United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Real-time Reverse Transcription Polymerase Chain Reaction Assay for the Detection of Senecavirus A RNA

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Table of Contents

1. Introduction

This Testing Protocol (PRO) describes a real-time reverse transcription polymerase chain reaction (qRT-PCR) for the detection and identity of Senecavirus A (SVA), also known as Seneca Valley Virus, in biological products. This PRO describes the qRT-PCR procedures described in the journal publication: Fowler, V. L., et al., "Development of a novel real-time RT-PCR assay to detect Seneca Valley virus-1 associated with emerging cases of vesicular disease in pigs." Journal of Virological Methods, 239 (2017): 34-37.

2. Materials

2.1 Equipment/instrumentation

NOTE: Equivalent equipment and instruments can be substituted for brand named items.

2.1.1 RNA extraction/ loading of template

1. Laminar flow biological safety cabinet (BSC) (NuAire Inc., Labgard)
2. Microcentrifuge (Eppendorf 5415 C)
3. Mini plate Spinner MPS 1000
4. Quick Spin Minifuge (6 x 1.5/2.0-mL microtube rotor)
5. Eppendorf 96-well PCR Cooler (stored at -20°C ± 2°C)
6. Eppendorf micropipettors (1000-µL, 200-µL, 100-µL, 20-µL, 10-µL, 2.5-µL)

NOTE: Separate micropipettors are assigned to individual BSCs and should not be interchanged to avoid contamination.

2.1.2 Preparation of the master mix

1. Laminar flow BSC
2. Eppendorf micropipettors (1000-µL, 200-µL, 100-µL, 20-µL, 10-µL, 2.5-µL)
3. Microcentrifuge tube rack
4. 96-Well PCR tube rack
5. Labtop cooler (stored at 4°C ± 2°C)
6. Quick Spin Minifuge (6 x 1.5/2.0-mL microtube rotor)
7. Vortexer

2.1.3 Real-time Reverse Transcriptase PCR run

1. Bio-Rad CFX96 C1000 Touch™ cycler
2. Bio-Rad computer software

2.2 Reagents/supplies

NOTE: Equivalent reagents and supplies can be substituted for brand named items.

2.2.1 Laboratory supplies

1. Sterile RNase-free aerosol-resistant pipette tips
2. Bio-Rad Multiplate 96-well PCR plates (high profile, unskirted, clear; Catalog #MLL9602)
3. Microtubes (1.5-mL or 2.0-mL)
4. 8-tube PCR strips (0.2-mL without caps, low profile, clear or white)
5. Bio-Rad flat cap optical strips (Catalog #TLS0803), optical film, or other optical PCR well caps
6. 10% hypochlorite solution (bleach)
7. 70% ethanol solution
8. Latex, vinyl, or nitrile powder-free disposable gloves
9. Nuclease-free water

2.2.2 RNA extraction reagents and worksheet

1. QIAamp Viral RNA Mini Kit (Catalog #52906) (store kit at 15°- 20°C)
2. Ethanol 200 proof molecular grade (for QIAamp Viral RNA mini kit)
3. Current version of **VIRTWS1052** (Qiagen RNA extraction worksheet)
4. Known positive and negative extraction controls (if used)

2.2.3 PCR reagents and worksheet for Fowler method

1. SuperScript™ III Platinum™ One-Step qRT-PCR Kit (500 reaction kit Catalog #11732-088; stored at -20°C ± 2°C)
2. Current version of **VIRTWS0129** (SVA qRT-PCR worksheet)
3. SVA Fowler primers and probes: SVV3D-F1, SVV3D-R1, SVV3D-Pr1

Primers are rehydrated to a concentration of 10 µM, 200µL are aliquoted into 1.5 mL microtubes, and stored in a -20°C ± 2°C freezer. The probe is rehydrated to a concentration of 10 µM and 20µL are aliquoted into 0.2 mL PCR strip tubes, capped for storage at -20°C ± 2°C, and protected from light.

Primer/ Probe Sequences:
SVV3D-F1: 5'-AGAATTTGGAAGCCATGCTCT-3'
SVV3D-R1: 5'- GAGCCAACATAGARACAGATTGC-3'
SVV3D-Pr1: 5'-FAM- TTCAAACCAGGAACACTACTCGAGA-BHQ1-3'

4. Standard plasmid control pSVV2 10^9 (see **Section 3.3** for preparation and usage of dilution series) (stored at -20°C ± 2°C)

3. Preparation for the Test

3.1 Personnel qualifications

The personnel performing this procedure must have experience and training in molecular techniques and qRT-PCR. This includes knowledge of the proper preparation, handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. When working with known or suspected live biological agents, a biological safety cabinet must be used and aseptic techniques must be followed.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturer's instructions and monitor for compliance with current corresponding Center for Veterinary Biologics (CVB) Standard Operating Procedures (SOPs). Refer to the operators manual for instructions on the use and programming of the Bio-Rad CFX96 C1000 Touch™ cycler. Use clean instruments and wear powder-free laboratory gloves when handling samples, reagents, chemicals, and when otherwise necessary. Program the thermocycler with the following program:

SVA qRT-PCR cycling parameters

Cycling Conditions		
Stage 1	60°C	30 min
Stage 2	95°C	10 min
Stage 3 50 Cycles	95°C	15 sec
	60°C	60 sec*

*optical reading occurs at the end of this step, every cycle

3.3 Preparation of standards

A BSC designated for work with SVA should be used when handling plasmid pSVV2. Before preparing the master mix, prepare a 10-fold serial dilution of plasmid pSVV2 in sterile nuclease-free water. Add 5uL of 10^9 stock pSVV2 to 45uL of water in individual microtubes down to 10^{-1}. Use 10^7 to 10^{-1} to create a standard curve.

Dilutions should be refrigerated (4°C ± 2°C) until the master mix has been aliquoted across the 96-well test plate or 8-tube PCR strips and the templates are ready to be loaded. The 10^9 stock plasmid is stored at -20°C ± 2°C.

3.4 Preparation of test samples

Template RNA is extracted and stored at -20°C ± 2°C until the time of use. Persons should not enter a clean area after working with SVA without changing clothes or wearing a disposable gown. Proper precautions should be taken to avoid SVA contamination of qRT-PCR master mix by designating clean areas to prepare the master mix, separate from areas where extracted samples, controls, and plasmids are handled.

4. Performance of the Test

4.1 RNA extraction

4.1.1 Thaw samples. Record sample information on the current version of **VIRTWS1052** (Qiagen RNA extraction worksheet).

4.1.2 Each set of extracted samples should contain a known SVA positive sample and a known SVA negative sample.

4.1.3 A BSC designated for RNA and SVA handling should be used for RNA extraction. Clean all work surfaces, pipettes, and pipette tip boxes with 10% bleach solution and let dry before working with RNA. After the 10% bleach solution is dry, wipe them down again with 70% ethanol solution. All tube racks used should be routinely soaked in 10% bleach solution for at least 10 minutes after being used and air-dried before storage.

4.1.4 Follow the Qiagen QIAamp Viral RNA Mini Kit spin protocol using carrier RNA.

4.1.5 The final elution of RNA can be done using nuclease-free water instead of the AE buffer provided in the kit.

4.1.6 Once the Qiagen extraction is complete, label each extraction tube and store the extracted DNA samples at -20°C ± 2°C until use in **Section 4.3**.

Example of an Extraction Label:
Q RNA Ext #1
Sample Name
Date/Initials

4.2 Preparation of the Fowler master mix

NOTE: Perform the following in a designated clean biological safety cabinet and keep all reagents on a cooler block (stored at -20°C ± 2°C) at all times.

4.2.1 Disinfect the biological safety cabinet with 70% alcohol prior to use.

4.2.2 Thaw reagents needed to prepare the master mix in a cooler block or on ice. The enzyme mix should remain in the freezer at -20°C ± 2°C and be added to the master mix last.

4.2.3 See the current version of **VIRTWS0129** (SVA qRT-PCR worksheet) to determine the correct volume of reagents needed for testing.

1. The number of samples being tested should include no-template controls, standards, and extracted samples. All test samples should be performed at least in duplicate.

2. Calculate the amount of master mix needed by determining the total number of reactions. **VIRTWS0129** autocalculates this for the user, plus one extra reaction volume.

3. Lightly mix thawed reagents for 10 seconds with a vortexer. Spin down with a Quick Spin Minifuge.

4. Add the master mix ingredients according to the table below. Add the SuperScript™ III RT/Platinum™ Taq Mix enzyme last and promptly replace the enzyme and all other reagents to -20°C ± 2°C for storage. This is the prepared master mix and should be immediately used.

Master Mix

Fowler method master mix			
Ingredient #	Ingredient Name	Concentration	Vol. (µL) 1 Rxn
1	Nuclease-free water	n/a	1.75
2	Reaction Mix	2x	12.50
3	Rox™ dye	n/a	0.50
4	Forward Primer	10uM	2.00
5	Reverse Primer	10uM	2.00
6	Probe	10uM	0.75
7	SuperScript™ III RT Platinum™ *Taq* enzyme mix	n/a	0.50
		Total Mastermix volume:	20.00

4.2.4 Dispense 20-µL of master mix into the required number of reaction wells of a 96-well PCR plate or 8-tube PCR strips and protect from light.

4.2.5 Clean the biological safety cabinet with 70% ethanol.

4.3 Loading of template

Note: Perform the following procedure in a biological safety cabinet designated for SVA exposure. Protect the master mix from light.

4.3.1 Disinfect the biological safety cabinet with 70% alcohol.

4.3.2 Thaw all extracted samples to be tested. Verify extracted sample identification with extraction worksheet **VIRTWS1052** and the SVA qRT-PCR worksheet **VIRTWS0129**.

4.3.3 Lightly mix the extracted samples with a vortexer for 10 seconds and spin down briefly using a Quick Spin Minifuge.

4.3.4 Add 5-µL of each template sample into their respective reaction wells, according to the SVA qRT-PCR worksheet **VIRTWS0129**, containing 20-µL of master mix.

4.3.5 Cover the wells with optical strip-caps or optical film once all samples are loaded. Do NOT vortex or shake the plate or tube strips.

4.3.6 Disinfect the biological safety cabinet with 10% bleach solution followed by 70% ethanol.

4.4 Loading samples into the Bio-Rad CFX96 C1000 Touch™ cycler

4.4.1 Before loading the plate into the cycler, spin the plate using the Mini Plate Spinner MPS1000.

4.4.2 Turn on the Bio-Rad CFX96 C1000 Touch™ cycler and allow the start-up process to complete before opening the block lid. After the start-up process is complete, log into the machine as an admin.

4.4.3 Open the block lid and place the 96-well PCR plate into place in the cycler. Close the lid.

4.4.4 Navigate to the Fowler protocol on the touch screen and select the protocol. Touch the "Run" button. Select "All Channels" or "SYBR/FAM" Scan Mode on the screen when prompted and name the test run, beginning with the date. Touch "OK" on the screen to start the run.

Example of test run name: Date Fowler Identifying name

4.5 Post qRT-PCR run

4.5.1 Exporting data from the thermocycler

1. On the touch screen, select "cancel run." The machine will take a moment to process the data and save it to its internal hard drive file.

2. When done processing, insert a USB device into the USB port and select "export run." The run data will be exported to the USB device. Cyclers can be set up to computers and computer networks and are capable of emailing run data to designated emails, if desired.

3. Open the block lid and remove the sample plate from the machine. Freeze the plate, if needed, at -20°C ± 2°C. Close the block lid and log out.

4. Turn the machine off.

4.5.2 Uploading data to computer the software

1. The data saved on the USB device, or emailed, will need to be opened on a computer that has the Bio-Rad CFX Manager software installed on it for data usage and interpretation.

2. After analyzing data, print off plate view report, standard curve plot, and the reaction curves chart for recordkeeping.

3. Save the run file(s) and all Excel data from the run into a dated and named file on the shared F-drive for electronic recordkeeping.

5. Interpretation of Test Results

5.1 Test results for the Fowler method for SVA detection

5.1.1 The Fowler method qRT-PCR assay is considered valid if:

1. The serially diluted standards, run at least in duplicate, create logarithmic curves. The following validity elements, combined with personnel knowledge regarding qRT-PCR theory and data, are used to interpret test results:

a. The r^2 value for the standard curve plot should be ≥ 0.98.

b. The slope of the standard curve plot should be between -3.0 and -3.7. A slope of -3.3 indicates 100% amplification efficiency.

c. It is permissible to remove outliers to achieve parts a. and b.

NOTE: Further criteria must be met for these validity elements to be achieved, including distance between Ct value in the dilution series and reproducibility between replicates. Expertise in qRT-PCR data analysis is essential, particularly regarding: Ct values and standard curves, Ct values between replicates, proper management of outliers, and the nature of the tested samples. More information can be found in the "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments."

5.1.2 When the qRT-PCR assay is valid, successfully amplified SVA RNA should have a Ct value ≤ to the pSVV2 10^1 dilution's Ct value. These samples are POSITIVE for SVA RNA.

5.1.3 If the qRT-PCR assay is valid and the test sample's Ct value is > the pSVV2 10^1 dilution's Ct value or undetected, SVA RNA is NOT DETECTED for the sample.

5.1.4 If the qRT-PCR assay is valid, replicates for each tested sample should be in agreement:

a. Test sample duplicates should have no more than a 3.3 Ct difference. 3.3 Ct represents one log of amplification.

b. Test sample duplicates must both fall within the parameters described in either **Section 5.1.2** or **5.1.3**.

If not in agreement, the results are INCONCLUSIVE.

5.1.5 If the qRT-PCR assay is valid for a test sample that has a Ct value but no inflection point on its graph of fluorescence, the sample is INCONCLUSIVE.

5.1.6 Results that do not conform to **5.1.2** to **5.1.5** are considered non-conforming. Non-conforming test samples, as determined by qualified personnel upon interpretation of the graphs of fluorescence, are determined INCONCLUSIVE.

5.1.7 All INCONCLUSIVE samples must be repeated in duplicate.

NOTE: Gel analysis may be performed on the qRT-PCR products to verify results, as needed. The Fowler primers create a 78bp amplicon. Use VIRTWS1071 to record E-gel information and results for CVB recordkeeping (use of 4% E-gels are recommended for this amplicon size instead of 2%).

6. Report of Test Results

Results of testing will be reviewed and signed by agent contact or designee. For firm testing, results may be entered into LSRTIS or the current reporting system and released to the Reviewer for distribution to the firm.

7. References

7.1 Fowler, V. L., et al., "Development of a novel real-time RT-PCR assay to detect Seneca Valley virus-1 associated with emerging cases of vesicular disease in pigs." Journal of Virological Methods, 239 (2017): 34-37.

7.2 CFX96 Touch™, CFX96 Touch Deep Well™, CFX Connect™, and CFX384 Touch™ Real-time PCR Detection Systems Instruction Manual. Bio-Rad, Hercules, California.

7.3 QIAamp Viral RNA Mini Kit manual. Qiagen, Inc., Valencia, California.

7.4 Bustin, Stephen A. et al. "The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments." Clinical Chemistry 55 (4) (2009): 611-622.