CONTROLLED//PROPIN//BASIC

United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Amplification of Extraneous Senecavirus Through Cell Culture

Date: March 4, 2026

Reference Number: CVB-PRO-0034.02

Contact: Center for Veterinary Biologics, 515-337-6100

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

INTERNAL USE ONLY

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may be suitable.

Table of Contents

1. Introduction

2. Materials

- **2.1 Equipment/instrumentation**
- **2.2 Supplies**
- **2.3 Reagents**

3. Preparation of Secondary CEFs

4. Preparation and Inoculation of Test Samples onto Secondary (2°) CEFs

- **4.1 Test samples**
- **4.2 Controls**

5. First, Second, and Third Passage of CEFs and Associated Harvest Samples

1. Introduction

Senecavirus A (SVA), also referred to as Seneca Valley virus, is an approximately 7.2 kb non-enveloped, single-stranded positive sense RNA virus in the family *Picornaviridae*. It was first isolated from pigs in 1988, but its identity was not confirmed until the early 2000's when it was incidentally found in laboratory cell lines. This testing protocol (PRO) describes the neutralization and passage of live veterinary biologicals in cell culture for the detection of Senecavirus.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Incubator, 37°C ± 2°C, humidified, with 5% CO_2

2.1.2 Water bath, 37°C ± 2°C

2.1.3 Microscope, inverted bright light

2.1.4 Vortex mixer

2.1.5 Magnetic stir bars (sterile) and a stir plate

2.1.6 Pipette-aid automatic pipettor (Drummond Scientific)

2.1.7 Eppendorf micropipettors (1000 µL, 200 µL, 100 µL)

2.1.8 Centrifuge (Beckman J6-MI, JS-4.2 rotor)

2.1.9 Hemocytometer

2.2 Supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Pipette tips

2.2.2 25-cm^2 and 75-cm^2 tissue culture flasks

2.2.3 Plastic funnel covered with four layers of fine gauze

2.2.4
2.2.5 15 mL centrifuge tubes

2.2.6 12 x 75-mm tubes

2.2.7 Disposable serological pipettes 1-, 5-, 10-, and 25-mL

2.2.8 Graduated cylinders, 25 mL, 50 mL, 100 mL, and 250 mL

2.2.9 Erlenmeyer flasks

2.2.10 Magnetic stir bar

2.3 Reagents

Equivalent reagents may be substituted. All reagents must be sterile.

2.3.1 Growth /Maintenance Medium (National Centers for Animal Health) (NCAH) Media #20010)

Medium 199 with Earles salts	1 L
Bacto tryptose phosphate broth	50 mL
$NaHCO_3$	1.5 g
Penicillin (potassium G)	100,000 units
Streptomycin sulfate	100 mg
Newborn calf serum (NCS) or equivalent	20-30mL
Fungizone (optional)	2 g

Adjust pH to 7.3 with $NaHCO_3$ solution
Store at 4°C ± 2°C

Note: Growth medium contains 3% NCS while maintenance medium contains 2% NCS.

2.3.2 0.01 M Phosphate buffered saline (PBS) (NCAH Media #30054)

1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)
0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)
8.5 g sodium chloride (NaCL)
Q.S. to 1000 mL with DI.

Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 2 N HCl.
Sterilize by autoclaving at 15 psi, 121°C ± 2°C for 35 ± 5 minutes.
Store at 4°C ± 2°C.

2.3.3 Trypsin-versene solution (NCAH Media #20005)

8 g sodium chloride (NaCl)
0.4 g potassium chloride (KCl)
1.0 g dextrose
0.58 g sodium bicarbonate (NaHCO3)
0.5 g irradiated trypsin
0.2 g Versene (EDTA)
0.4 mL phenol red (0.5%)
Q.S. to 1000 mL with DI

2.3.4 Senecavirus positive control, (SVV-15-031348) diluted for use

2.3.5 Monospecific, neutralizing antisera, free of Senecavirus antibodies and specific to the agent(s) under test

2.3.6 Tissue culture monolayers of secondary chicken embryo fibroblasts (2° CEFs)

3. Preparation of Secondary CEFs

Secondary CEF cultures can be prepared in the following manner:

3.1 Aseptically decant the medium from one roller bottle of primary CEFs, removing all remaining media with a 5-mL pipette, and add 15 mL of prewarmed (37°C) trypsin-versene solution. Rotate the bottle until the cells begin to detach, approximately 1-4 minutes. Decant the trypsin-versene and, with an open hand, strike the side of the roller bottle until most of the cells become detached. Rinse the inside of the roller bottle with 15 mL of growth medium and swirl. Pipette the cell suspension into an empty Erlenmeyer flask. Repeat twice more for a total of three rinses. Repeat the above steps for each additional roller bottle.

3.2 Pour the combined cell suspension from the Erlenmeyer flask through a funnel covered with four layers of sterile, fine gauze into a second Erlenmeyer flask containing a sterile stir bar and 75 mL of growth medium for each roller bottle used. Then rinse the funnel with an additional 75 mL of medium for each roller bottle used. Thoroughly mix the cell suspension.

3.3 Quantitate the cell count of the suspension using a hemocytometer. Adjust the volume so that the cell concentration is approximately 100,000 – 150,000 cells per mL.

3.4 Plant the secondary cell suspension into the vessels to be used for the test using 10 mL of the suspension for a 25-cm^2 flask or 25 mL for a 75 cm^2 flask. Incubate the cultures in a 37°C humidified incubator containing 5% CO_2.

3.5 Allow the cell sheets to attach and grow for approximately 18 hours. The monolayers should be 60-80% confluent for use.

One day prior to test initiation (first passage) and one day prior to the second and third passages, seed one 25-cm^2 cell culture flask for each test sample (serial, Master Seed virus (MSV), or Master Cell stock (MCS)) and three 25-cm^2 cell culture flask (one for each un-inoculated cell control, antisera control, and positive control).

4. Preparation and Inoculation of Test Samples onto Secondary (2°) CEFs.

This protocol is designed to detect extraneous Senecavirus in modified live virus veterinary vaccines, nucleic acid veterinary vaccines, candidate MSV and candidate MCS that have been manufactured using ingredients derived from swine. If the test materials are diluted prior to inoculation, medium without serum can be used for the dilution.

4.1 Test samples

Process samples in the following order: negative control, antisera control, test samples, and finally the positive control.

4.1.1 The material under test may be neutralized using an appropriate heat inactivated, monospecific antisera in the following manner: 1 mL of test sample is combined with 1 mL of antisera in a single receptacle, mixed thoroughly, and placed into a 37°C humidified incubator set at 37°C containing 5% CO_2 for approximately 1 hour. The test/antisera mixture is mixed again at approximately 30 minutes.

4.1.2 Just prior to inoculation, remove or decant the maintenance medium from the culture flasks and rinse each monolayer with 5 mL of 0.01 M (PBS).

4.1.3 Remove or decant the PBS and then add the entire volume of test or test/antisera mixture to the rinsed CEF monolayer. For instance, a MSV is typically neutralized by combining 1 mL of antisera with 1 mL of MSV. This entire volume is added to the monolayer. Vaccines are handled in a similar manner, such that the volume of antisera or combinations of antisera required to neutralize the test is then added to the monolayer.

4.1.4 Allow the test or test/antisera mixture to adsorb on the CEF monolayer in a humidified 37°C incubator containing 5% CO_2 for 1 hour.

4.1.5 Following the adsorption, rinse each monolayer with 10 mL of maintenance medium and discard. Add 10 mL of maintenance medium to each 25 cm^2 test flask and return to a humidified 37°C incubator 37°C ± 2°C containing 5% CO_2. Observe the monolayers 2-4 hours after inoculation for

cytotoxicity. If monolayers show cytotoxicity, remove the medium and replace with fresh maintenance medium.

4.2 Controls

With each test series, maintain an uninoculated CEF flask serving as a cell culture negative control, a flask inoculated with monospecific test antisera serving as a control to evaluate cytotoxicity due to the antisera, and a diluted sample of Senecavirus serving as the positive control.

5. First, Second, and Third Passage and Associated Harvest Samples

5.1 At each passage, examine each flask microscopically with an inverted light microscope for microbial contamination and any signs of cytopathic effects (CPE) or cytotoxicity. Record observations of test and control monolayers. CPE includes cell rounding, cell lysis, and partial destruction of cell monolayer and may be visible on the CEF monolayer within 24-48 hours of inoculation.

Note: It is imperative that cultures are examined between 22-28 hours post-inoculation. CPE due to Senecavirus is most easily observed in young established CEF monolayer no older than 48 hours.

At approximately 48-72 hours, passage the monolayers in the following manner:

5.1.1 Freeze all flasks at -70°C ± 5°C for a minimum of 60 minutes. Thaw each flask at room temperature and shake contents. All flasks undergo three freeze/thaw cycles. Flasks may be maintained at -70°C ± 5°C until the next passage.

5.1.2 Following the three freeze/thaw cycles, aspirate and dispense the cell suspension against the wall of the cell culture flask 4-5 times. Aseptically transfer the flask contents to a sterile 15 mL centrifuge tube and centrifuge at approximately 1136 x g (2000 rpms) for 10 minutes at 4°C.

5.1.3 **Cell passage:** Pipette 1.0 mL of the supernatant fluid from the first passage of the test sample into a new 25 cm^2 flask of CEFs. Incubate cultures in a 37°C humidified incubator containing 5% CO_2 for 48-72 hours.

5.1.4 **Samples for testing:** Pipette the remaining volume of supernatant fluid from the centrifuged test sample into two sterile, labeled (date of harvest) 12x75 mm test tubes. If the samples are not tested (**Section 5.1.6**) on the day of harvest, they are stored at -70°C.

5.1.5 The second and third passes are performed as previously described in **Sections 5.1.1 through 5.1.4** and the samples are appropriately labeled.

5.1.6 One or more of the CEF passages are tested for the presence or absence of Senecavirus using one set of the samples from **Section 5.1.4** in a real time polymerase chain reaction (RT-PCR) and/or indirect fluorescent antibody (IFA).

5.1.7 The second set of samples from **Section 5.1.4** is retained at -70°C.