers containing select agents are with the RO (Angela Bransfield) or the PI (Dr. Philip Armstrong).
Biosafety

A. Biological Hazards: Infectious supernatants and mosquito pools contain infectious viruses. Exposure may occur by direct contact with broken skin or mucous membranes, or by inhalation of infectious aerosols

B. Personal Protective Equipment: Laboratory gown, gloves, cryogloves

C. Engineering Controls: N/A

D. Handling Requirements: All material are sealed in vials and stored in numbered storage boxes. The identity and location of stored samples are recorded on datasheets which are used to update electronic databases.

E. Waste Management: N/A

F. Decontamination Procedures: Affected work surfaces are decontaminated with 70% alcohol.

G. Emergency Procedures: In the event of a splash, spill, or release incident immediately evacuate personnel from the affected area. Remove any splashed clothing and PPE. Rapidly wash affected body parts with iodine soap and water or another disinfectant. Label spill area off limits for 30 minutes and notify theRO or supervisor as soon as possible.

Materials
Samples- Infectious supernatants, virus-positive mosquito pools, and/or RNA extracts
Tube racks
Storage boxes

Protocol
For select agents, obtain keys to freezer from RO or PI. Only authorized persons should handle mosquito pools, cultures, or the RNA of select agents.

<u>Adding samples</u>
1. Find the appropriate inventory sheet for your sample. Separate sheets are maintained for RNA extractions, infectious supernatants, and mosquito pools and may be found on clipboards inside the freezer room. Each sheet represents the contents of one freezer box divided into a 10x10 grid to correspond to the box's dividers.

2. Record the following information for each sample in the corresponding storage space for the box:
   Sample identification number
   Passage number
   Amount
   Date frozen
   Identification of isolate (if known)

3. Place sample in the appropriate box, according to its recorded position, and place the box in the appropriate -80^{o} C freezer.

Removing samples

1. Find the data sheet labeled "Disposition of Infectious Isolates in the Possession of the CAES, Johnson-Horsfall Laboratory, Room 30".

2. Record the following information for each sample removed.
    - Freezer number
    - Location in freezer
    - Sample identification number
    - Identification of isolate
    - Host source
    - Date acquired
    - Quantity
    - Date removed
    - Quantity removed
    - Action taken
    - Details on use
    - Replacement of aliquot, if applicable
    - Name/signature
3. Retrieve sample from the appropriate box and position stored in the -80^{o} C freezer.

Appendix B: SOPs for reagent/primer/gel/media/culture creation and/or maintenance

Connecticut Agricultural Experiment Station **Protocol 4.1**
BSL3 Virus Laboratory

Title: 1X Dulbecco's PBS Cell Wash recipe
Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide a detailed recipe and instructions for mixing 1X Dulbecco's PBS cell wash solution.
Scope: This procedure applies to the preparation of 1X Dulbecco's PBS cell wash solution in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Specific training to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: N/A
B. Personal Protective Equipment: Laboratory gown, gloves, goggles or face shield
C. Engineering Controls: Biosafety cabinet (BSC)
D. Handling Requirements: PBS is filtered and then aliquoted in the biosafety cabinet to insure sterility.
E. Waste Management: Filter unit is placed in clear trash bags and pipettes are placed in the 10% bleach container inside the hood.
F. Decontamination Procedures: The work surface of the biosafety cabinet is decontaminated with 70% alcohol.
G. Emergency Procedures: N/A

Materials
.2µm Nalgene filter unit
500 mL dH_2O
50 mL 10X Dulbecco's PBS
Graduated cylinder
pH meter

Procedure
A. Pour 50 mL 10X Dulbecco's PBS into graduated cylinder.
B. Add 400 mL dH_2O.
D. Adjust pH to 7.1 with Sodium Hydroxide, NaOH
E. Bring to 500 mL of volume with dH_2O.
F. Filter with 0.2µm Nalgene vacuum filter unit.
The following procedures are performed in the biosafety cabinet:
G. Wipe work surfaces inside the biosafety cabinet with 70% alcohol
H. Aseptically aliquot 5.0 mL into 8.0 mL blue screw cap tubes.
I. Decontaminate the biosafety cabinet again with 70% alcohol.
J. Store aliquots of PBS in refrigerator (4°C).

Connecticut Agricultural Experiment Station **Protocol 4.2**
BSL3 Virus laboratory

Title: PBS-G diluent and PBS-G complete recipes.

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide a detailed protocol for PBS-G diluent and PBS-G complete.
Scope: This procedure applies to the preparation of PBS-G in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: N/A
B. Personal Protective Equipment: Laboratory gown, gloves, goggles or face shield
C. Engineering Controls: Biosafety cabinet
D. Handling Requirements: PBS-G is prepared in the biosafety cabinet to insure sterility.
E. Waste Management: Pipettes are placed in the 10% bleach container inside the hood. Disposable vials and tubes are discarded in biohazard bags
F. Decontamination Procedures: The work surface of the biosafety cabinet is decontaminated with 70% alcohol.
G. Emergency Procedures: N/A

Reagents
NaCl
KCl
Na_2HPO_4
KH_2PO_4
dH_2O
Phenol Red (see protocol 4.3)
Gelatin
Rabbit serum
Anti-biotic/mycotic

Materials
Beaker
100 mL bottles
8.0 mL blue screw cap tubes

Procedure for making PBS-G diluent
A. Use the following table to determine the appropriate quantities of reagent to make 1, 2, or 4 liters of PBS-G diluent:

Reagent	1 Liter	2 Liters	4 Liters
NaCl	8.0 g	16.0 g	32.0 g
KCl	0.2 g	0.4 g	0.8 g
Na_2HPO_4	1.15 g	2.3 g	4.6 g
KH_2PO_4	.2 g	0.4 g	0.8 g
dH_2O	1 000 mL	2 000 mL	4000 mL

*Phenol Red	2.0 mL	4.0 mL	8.0 mL
Gelatin	5.0 g	10.0 g	20.0 g

*Refer to Phenol Red recipe and mixing instructions protocol 4.3.

B. Add dH_2O to a large flask.
C. Remove 300 mL of dH_2O for each 1 L of PBS-G you are making. Pour removed dH_2O into a beaker.
D. Add the NaCl, KCl, Na_2HPO_4, and KH_2PO_4, to the dH_2O remaining in the flask. Use a stir bar to mix.
E. Once the reagents are fully dissolved, remove the flask from the stir plate. Put the beaker with the removed dH_2O on the hot plate with a stir bar. Mix slowly, and heat the water to a near boil.

- While the water is heating, measure out the gelatin and aliquot the phenol red in a BSC. Take the rabbit serum out of the freezer to thaw.

F. Once the water is hot (near boil), add the gelatin. Turn heat down so that it does not boil. Wait for the gelatin to completely dissolve (although some may remain on the top of the water).
G. Pour the gelatin mixture back into the flask with the reagent mixture. Wait for the hot plate to cool down, then turn on the mixing magnet and add the aliquot of phenol red.
H. Measure the pH. Use NaOH or HCl to adjust until the pH is between 7.1-7.4. Usually the pH will not have to be adjusted.
I. Aliquot 200 mL of PBS-G into bottles.
J. Autoclave bottles of PBS-G (with loosened caps) on liquid cycle.
K. Store in 4°C refrigerator.

Procedure for making PBS-G Complete for Virus Isolation.

A. Use the following table to determine the appropriate quantities of reagents to make 100 mL, 500 mL, or 200 mL PBS-G complete:

Reagent	**Quantity PBS-G Complete**		
	100 mL	**500 mL**	**200 mL**
*Rabbit Serum	42.8 mL	214.0 mL	86.0 mL
Anti-biotic/mycotic	1.4 mL	7.0 mL	2.8 mL

*Rabbit serum must be heat inactivated before use. Once thawed, place the bottle of rabbit serum in a 56°C hot water bath for 30 minutes. Label the bottle with "Heat Inactivated", the date, and your initials when placing it back in the refrigerator.

The following procedures are performed in the biosafety cabinet:

B. Wipe work surfaces inside the biosafety cabinet with 70% alcohol
C. Place PBS-G diluent 200 mL bottles inside Class II Type A Biosafety cabinet.
D. Aseptically add appropriate quantities of rabbit serum and anti-biotic/mycotic to each bottle.
E. Anti-biotic/mycotic aliquots are stored in 8.0 mL blue screw-cap tubes at –20°C. Thawed rabbit serum bottles are stored in 4°C refrigerator.
F. Decontaminate the biosafety cabinet again with 70% alcohol.
G. Store PBS-G in 4°C refrigerator.

Connecticut Agricultural Experiment Station **Protocol 4.3**
BSL3 Virus Laboratory

Title: Phenol Red 0.5%

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide a detailed protocol for preparation of 0.5% Phenol Red.
Scope: This procedure applies to the preparation of Phenol Red in the BSL3 laboratory at the Connecticut Agricultural Experiment Station.
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: N/A
B. Personal Protective Equipment: Laboratory gown, gloves, goggles or face shield
C. Engineering Controls: Biosafety cabinet (BSC)
D. Handling Requirements: Phenol red is aliquoted in the biosafety cabinet to ensure sterility.
E. Waste Management: Pipettes are placed in the 10% bleach container inside the hood. Filter unit is discarded in white trash bags.
F. Decontamination Procedures: The work surface of the biosafety cabinet is decontaminated with 70% alcohol.
G. Emergency Procedures: N/A

Materials / Reagents
Phenol Red powder
dH_2O
.45 µm membrane filter
8.0 mL blue screw cap tubes

Procedure
1. Measure out 0.5 g phenol red powder in a weigh boat.
2. Add powder to 100 mL dH_2O with a stir bar
3. The powder *will not* completely dissolve.
4. Filter through 0.45 µm membrane filter unit.

The following procedures are performed in the biosafety cabinet:
5. Decontaminate work surfaces with 70% alcohol and aseptically aliquot solution into 50 mL conical tubes.
6. Decontaminate work surfaces of biosafety cabinet with 70% alcohol again.
7. Wrap lid of conical tube with parafilm and store in 4°C refrigerator

Connecticut Agricultural Experiment Station **Protocol 4.4**
BSL3 Virus Laboratory

Title: Preparation of Vero cell growth media (VCGM)

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide a detailed recipe and instructions for mixing VCGM.
Scope: This procedure applies to the preparation of VCGM for the Vero cell line in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: N/A
B. Personal Protective Equipment: Laboratory gown, gloves, goggles or face shield
C. Engineering Controls: Biosafety cabinet (BSC)
D. Handling Requirements: Media is autoclaved and then supplemented with additional reagents in the biosafety cabinet to insure sterility.
E. Waste Management: Pipets are placed in 10% bleach container inside the hood. Waste material is collected in a small red bag inside the BSC and then discarded into the waste basket.
F. Decontamination Procedures: The work surface of the biosafety cabinet is decontaminated with 70% alcohol.
G. Emergency Procedures: N/A

Materials:

Material	**Vendor**	**Catalog Number**
Fetal Bovine Serum (FBS)	Gibco – Invitrogen Corporation	16140-071
Anti-biotic/mycotic	Gibco – Invitrogen Corporation	15240-062
Sodium Bicarbonate $NaHCO_3$, 7.5% Soln.	Gibco – Invitrogen Corporation	25080-081
L-Glutamine 200mM 100x	Gibco Invitrogen Corporation	25030-164
Minimal Essential Media (αMEM)	Gibco – Invitrogen Corporation	11700-077
DH_2O	Filtered in-house	N/A

Procedures:

A. Minimal Essential Media
1. Use the following table to add appropriate quantity of membrane powder (located in 4°C refrigerator desiccator) to dH_2O to create the unsupplemented MEM:

	I Liter	**2 Liters**	**4 Liters**
MEM	9.4g	18.8g	37.6g
DH_2O	1000 mL	2000 mL	4000 mL

The pH of a newly opened bottle of MEM should be 4.1-4.2.

2. Aliquot the above solution into sterile, orange capped, 500 mL media bottles.

3. Autoclave for 15 minutes at 121°C (liquid cycle).
4. Once solution is cooled to RT, aseptically (perform step in BSC) add FBS, L-Glutamine, anti-biotic/mycotic, and sodium bicarbonate (see section B).
5. Shake mixture well.
6. Store in 4°C refrigerator.

B. Supplementing the Minimal Essential Media Solution.

The following procedures are performed in the BSC:

1. Work surfaces inside the BSC must be wiped down with 70% alcohol before supplementing media.
2. To 500 mL of MEM solution add the following:

FBS	26.0 mL
L-Glutamine	5.2 mL
Anti-biotic/mycotic	5.2 mL
Sodium bicarbonate, $NaHCO_3$	10.4 mL

a. Add 26.0 mL FBS with a 25 ml serological pipettor
b. 5.2 mL L-glutamine and anti-biotic/mycotic had earlier been aseptically aliquoted into 8.0 mL blue screw cap tubes and stored in a designated –20°C freezer.

4. Decontaminate the biosafety cabinet again with 70% alcohol.

Connecticut Agricultural Experiment Station **Protocol 2.1**

BSL3 Virus laboratory

Title: Seeding Small Tissue Culture Flasks with Vero Cells

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015

Author(s): Philip Armstrong, Shannon Finan, Michael Misencik

Purpose: To provide detailed instructions for seeding small tissue culture flasks with Vero cells.

Scope: This procedure applies to seeding small tissue culture flasks with Vero Cells in the BSL3 laboratory at the Connecticut Agricultural Experiment Station

Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.

Biosafety

A. Biological Hazards: Vero cell cultures are not known to cause human disease.

B. Personal Protective Equipment: Laboratory gown, gloves

C. Engineering Controls: Biosafety cabinet

D. Handling Requirements: Cells are prepared in the biosafety cabinet to insure sterility.

E. Waste Management: Pipettes are placed in the 10% bleach container inside the hood. Disposable vials and tubes are discarded in biohazard bags

F. Decontamination Procedures: The work surface of the biosafety cabinet is decontaminated with 70% alcohol.

G. Emergency Procedures: N/A

Materials:

Vero cell growth media (MEM, 5%FBS; protocol 4.4 for preparation)

1X Dulbecco's PBS (aseptically divided into aliquots of appropriate volume in Falcon 352027 tubes, prior to the procedure, kept refrigerated)

1X Trypsin-EDTA (aseptically divided into aliquots of appropriate volume in Falcon 352027 tubes, prior to the procedure and frozen)

One (1) large flask (750 mL) of vero cells

Two (2) packets of small tissue culture flasks (50 mL, 20/case)

Procedure

A. From an incubator, select a large tissue culture flask with a confluent layer of vero cells.

1. Cells must not be older than one (1) week as they may become overgrown.
2. Check cell morphology and density under an inverted light microscope.

The following procedures are performed inside a biosafety cabinet:

B. Wipe work surfaces inside the biosafety cabinet with 70% alcohol.

C. Decant culture media into waste beaker containing bleach.

D. Wash vero cells by adding a 5 mL aliquot of PBS and swirl over layer of cells and along sides of flask making sure to thoroughly cover entire monolayer.

E. Decant PBS into waste beaker.

F. Thaw and add a 2.5 mL aliquot of Trypsin and swirl over layer of cells making sure to thoroughly cover entire monolayer.

G. Incubate flask in incubator for five (5) minutes at 37°C, 5% CO_2.

H. Gently shake and swirl flask to remove cells from bottom surface. The cells should slide off easily; incubate one (1) additional minute if cells do not easily slide off. Tap the bottom and sides of the flask to dislodge any remaining cells.

I. Add 5 mL of growth media and titurate 15-20 times or until cells are thoroughly broken up.

J. Add an additional 15 mL growth media for a total volume of 22.5 mL and mix well.

K. Stand up the small flasks in two trays (20/tray). With the necks pointing to the left, label top side of the small tissue culture flasks (40) with the letter of the mother flask being used and the day of the month.
L. Place the trays inside the biosafety cabinet, one tray in front of the other, and remove flask caps.
M. Dispense 4 mL of vero cell growth media into each small flask
N. Dispense 0.5 mL cells into each of the 40 flasks. Replace flask caps.
O. Add media to the mother flask to bring the total volume up to the 50 mL line. Replace mother flask in the incubator at 37^{o} C, 5% $CO_{2.}$
P. Remove the small flasks from the trays in stacks of five. Check that the entire bottom of each flask is covered with media/cells when the flasks are lying down (neck of flask should be tilting upwards); gently swirl flasks if flask bottoms are not covered.
Q. Place flasks in stacks in the incubator at 37^{o} C, 5% CO_2 and grow overnight until confluent.
R. Decontaminate the biosafety cabinet with 70% alcohol.

Notes
Cells are passed 1-2 times per week.

After the seventh (7) or eighth (8) passage into smaller flasks, the culture flask should be discarded and a new flask set up to replace it.
Split large flasks if cells are not used within 7 days. Follow steps A-G above. Discard 20 mL of cells and bring remainder to 50 mL of volume with growth media.

Connecticut Agricultural Experiment Station **Protocol 2.2**
BSL3 Virus Laboratory

Title: Maintaining Vero Cells Growing in Large Tissue Culture Flasks

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions for maintaining large Vero cell culture flasks
Scope: This procedure applies to maintenance of large flasks of Vero cells in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: Vero cell cultures are not known to cause human disease.
B. Personal Protective Equipment: Laboratory gown, gloves
C. Engineering Controls: Biosafety cabinet
D. Handling Requirements: Cells are prepared in the biosafety cabinet to insure sterility.
E. Waste Management: Pipettes are placed in the 10% bleach container inside the hood. Disposable vials and tubes are discarded in biohazard bags
F. Decontamination Procedures: The work surface of the biosafety cabinet is decontaminated with 70% alcohol.
G. Emergency Procedures: N/A

Materials

- Vero cell growth media (MEM, 5%FBS; protocol 4.4 for preparation)
- 1X Dulbecco's PBS (aseptically divided into aliquots of appropriate volume in Falcon 352027 tubes, prior to the procedure, kept refrigerated)
- 1X Trypsin-EDTA (aseptically divided into aliquots of appropriate volume in Falcon 352027 tubes, prior to the procedure, and frozen)
- One (1) large tissue culture flask (750 mL) of confluent Vero cells
- Four (4) large tissue culture flasks (750 mL) labeled with the date.

Procedure

A. From an incubator, select a large tissue culture flask with a confluent layer of vero cells.
    1. Cells must not be older than one (1) week as they may become overgrown.
    2. Check cell morphology and density under an inverted light microscope.

The following procedures are performed inside a biosafety cabinet:

B. Wipe work surfaces inside the biosafety cabinets with 70% alcohol.
C. Decant culture media into waste beaker containing bleach.
D. Wash vero cells by adding a 5 mL aliquot of PBS and swirl over layer of cells and along sides of flask making sure to thoroughly cover entire monolayer.
E. Decant PBS into waste beaker.
F. Thaw and add a 2.5 mL aliquot of Trypsin and swirl over layer of cells making sure to thoroughly cover entire monolayer.
G. Incubate flask in incubator for five (5) minutes at 37°C, 5% CO_2.
H. Gently shake and swirl flask to remove cells from bottom surface. The cells should slide off easily; incubate one (1) additional minute if cells do not easily slide off. Tap the bottom and sides of the flask to dislodge any remaining cells.
I. Add 5 mL of growth media and titurate 15-20 times or until cells are thoroughly broken up.

J. Add an additional 15 mL growth media for a total volume of 22.5 mL and mix well.
K. Dispense 5 mL of cells into each of the four (4) large tissue culture flasks. If more mother flasks are needed, 2.5 mL of cells can be dispenses into each of 8 large flasks. Approximately 2.5 mL of cells should remain in original flask.
L. Add approximately 50 mL volume of growth media to each flask.
M. Return flasks to incubator at 37°C, 5% CO_2.
N. Decontaminate the biosafety cabinet with 70% alcohol.

Appendix C: *SOP for primary analytical methods*

Connecticut Agricultural Experiment Station **Protocol 2.3**
BSL3 Virus Laboratory

Title: Processing Mosquito Pools for Virus Testing

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions for preparing mosquito pools for virus testing.
Scope: This procedure applies to the preparation of mosquito pools for virus testing in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Laboratory Hazards: Frozen mosquitoes are dead but may be infected by pathogenic viruses. Exposure may occur by direct contact with broken skin or mucous membranes, or by inhalation of infectious aerosols
B. Personal Protective Equipment: Laboratory gown, gloves, respirator
C. Engineering Controls: Biosafety cabinet (BSC)
D. Handling Requirements: Tubes containing dead mosquitoes are opened and closed only within the BSC. Mosquitoes are homogenized in solution using a mixer-mill placed inside the BSC. Mosquito homogenates are centrifuged in a refrigerated, aerosol-tight microcentrifuge on the benchtop. To guard against aerosol production within the rotor of the centrifuge, the operator must wear a respirator while retrieving the samples from the centrifuge.
E. Waste Management: Pipette tips are placed in a 10% bleach container inside the hood.
F. Decontamination Procedures: The centrifuge, centrifuge rotor, Mixer Mill cassettes, the BSC, and affected work surfaces are decontaminated with 70% alcohol after use.
G. Emergency Procedures: In the event of a splash, spill, or release incident immediately evacuate personnel from the affected area. Remove any splashed clothing and PPE. Rapidly wash affected body parts with iodine soap and water or another disinfectant. Label spill area off limits for 30 minutes and notify the RO or supervisor as soon as possible.

Materials/Reagents
Mosquito pools on dry ice
Two (2) ice racks
PBS-G (protocol 4.2 for preparation)
Mixer Mill with cassettes
Sterile beaker
Procedure

Mosquito team in Slate Laboratory identifies the mosquitoes brought in from the different sites around CT. They group them according to species and location. Each grouping is considered a pool. A pool may consist of 1-50 mosquitoes. They are placed in **2 ml tubes with a BB**. Once the mosquitoes are ready for testing, they are put in a bag with the date and site written on the side. All the pools for that day are placed in a larger bag, delivered to us, and placed in the Revco (-80^{o}C) freezer. We usually test the mosquitoes the day after they are identified. We print a list from the database for the day's mosquitoes, which we use to record which pools are tested. Each pool is given a number. At the start of the season, we start numbering from #1. The sheets are all kept in a binder labeled "Mosquito Surveillance, year."

1. Retrieve the mosquito pools from the freezer.
2. Keep the mosquitoes on dry ice in a styrofoam cooler.

3. Label the tubes with the appropriate number from printed list.
4. Place 24 labeled tubes of mosquitoes into each ice rack.
5. Place the full ice racks into the refrigerator to keep them cool before grinding.

Steps 6-16 are performed inside a BSC:

6. Wipe work surfaces inside the BSC with 70% alcohol.
7. Pour an appropriate working volume of PBS-G into a sterile beaker.
8. Add 1 mL PBS-G to each tube of mosquitoes.
9. Place 24 tubes into each cassette and push the tops down securely.
10. If there are less than 48 pools to be ground, distribute the tubes evenly between the 2 cassettes (much like balancing a centrifuge).
11. Place the lid securely over the cassette so that the "Qiagen" logo on the lid and cassette are in the same orientation.
12. Place the cassette into clamps of the Mixer Mill so that the "Qiagen" logo is at the top of the cassette.
13. Tighten the cassettes into place with the clamp screw and lock with buckle. Lower the Plexiglas cover.
14. Turn on the Mixer Mill with the switch located at the lower left of the instrument's rear and verify that the settings match the following:

    Time = 4 minutes
    Frequency = 25 cycles/second

15. Push "Start"; the instrument will stop automatically at completion of the run. Occasionally, if the tubes are not balanced properly or if the cassettes are not aligned inside the clamps correctly, the instrument might visibly shake and/or become very loud. It is then necessary to stop the mill and reposition the tubes and/or cassettes properly.
16. Remove cassettes from instrument and return tubes to the ice racks. Remove ice racks with tubes from BSC and load refrigerated centrifuges with samples.
17. Centrifuge tubes for 7 minutes at 7,000 rpm at 4°C.
18. After the centrifuge stops, the operator must wear a respirator while removing the lid.
19. Place the tubes back into the ice racks (20 tubes per rack) for inoculation into Vero cells.
20. Decontaminate the BSC, the centrifuge, centrifuge rotor and the adjacent bench top area with 70% alcohol.

Connecticut Agricultural Experiment Station **Protocol 2.4**
BSL3 Virus Laboratory

Title: Inoculating Vero cell cultures with sample homogenates

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions for inoculating Vero cells with mosquito pool homogenates.
Scope: This procedure applies to the inoculation of Vero cells with mosquito pool preparations in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: Processed mosquito pools may be infected by pathogenic viruses. Exposure may occur by direct contact with broken skin or mucous membranes, or by inhalation of infectious aerosols
B. Personal Protective Equipment: Laboratory gown, gloves
C. Engineering Controls: Biosafety cabinet (BSC)
D. Handling Requirements: The aqueous phase of processed mosquito pools are added to monolayer of Vero cells growing in tissue culture flasks. These tasks are performed within the BSC.
E. Waste Management: Pipettes and pipette tips are placed in 10% bleach container inside the BSC.
F. Decontamination Procedures: The BSC and affected work surfaces are decontaminated with 70% alcohol. Waste is decontaminated with 10% bleach prior to autoclaving.
G. Emergency Procedures: In the event of a splash, spill, or release incident immediately evacuate personnel from the affected area. Remove any splashed clothing and PPE. Rapidly wash affected body parts with iodine soap and water or another disinfectant. Label spill area off limits for 30 minutes and notify the RO or supervisor as soon as possible.

Materials
Processed mosquito pools
Small tissue culture flasks (50 mL) of confluent Vero cells
Vero cell growth media (MEM, 5%FBS; protocol 4.4 for preparation)
Waste beaker with bleach

Procedure

1. Use the inverted microscope to check two tissue culture flasks from each series to ensure adequate confluency and cell health; should see an approximate 80% coverage.
2. Cells that have not reached appropriate confluency should be inoculated last.
3. Line up appropriate number of tissue culture flasks (usually 20) and label each with the corresponding mosquito pool number located on the tube.

The following procedures are performed in a BSC.

4. Wipe work surfaces inside the biosafety cabinets with 70% alcohol
5. Decant most of the media from tissue culture flasks into waste beaker; leave enough media in the flask to completely coat cell monolayer and leave caps loose but do not remove.
6. Draw up 100µL of supernatant from microcentrifuge tubes containing processed mosquito pools and dispense into corresponding culture flask.
7. Take care not to allow contact between pipet tip and culture flask neck or lip.
8. Replace and tighten culture flask cap.
9. Place culture flasks on shaker for approximately five (5) minutes at 88-100 rpm.
10. Place culture flasks upright in trays and place the trays in the BSC.
11. Uncap 3 culture flasks at one time, dispense 4 mL growth media into each flask, and replace caps.

12. Be sure that the pipette tip does not contact the culture flask neck or lip. Change pipette tips as necessary if contact is made with the flask.
13. Repeat Step 11 for the remaining culture flasks.
14. Gently swirl media inside flask(s) to ensure even surface coverage.
15. Incubate culture flasks at 37°C, 5% CO_2.
16. Wipe the BSC and affected work surfaces with 70% alcohol
17. Place the mosquito pools in order inside a box labeled with the following information:
    Mosquito (Sample Type)
    Year
    Tube Numbers e.g. 101-200
18. Place box into designated –80°C Revco freezer.

Connecticut Agricultural Experiment Station **Protocol 2.5**
BSL3 Virus Laboratory

Title: Screening and harvesting cell cultures

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions for screening Vero cells for cytopathic viruses and harvesting virus-infected cultures.
Scope: This procedure applies to the screening and harvesting of virus-infected Vero cell cultures in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: Vero cell cultures may be infected by pathogenic viruses. Harvesting cultures involves working with amplified virus. Exposure may occur by direct contact with broken skin or mucous membranes, or by inhalation of infectious aerosols
B. Personal Protective Equipment: Laboratory gown, gloves
C. Engineering Controls: Biosafety cabinet (BSC)
D. Handling Requirements: Cell cultures are monitored for signs of viral infection and positive cultures are harvested in the BSC. Virus cultures are sealed in cryotubes and stored in -80^{o} C freezers.
E. Waste Management: Pipettes are placed in 10% bleach container inside the BSC.
F. Decontamination Procedures: The BSC and affected work surfaces are decontaminated with 70% alcohol.
G. Emergency Procedures: In the event of a splash, spill, or release incident immediately evacuate personnel from the affected area. Remove any splashed clothing and PPE. Rapidly wash affected body parts with iodine soap and water or another disinfectant. Label spill area off limits for 30 minutes and notify the RO or supervisor as soon as possible.

Materials
Inoculated tissue culture flasks
Serological pipettes
Cryotubes
Tube racks

Procedure
1. Inoculated tissue culture flasks are maintained in incubators and screened for signs of viral infection, cytopathic effect (CPE), from 3-7 days post-inoculation.
2. Visually inspect cell cultures for CPE and/or contamination by tilting the flasks and examining the media pools at the bottom of the flask. The media will appear cloudy as the cells deteriorate during CPE or due to growth of yeast, fungus, or bacterial contamination.
3. Re-examine cell cultures that exhibit cloudy media under an inverted microscope. Viruses will cause cells to become tattered and break loose from the culture flasks. Cell cultures with visible contaminants e.g. yeast, bacteria, fungus or heavy mosquito debris are re-tested by filtering the original sample in a 0.22uM filter (see protocol 2.6).
<u>The following harvesting procedures are performed inside a BSC</u>
4. Wipe work surfaces inside the biosafety cabinets with 70% alcohol.
5. Record the “sample ID number”, “passage history”, "volume", and “date” of the CPE positive culture on the side of cryotubes.
6. Aseptically dispense 1-2 mLs of media from positive cell cultures into each labeled cryotube using a serological pipette (usually a 5 mL pipette).
7. Seal cryotubes and decontaminate the BSC again with 70% alcohol when finished harvesting cultures.
8. Place virus cultures into designated boxes in the -80^{o} C freezer, and record new information on the inventory list.

Title: Retesting mosquito pool homogenates by syringe filtration

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015

Author(s): Philip Armstrong, Shannon Finan, Michael Misencik

Purpose: To provide detailed instructions for retesting contaminated mosquito pools by passing material through a 0.22 uM syringe filter prior to inoculating Vero cell cultures.

Scope: This procedure applies to the retesting of contaminated mosquito pool homogenates by syringe filtration in the BSL3 laboratory at the Connecticut Agricultural Experiment Station

Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Specific training to this protocol is provided by the direct supervisor.

Biosafety

A. Biological Hazards: Processed mosquito pools may be infected by pathogenic viruses. Exposure may occur by direct contact with broken skin or mucous membranes, or by inhalation of infectious aerosols. Clogged syringe filters may cause a spray of potentially infectious material if too much pressure is applied.

B. Personal Protective Equipment: Laboratory gown, gloves

C. Engineering Controls: Biosafety cabinet (BSC)

D. Handling Requirements: The aqueous phase of processed mosquito pools are passed through a 0.22 uM syringe-filter on to monolayer of Vero cells growing in tissue culture flasks. These tasks are performed within the BSC.

E. Waste Management: Pipettes and syringe filters are placed in 10% bleach container inside the BSC.

F. Decontamination Procedures: The BSC and affected work surfaces are decontaminated with 70% isopropanol. Liquid waste is decontaminated with 10% bleach.

G. Emergency Procedures: In the event of a splash, spill, or release incident immediately evacuate personnel from the affected area. Remove any splashed clothing and PPE. Rapidly wash affected body parts with iodine soap and water or another disinfectant. Label spill area off limits for 30 minutes and notify the RO or supervisor as soon as possible.

Materials

Mosquito sample supernatant(s)
50 mL culture flasks of Vero cells
Growth media (MEM, 5% FBS; see protocol 4.4 for preparation)
1000 µL pipet tips
1000 µL pipettor
Small sterile beaker
Waste beaker with bleach
Sterile .22 micron millex GP syringe driven filter unit
Sterile single use 1 mL syringe with tuberculin slip tip (no needle)

Procedure

1. Use the inverted microscope to check two tissue culture flasks from each series to ensure adequate confluency and cell health; should see approximately 80% coverage.

2. Retrieve mosquito pools from the freezer for retesting and thaw them in hot water.

3. Line up appropriate number of tissue culture flasks and label each with the corresponding mosquito pool number located on the tube. Label the top of each “0.22” to indicate that it was filtered.

The following procedures are performed in a BSC.

4. Wipe work surfaces inside the biosafety cabinets with 70% alcohol

5. Obtain a small, sterile beaker, place in biosafety cabinet and fill with PBS-G.

6. Fill tubes containing processed mosquito pools with PBS-G.

7. Decant most of the media from tissue culture flasks into waste beaker of bleach; leave enough media in the flask to completely coat cell monolayer and leave caps loose but do not remove.
8. Pull back plunger to fill syringe with 0.5 mL air. Then aspirate 0.5 mL of mosquito pool homogenate into syringe.
9. Insert syringe tip into filter unit.
10. Slowly dispense mosquito homogenate through filter into the corresponding culture flask. Do not force through.
11. Replace and tighten culture flask cap.
12. Place culture flasks on shaker for five 5 minutes at 88-100 rpm.
13. Place culture flasks back into biosafety cabinet.
14. Uncap 3 culture flasks at one time, dispense 4 mL growth media, and replace caps.
15. Be sure that the pipette tip does not contact the culture flask neck or lip.
16. Repeat Step 11 for the remaining culture flasks.
17. Gently swirl media inside flask(s) to ensure even surface coverage.
18. Incubate culture flasks at 37°C, 5% CO_2.
19. Wipe the BSC and affected work surfaces with 70% alcohol when finished.

Connecticut Agricultural Experiment Station **Protocol 3.1**
BSL3 Virus Laboratory

Title: Extraction of Viral RNA Using Qiagen Kit

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions for extracting viral RNA from mosquito pools and virus cultures
Scope: This procedure applies to the extraction of viral RNA from mosquito pools and virus cultures in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: Mosquito pools and cultures may be infected by pathogenic viruses. Exposure may occur by direct contact with broken skin or mucous membranes and inhalation of infectious aerosols
B. Personal Protective Equipment: Laboratory gown, gloves
C. Engineering Controls: Biosafety cabinet (BSC)
D. Handling Requirements: The aqueous phase of processed mosquito pools and virus cultures are added to virus lysis buffer (AVL buffer) in the BSC. The sample is then vortexed and incubated for 10 minutes to inactivate infectious virus.
E. Waste Management: Pipette tips and tubes are placed in 10% bleach container inside the BSC until virus has become lysed, or collected in deli cups and then autoclaved.
F. Decontamination Procedures: The BSC and affected work surfaces are decontaminated with 70% alcohol.
G. Emergency Procedures: In the event of a splash, spill, or release incident immediately evacuate personnel from the affected area. Remove any splashed clothing and PPE. Rapidly wash affected body parts with iodine soap and water or another disinfectant. Label spill area off limits for 30 minutes and notify the RO or supervisor as soon as possible.

References:
Protocol adapted from QIAamp Viral RNA Mini Kit Protocol (Qiagen, Valencia, CA)
Blow JA, Dohm DJ, Negley DL, Mores CN (2004) Virus inactivation by nucleic acid extraction reagents. J Virol Methods 119(2): 195-8.

Materials
1.5 mL microcentrifuge tubes
Mini spin columns
Collection tubes
Microcentrifuge tube rack(s)
100 and 1000 µL pipet tips
100 and 1000 µL pipettor (RNA extraction dedicated)
Vortex
Microcentrifuge (RNA extraction dedicated)
Sterile water
Waste container

Reagents and Procedures for Preparation

A. Buffer AVL
Add 1 mL Buffer AVL to one tube lyophilized carrier RNA

Shake for 30 seconds to dissolve carrier RNA
Transfer contents back to Buffer AVL bottle and shake for 30 seconds.
AVL/RNA solution can be stored at RT for ≤ 2 weeks.
For long-term storage, aliquot into 15 mL sterile tubes and refrigerate at 4°C ≤ 6 months.
Heat gently (<80°C) prior to use.

B. Ethanol

Pour 95% EtOH into a sterile 50 mL tube.

C. Buffer AW1

Add 125 mL 95% EtOH to AW1 concentrate.
Store at RT for ≤ 1 year.

D. Buffer AW2

Add 160 mL 95% EtOH to AW2 concentrate.
Store at RT for ≤1 year.

Procedure for RNA Extraction

*NOTE: To prevent the inadvertent contamination of nucleic acids during this procedure, use dedicated pipettors and filter tips for each step. Change gloves frequently. Perform RNA extraction on sterile water or a known negative sample to monitor for contamination during the RNA extraction process.

1. Label 1.5 mL microcentrifuge tube and one spin column + collection tube for each specimen.
2. Add 280 µL of AVL Buffer to the labeled 1.5 mL tube.

Steps 3-7 are performed in the BSC:

3. Wipe work surfaces inside the BSC with 70% alcohol.
4. Add 70 µl of virus culture or mosquito pool supernatant to the 1.5 mL tube containing AVL Buffer.
5. Use 70 ul of sterile water instead of supernatant for the negative extraction control.
6. Vortex for 5-10 seconds.
7. Incubate sample for at least 10 minutes at RT.
8. Pulse centrifuge to remove liquid droplets from lid.
9. Add 280 µL 95% EtOH to same tube.
10. Vortex and pulse centrifuge.
11. Add 630 µL of mixture to spin column and centrifuge for 1 minute at 8000 rpm. Discard collection tube and replace with a fresh collection tube.
12. Add 500 µL Buffer AW1 to spin column.
13. Centrifuge for 1 minute at 8000 rpm. Discard collection tube and replace with fresh collection tube.
14. Add 500 µL AW2 to spin column.
15. Centrifuge for 3 minutes at 13,200 rpm. Discard collection tube and replace with a second labeled 1.5 mL microcentrifuge tube.
16. Add 70 µL AVE or TE to elute RNA off spin column. Incubate at RT for 1 minute and centrifuge for 1 minute at 8000 rpm.

Connecticut Agricultural Experiment Station **Protocol 3.3**
BSL3 Virus laboratory

Title: Detection of bunyaviruses by conventional RT-PCR

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions for screening samples for bunyaviruses (California and Bunyamwera serogroups) by conventional RT-PCR. Bunyaviruses known to occur in Connecticut include Jamestown Canyon, Trivittatus, La Crosse, Cache Valley, and Potosi virus.
Scope: This procedure applies to detection of bunyaviruses by conventional RT-PCR in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Specific training to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: None specific to protocol. Extracted RNA preparations are not infectious.
B. Personal Protective Equipment: Laboratory gown, gloves
C. Engineering Controls: N/A
D. Handling Requirements: PCR reactions are set up using dedicated pipettors and filter tips in room 31.
E. Waste Management: Pipette tips and tubes are discarded in plastic waste receptacles and then autoclaved with laboratory trash.
F. Decontamination Procedures: The work surface is wiped-down with 10% bleach.
G. Emergency Procedures: N/A

Kits
Titan RT-PCR System (Roche Diagnostics, item#18555476)

Reagents
5X RT-PCR Buffer
Enzyme mix
DEPC-treated RNase free water
DTT-solution
dNTP's (10 mM)
RNAse Inhibitor (Applied Biosystems, item# N8080119)
PCR primers
RNA preparations
Positive control RNA

Materials
White freezer rack or ice bucket
Blue freezer rack
Microcentrifuge tubes
Filter tips

Proceedure
1) In the PCR room (room 31), prepare all reactions on ice or in blue-freezer racks. Pre-heat thermal cycler to 85°C.
2) Master Mix I

Reagent	µl/rxn	Master-mix I (µl)
Water	9.5	_____
dNTP's (10 mM)	4	_____
DTT-solution (100mM)	2.5	_____

BUNS+new (20 uM)	1	_____
BUNS-new (20 uM)	1	_____
Total	18	_____

3) Aliquot 18 µl master mix I per tube + 2 µl RNA.
4) Transfer tubes to heating block at 85°C. Incubate for 5 min, then place tubes on ice or blue-freezer racks.
5) Master Mix II

Reagent	µl/rxn	Master-mix II (µl)
Water	18.5	_____
5X RT-PCR Buffer	10	_____
Titan enzyme mix	1	_____
RNase Inhibitor (20U/ul)	0.5	_____
Total	30	_____

6) Add 30 ul of master mix II (chilled) per tube.
7) Remove items from the PCR set-up room and wipe down work surface with 10% bleach.
8) Start appropriate thermocycling program and transfer tubes to heating block when it reaches 50°C.

Primers*	Size	Thermocycling
BUNS+new/BUNS-new	~950	1 cycle of 50 °C-30 min and 94 °C-2min
S-segment		10 cycles of 94°C-15 sec, 55 °C-30 sec, and 68°C-1min
		25 cycles of 94 °C-15 sec, 55 °C -30 sec, and 68°C-1 min + 5 sec per cycle
		1 cycle of 68 °C for 7 min

Primer Sequences for Bunyaviruses

BUNS+new (TGACCAGTAGTGTACTCCAC)

BUNS-new (CAAGCAGTAGTGTGCTCCAC)

Connecticut Agricultural Experiment Station **Protocol 3.4**
BSL3 Virus laboratory

Title: Detection of West Nile, EEE, or Highlands J virus by real-time RT-PCR

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions for screening RNA preparations for West Nile, eastern equine encephalitis, or Highlands J virus by real-time RT-PCR.
Scope: This procedure applies to testing samples for viruses by real-time RT-PCR in the BSL3 laboratory at the Connecticut Agricultural Experiment Station
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Training specific to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: None specific to protocol. Extracted RNA preparations are not infectious.
B. Personal Protective Equipment: Laboratory gown, gloves
C. Engineering Controls: N/A
D. Handling Requirements: PCR reactions are set up using dedicated pipettors, filter tips in room 31.
E. Waste Management: Pipette tips and tubes are discarded in plastic waste receptacles and then autoclaved with laboratory trash.
F. Decontamination Procedures: The work surface is wiped-down with 10% bleach.
G. Emergency Procedures: N/A

Kits
Taqman one-step RT-PCR MM Reagents Kit (Applied Biosystems item#4304437)
Reagents
2X PE Ready Mix
Enzyme mix
DEPC-treated RNase free water
PCR primers and probes
RNA preparations
Positive control RNA

Materials
White Ice rack or ice bucket
Metal Rack for smart cycler tubes
Microcentrifuge tubes
Filter tips

Procedure
1. In the PCR set-up room, prepare master mix for number of samples to be tested:

Reagent	µl/rxn	Master-mix (µl)
RNAse free water	8.9	_____
2X RE Ready Mix	12.5	_____
Primer 1 (100 uM)	0.25	_____
Primer 2 (100 uM)	0.25	_____
Probe (25 uM)	0.15	_____

Enzyme (PE Kit)	0.5	_____
Total	22.55	_____

2. Aliquot 22.5 µl of master mix into each Smart Cycler tube and add 2.5 µl of template RNA.
3. Centrifuge tubes with the hinge facing down for 5 seconds.
4. Remove items from the PCR set-up room and wipe down work surface with 10% bleach.
5. Place tubes in Smart Cycler with the hinge to the back.
6. Click "CREATE RUN" and then "ADD REMOVE SITES".
7. Choose the WNV protocol in the top left window and select the appropriate number of sites for your run. Once they are selected, click the right arrow to activate these sites for the PCR run.

8. Thermocycling:	50°C	1800 sec	1 cycle
	95°C	600 sec	
	95°C	15 sec	45 cycles
	60°C	60 sec	

West Nile virus primer/probe sets:

1. WNV10533+ WNV10625 + WNV10560probe
2. WNV1160 +WNV1229C/WNV1186
3. WNV10668+WNV11770/WNV10692

Eastern equine encephalitis virus primer/probe sets:

1. EEE1858+EEE1926CN/EEE1881
2. EEE 411F+EEE527R/EEE463

Highlands J virus primer/probe sets:

1. HJ-E1fwd + HJ-E1rev + HJ-E1probe

Connecticut Agricultural Experiment Station **Protocol 3.6**
BSL3 Virus Laboratory

Title: Gel Electrophoresis

Date Issued: 6 May 2014 **Next Revision Date:** 6 May 2015
Author(s): Philip Armstrong, Shannon Finan, Michael Misencik
Purpose: To provide detailed instructions to run gel electrophoresis.
Scope: This procedure applies to running gel electrophoresis including buffer and gel preparation, running, staining, and photographing gel and management and disposal of Ethidium Bromide (EtBr) and Ethidium Bromide contaminated materials.
Training Requirements: Workers performing this procedure must complete biosafety training and attend annual training sessions conducted by the CAES responsible official (A. Bransfield). Specific training to this protocol is provided by the direct supervisor.
Biosafety

A. Biological Hazards: Ethidium Bromide is mutagenic and moderately toxic.

B. Personal Protective Equipment: Laboratory gown, gloves

C. Engineering Controls: N/A

D. Handling Requirements: All Ethidium Bromide solutions, contaminated materials, and work supplies are restricted to an "EtBr Only Area" bench.

E. Waste Management: Ethidium Bromide solution is filtered and discarded in general laboratory sink drain. Ethidium Bromide contaminated materials are wrapped in aluminum foil and discarded and autoclaved with biohazard waste.

F. Decontamination Procedures: Work surfaces are decontaminated with 70% Isopropanol.

G. Emergency Procedures: N/A

Materials
Pipettes
Pipet tips
Parafilm
Mupid-21 mini-gel electrophoresis system
-gel maker set
-mini gel migration trough
-power supply
Owl Scientific gel migration trough
-gel maker tray
-gel migration trough
-power supply
Invitrogen UltraPure™ Agarose
FisherBioReagents Ethidium Bromide, 1.0 % Biotech grade
Promega 100bp DNA Ladder
Promega 1kb DNA Ladder
Promega Blue/Orange 6X Loading dye
Ethidium Bromide-only staining box
Canon PowerShot A590 Camera
Fotodyne FOTO/Analyst Apprentice

Canon Selphy Printer
Canon Selphy Color Ink/Paper set KP-36IP
Fisher Scientific Biotech Electrophoresis Systems 312nm Variable Intensity Transilluminator
Whatman Extractor Ethidium Bromide Waste Reduction System
Nalgene Hand-Operated Vacuum Pump

Procedure

I. Preparation of Solutions and Buffers

A. 20X Tris Borate Buffer (TBE) 1 Liter

1. Tris Base - 216.0g
   Boric Acid - 110.0 g
   0.5 M EDTA, pH 8.0 - 80.0 ml
   a. Dissolve Tris base and Boric Acid in about 600.0 ml distilled H_2O.
   b. Add EDTA.
   c. Bring volume to 1 liter with distilled H_2O.
   d. Autoclave.

B. 0.5 M EDTA pH 8.0 500.0 ml

1. EDTA - 93.0g
   NaOH - 10g
   a. Add 10.0 g solid NaOH to the EDTA.
   b. Adjust pH to 8.0 with 2N NaOH after the solid NaOH is in solution.
   c. Autoclave.

C. 1X TBE Solution

1. Add 50.0 ml 20X TBE to 1.0 L bottle and bring to 1.0 L of volume with distilled H_2O.

D. Ethidium Bromide Solution

1. Add 10.0 µL EtBr to 200.0 ml distilled H_2O.

E. Agarose Gel

1. Measure and add appropriate amount of agarose to small flask.
2. Bring to volume with 1X TBE solution.
   a. EXAMPLE: For 150.0 ml of 1.5% agarose solution; calculate 150 x .015 = 2.25 g agarose to 150 ml TBE
   For 150.0 ml of 2.0 % agarose solution; calculate 150 x .02 = 3.0 g agarose to 150 ml TBE
3. Microwave mixture, swirling frequently, until all agarose is in solution.
4. Pour solution into gel maker tray with combs and allow to cool and polymerize, about 30 minutes.

II. Running, Staining, and Photographing the Gel

A. Running the Gel

1. Prepare templates
   a. On a piece of parafilm or in a tube, combine 4.0µL loading dye with 6.0µL template
2. Prepare Gel Migration trough

a. Place gel inside trough so that template will run negative to positive. Negative and positive ends are indicated on the trough.
b. Add enough 1X TBE to trough to completely cover gel.

3. With gel loading pipette tips, aspirate 10.0 µL of template/loading dye and dispense into well.
4. Fasten lid to trough and connect power supply.
   a. Power supply should be set to 100V and running negative to positive.
   b. Run for 30.0 minutes.

B. Staining the Gel

1. Pour 200.0 ml EtBr solution into EtBr-only staining box and add gel.
2. Stain for 15-20 minutes swirling frequently.
3. Discard EtBr solution after 3 uses (see section III. Management and Disposal of Ethidium Bromide and Ethidium Bromide Contaminated Materials)

C. Photographing the Gel

1. Remove gel from EtBr solution and place on transilluminator.
2. Place hood with camera over the gel and turn transilluminator on.
3. Turn camera on by pressing the Power button on the top of the camera. Be sure that camera is in Photo mode.
4. Frame and focus the gel
   a. Press the MF button and select MACRO
   b. Use the Zoom Lever to zoom in and out on the gel. The camera will automatically focus.
5. Press the shutter button fully.
6. View Saved Images
   a. Slide Mode button to Playback mode.
   b. View saved images by pressing the left and right scrolling buttons.
7. Print Image
   a. Turn printer on
   b. Press the Print button (illuminated in blue at top right).

III. Management and Disposal of Ethidium Bromide and Ethidium Bromide Contaminated Materials

A. Management of Ethidium Bromide
   1. EtBr solution is kept in a 500.0 ml light blocked glass bottle.
   2. EtBr solution is discarded after three (3) uses as indicated by tick marks on the label.
      a. Used EtBr solution is stored in a 1.0L glass bottle marked “EtBr Waste” until ready for filtration.

B. Disposal of Ethidium Bromide Solution and Contaminated Materials
   1. EtBr solution is filtered and discarded into laboratory sink.
      a. Place filter unit over mouth of a 1.0 L bottle designated for EtBr filtration.
      b. Connect hand-operated vacuum pump to filtration unit.
      c. Carefully pour EtBr solution into filter unit reservoir.
      d. Begin filtering by depressing the vacuum pump handle intermittently.
      e. The EtBr filtrate can be poured down the general laboratory drain.
   2. Ethidium Bromide contaminated materials are discarded and autoclaved with biohazard waste.

Appendix D: SOP for audit

LABORATORY BIOSAFETY CHECKLIST

Principal Investigator (PI) _____ Inspection Date_____

Bldg _____Room(s) _____

Phone_____

Biosafety Level _____ Biosafety Exam Officer_____

GENERAL INFORMATION:

Present Use: cell culture rDNA infectious agents animals human material

Infectious agents in use:_____

Select agents in storage:_____

Brief description of experiments:_____

_____

_____

List of authorized users:_____

_____

_____

Supervisors who oversee activities in BSL3 Laboratory:_____

_____

RO:_____ ARO:_____

_____

MEDICAL SURVEILLANCE:

Is a baseline serum stored for all workers in BL3?	yes	no
Is immunization, if available, offered to workers?	yes	no
Do immunosuppressed researchers (i.e. pregnant, on steroid or cytotoxic drug treatment) consult with PI about reassignment outside the lab?	yes	no
Do workers with cuts, abrasions, dermatitis, eczema, or other form of compromised skin condition consult with PI before beginning work?	yes	no
Are workers restricted from working unless waterproof bandages can appropriately cover the affected area?	yes	no

---

TRANSPORT/RECEIVING OF BSL3 MATERIALS:

Are all materials packaged for transport inside the biological safety cabinet?	yes	no
Are Federal Regulations for shipping and receiving infectious agents followed?	yes	no

Workers trained for dangerous goods shipping _____

Are prescribed procedures for shipping and receiving of Select Agents followed?	yes	no
Are packages of infectious materials opened in biosafety cabinet?	yes	no

INVENTORY:

Inventory log sheet maintained for all Select Agents?	yes	no
Each activity involving a Select Agent recorded on the inventory log sheet?	yes	no
Inventory log securely maintained and access restricted to authorized personnel?	yes	no
Copy of inventory log on file with ARO?	yes	no
Inventory log verified accurate and up to date?	yes	no
Evidence of tampering or alterations on inventory log?	yes	no

LABORATORY SECURITY:

A) Physical Security

Maintenance & custodial workers with security clearance from Dept of Justice (DOJ)? Names of cleared personnel_____	yes	no
Proximity card required for general entry to building?	yes	no
Is lab separated from unrestricted traffic flow?	yes	no
Are door signs posted detailing biosafety level, entry requirements, and names and locations of PI and other emergency contacts?	yes	no
Is the use or storage of Select Agents revealed on the lab door sign?	yes	no
Are there two sets of self-closing doors that create an airlock?	yes	no
Are entry doors kept closed and locked?	yes	no
Is access to lab restricted to authorized personnel only?	yes	no
Is proximity card access required for restricted lab entry?	yes	no
Is each lab entry and exit recorded on written sign-in/sign-out log?	yes	no
Is there an electronic record of each lab entry and exit filed with Department of Administrative Services: Statewide Security Unit?	yes	no
Are sign-in logs reconciled with electronic door entry records?	yes	no
Are Entry/Exit access records on file with RO/ARO for at least 3 years?	yes	no
Are secured freezers used for primary storage of Select Agents?	yes	no
Are freezers, refrigerators, cabinets, and other Select Agent storage units always locked when not under direct view of workers?	yes	no
Are all keys for Select Agent storage units kept in secure location with access restricted to authorized personnel?	yes	no

B) Visitor Policy

Is unauthorized visitor access restricted?	yes	no
Is a Select Agent awareness and security training session provided to all visitors or other non DOJ-authorized personnel before entry?	yes	no
Is each visitor entry and exit recorded and confirmed by the signature of an authorized personnel?	yes	no
Are unauthorized maintenance/custodial staff allowed access?	yes	no
If yes, are maintenance/custodial personnel accompanied at all times during their visit by DOJ-authorized personnel?	yes	no
Are both short-term visitors and long-term visitors (e.g., non DOJ-authorized summer helpers) accompanied at all times during their visit by DOJ-authorized personnel?	yes	no
Have long-term visitors received special training from the RO?	yes	no
Are bags, boxes or other storage containers brought by all visitors into or out of the facility inspected before entry or exit?	yes	no

C) Security Program Postings, Incident and Emergency Response Protocols

Are copies of relevant manuals (i.e., Laboratory Training Manual (Biosafety Plan, Incident Response Plan, and Security Plan) Blood-borne Pathogens, MSDS information) present within the facility or immediately available to personnel?	yes	no
Is an Exposure Response Poster placed in a visible location within the lab?	yes	no
Is a BSL3 Spill Response Poster placed in a visible location within the lab?	yes	no
Is a Select Agent Emergency Evacuation response poster posted within the lab?	yes	no
Are Fire Evacuation plans posted in the hallway outside the laboratory?	yes	no
Is Security Training documented annually for all Select Agent personnel?	yes	no
Are BSL3 lab inspections documented at least once per year for the facility?	yes	no
Does a Safety/Biosafety committee meet annually for BSL3 facilities?	yes	no
Are standard operating procedures present within the facility?	yes	no

D) Reporting Requirements

Are all personnel aware of requirement to immediately report loss of proximity card to RO or ARO?	yes	no
Are all personnel aware of requirement to report all suspicious events (packages, people, or activities) immediately to RO or ARO and Police?	yes	no
Are all personnel aware of the requirement to immediately report any loss, theft, or release of Select Agents in or out of the laboratory to RO or ARO?	yes	no
Are all personnel aware of requirement to immediately report any discrepancy of Select Agent Inventory log immediately to RO or ARO?	yes	no
Are all personnel aware that Federal approval is required prior to the transfer of a Select Agent to any other entity (at CAES or off-campus)?	yes	no
Are all personnel aware of the requirement of immediately reporting any exposure incidents involving Select Agents to RO or ARO and Employee Health?	yes	no

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LABORATORY FACILITIES:

A) General Equipment/Facilities

Date of most recent routine exhaust fan maintenance _____

Can lab personnel verify digital pressure gauge readings prior to entry into lab? yes no

Verification by: Kimwipe strips by intake vents _____

Dry ice vapor flow at doors _____ Date lasted tested _____

Digital Pressure Gauge Readings at time of inspection:

Room 023 (corridor) _____ Room 030 freezer _____ Room 027 (Vector) _____

Room 028 (Vector) _____ Room 024 autoclave _____ Room 026 (Animal) _____

Room 026A (Animal) _____ Room 026B (Animal) _____ Room 026C (Animal) _____

Date of calibration of digital pressure gauges _____

Date of certification of HVAC prefilters and HEPA filters: _____

Magnahelic gauge #1 reading: Prefilter: _____ HEPA filter: _____

Magnahelic gauge #2 reading: Prefilter: _____ HEPA filter: _____

Magnahelic gauge #3 reading: Prefilter: _____ HEPA filter: _____

Date of most recent fire alarm tests _____

Is a foot, elbow, or automatically operated sink available near exit door?	yes	no
Is there a supply of soap and paper towels?	yes	no
Are interior surfaces water resistant?	yes	no
Are floor, walls, and ceiling impervious?	yes	no
Are joints and penetrations sealed or capable of sealing?	yes	no
Is lighting adequate and flush against ceiling?	yes	no

Are windows closed and sealed? yes no
Is there an autoclave for decontamination of biological waste? Where? _____ yes no
Are autoclaving procedures/parameters verified? yes no

What are the verification methods?_____
Are eyewashes available within the laboratory? yes no
Are eyewashes flushed regularly? _____
Is there a system of communication from the lab to the outside? yes no
Is emergency power available for the lab or equipment? yes no
Is emergency power system tested periodically? yes no
Are written records available documenting emergency power testing? yes no
Date of most recent test _____
Have back-up locations been identified for storage or use in the event of equipment failure? yes no
If so, are materials transported in a non-breakable, labeled, leak-proof, plastic container? yes no
Is required equipment located within the lab? yes no
Centrifuge _____
Shaker _____
Sonicator _____
Other _____
Are containment devices used (safety buckets, sealed rotors, or sealed tubes) or is the equipment placed inside the biological safety cabinet during use? yes no
If no, do personnel use appropriate PPE? yes no
Are aerosol-tight rotor lids replaced periodically on centrifuge rotors? yes no
Date rotor lids were last replaced _____
Type of vacuum system: central pump aspirator
Are vacuum traps and filters used to protect the vacuum system? yes no
Are agents stored in a secure location with limited access? yes no
Is the biohazard label placed on equipment used for work with these agents or storing them? yes no
If liquid N_2 used, is storage limited to vapor phase only? yes no
What disinfectants are available?
70% ethanol_____
70% isopropanol_____
1:10 bleach_____
Betadine Iodine Scrub_____
Other_____
Are work surfaces (biological safety cabinet) and lab equipment decontaminated after all experiments? yes no
Is a pest control program in effect? yes no
Are all non-experimental insects and rodents present in the lab reported? yes no

Monitors used for pest surveillance?_____
Are pipettors available (mouth pipetting is prohibited)? yes no
Is there a policy for safe handling of sharps? yes no
Eating, drinking, smoking, and food storage not permitted in lab? yes no

B) Animal Rooms

Are the animal/insect rooms clean and easy to clean? yes no
Are the animal rooms adequate for containing the animals? yes no
Do workers wear respiratory and face protection when appropriate? yes no

C) Biological Safety Cabinet

Is there at least one biological safety cabinet (BSC) available in the laboratory? yes no
Are all manipulations performed inside a BSC or other

physical containment device?	yes	no
Are all supplies placed inside the BSC prior to initiation of work?	yes	no
Is all waste collected inside the BSC?	yes	no
Is the waste bag sealed and wiped down with disinfectant prior to removal?	yes	no
Are all items wiped down with disinfectant prior to removal from BSC?	yes	no
Is the interior of the BSC wiped after experiments (grills, work surface, sides, rear wall, and inside front view screen)?	yes	no
Is/Are the BSC(s) certified at least annually?	yes	no

Room #____ BSC Serial No. _____ Date of Certification _____

Room #____ BSC Serial No. _____ Date of Certification _____

Room #____ BSC Serial No. _____ Date of Certification _____

Room #____ BSC Serial No. _____ Date of Certification _____

D) Centrifugation Equipment

Are centrifuge tubes placed into and removed from safety cups and sealed rotors inside the biological safety cabinet?	yes	no
Are tubes and bottles checked for deformities before each use?	yes	no
Are O-rings changed if cracked, worn or missing?	yes	no
Are primary containers limited to 3/4 full?	yes	no
Is the air evacuated through a vacuum trap and filter after each run?	yes	no
Is respiratory protection worn when opening the centrifuge?	yes	no
Are safety cups and rotors disinfected after each use?	yes	no
Is the centrifuge interior decontaminated after each use?	yes	no

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OPERATIONAL PROCEDURES

A) Work Practices/Training

Do workers have education and experience with infectious agents that may be present in the BSL3 lab?	yes	no
Does PI/laboratory director ensure that workers have been trained in the safe handling of these agents?	yes	no
Do workers receive universal handling precaution training?	yes	no
If receive precaution training, date of last training: _____		
Are all accidents, exposures, and spills reported to the PI or RO?	yes	no
Are there written procedures for:		
-cleaning and decontaminating the laboratory and equipment?	yes	no
-spill, accident, and exposure incident response?	yes	no
-standard operating procedures?	yes	no
Is a biological spill kit available?	yes	no
Has plastic been substituted for glass wherever feasible?	yes	no
Are emergency response procedures posted for spills and accidents?	yes	no
Do workers practice response procedures on a regular schedule?	yes	no
If so, date of last practice: _____		
Do lab workers know the proper response in event of a fire?	yes	no
Is fire extinguisher available and up to date?	yes	no
Is fire alarm audible from within BL3 Lab?	yes	no
Are supplies of gloves, back-fastening gowns, tyvek suits, face, respiratory, or other protection available?	yes	no
Is protective eyewear used in the laboratory?	yes	no
Are required PPE items worn for all work with infectious agents?	yes	no
Do workers follow the required entry and exit requirements?	yes	no
Do workers check airflow, bring in supplies, don PPE, and make sure		

door is closed behind them before entering the facility? yes no

Do workers remove PPE, then step into clean area, wash hands thoroughly, and ensure that door is closed securely prior to leaving the facility? yes no

Is there an appropriate storage and dressing area? yes no

Are hands washed each time gloves are removed? yes no

B) Decontamination and Disposal of Biological Waste

Are all containers of biological waste clearly labeled? yes no

Is all waste within the biological safety cabinet discarded? yes no

Is the outside of the bag wiped down with disinfectant before removal? yes no

Is waste transported to the autoclave in a durable, leak-proof container? yes no

Is infectious waste autoclaved by the end of each work day? yes no

Method for decontamination of laboratory waste?

Type of waste	Method	Contact time
Medical: plastics		
liquids		
animals		
other		
Non-medical: recyclable glassware		
liquids		
other		

ADDITIONAL COMMENTS/NOTES:_____

Signature of Inspector_____ **Date**_____