(TRIPLEX) ONE-STEP REAL-TIME RT-PCR FOR THE DETECTION OF THE VIRAL RNA OF SENECAVIRUS A

1. PURPOSE AND SCOPE

This SOP describes the procedure for the detection of Senecavirus A (SVA) RNA using a (triplex) real-time RT-PCR. This procedure is applicable for RNA extracted from liquid or tissue samples from susceptible animals.
Infection with Senecavirus A is a differential diagnosis for infection with foot-and-mouth disease virus (FMDV). Therefore, it is essential that any sample suspected of vesicular disease is first and foremost tested for FMDV.
Further, any suspected sample must be collected taking into account all applicable legal and biosafety regulations. Suspected samples must be packed according to PI 650 of the IATA Dangerous Goods Regulations and send to the laboratory as “Biological substance, category B” (UN 3373). However, not or insufficiently applying these legal and biosafety regulations has no influence on the analysis results.

2. NORMATIVE REFERENCES

Bibliographic references:

1. *Fowler VL, Ransburgh RH, Poulsen EG, Wadsworth J, King DP, Mioulet V, Knowles NJ, Williamson S, Liu X, Anderson GA, Fang Y, Bai J*. Development of a novel real-time RT-PCR assay to detect Seneca Valley virus-1 associated with emerging cases of vesicular disease in pigs. *J Virol Methods 2017 Jan;239:34-37.* doi: 10.1016/j.jviromet.2016.10.012

2. *Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K.* Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *J Virol Methods. 2007 Mar;140(1-2):115-123*. doi: 10.1016/j.jviromet.2006.11.007

3. *Vandenbussche F, Vandemeulebroucke E, De Clercq K.* Simultaneous detection of bluetongue virus RNA, internal control GAPDH mRNA, and external control synthetic RNA by multiplex real-time PCR. *Methods Mol Biol. 2010;630:97-108.* doi: 10.1007/978-1-60761-629-0_7

4. *Vandemeulebroucke E, De Clercq K, Van der Stede Y, Vandenbussche F.* A proposed validation method for automated nucleic acid extraction and RT-qPCR analysis: an example using Bluetongue virus. *J Virol Methods. 2010 Apr;165(1):76-82.* doi: 10.1016/j.jviromet.2010.01.007

5. *Vandenbussche F, Lefebvre DJ, De Leeuw I, Van Borm S, De Clercq K.* Laboratory validation of two real-time RT-PCR methods with 5'-tailed primers for an enhanced detection of foot-and-mouth disease virus. *J Virol Methods. 2017 Aug;246:90-94.* doi: 10.1016/j.jviromet.2017.04.014

3. DEFINITIONS

rtRT-PCR = real-time reverse transcription polymerase chain reaction
IC = internal control
IC-ACTB = internal β-actin control
EC = external control

EC-EXTR = external control of extraction
NPC = negative PCR control
PPC = positive PCR control
NEC = negative extraction control
Pf = forward primer
Pr = reverse primer
Tp = target probe
UNDET = undetermined

Samples for quality control:
Negative PCR control (NPC)
The NPC is DEPC-treated water, tested simultaneously with the samples.

Positive PCR control (PPC)
The positive control is diluted and extracted virus tested simultaneously with the samples.
Alternatively, the positive control can consist of synthetic RNA tested simultaneously with the samples.
The performance of the PPC is monitored using Shewhart control charts.

Negative extraction control (NEC)
The NEC is DEPC-treated water, to which the EC was added before RNA extraction. The NEC is extracted and tested simultaneously with the samples. The EC consists of synthetic RNA.

4. PRINCIPLE OF THE METHOD

A triplex real-time RT-PCR is used to detect RNA from Senecavirus A (SVA), formerly called Seneca Valley virus-1 (SVV-1) in cells, fluids or tissues from susceptible animals, mainly pigs and wild boars. It consists of an SVA real-time RT-PCR with beta-actin (ACTB) as in internal control (IC) and synthetic RNA as an external control (EC) and uses primers specific to SVA, ACTB and EC. PCR products are detected in real-time with sequence-specific probes consisting of oligonucleotides that are labelled with a fluorescent reporter. During the PCR the probes are degraded by the polymerase and the reporters are released. Fluorescence is measured by the real-time PCR thermocycler.
Since the IC consists of endogenous RNA, a negative IC-ACTB result is indicative of poor sample quality. However, a suboptimal IC-ACTB result can also be due to improper RNA extraction and/or PCR inhibition. To rule out this possibility, a fixed amount of external extraction control (EC-EXTR) is added to each sample prior to extraction which specifically allows monitoring of the extraction and PCR efficiency.
The described rtRT-PCR can also be performed as a duplex for SVA and ACTB or as a singleplex for SVA only, although it is advised to always use an internal control. In these cases it is not necessary to supplement with extra Taq polymerase.

5. EQUIPMENT AND MATERIALS

- Latex/nitril examination gloves
- Class II microbiological safety cabinet (MSC) A for primer mix and PCR
- Class II microbiological safety cabinet (MSC) B for samples
- Cold pan
- Crushed ice
- Centrifuge for microtubes
- Centrifuge for PCR plates

- Vibration shaker
- Pipetting and micro-pipetting materials
- 10, 20, 200 and 1000 µl RNase- and DNase-free filter tips
- 0.2 ml, 1.5 ml and 2 ml RNase- and DNase-free microtubes
- Plastic consumables (microplates, microtubes, rods, etc.) compatible with the real-time thermocycler
- Sealing foil
- Real-time thermocycler (the present SOP was optimized on a Roche LightCycler 480 II)
- DNA remover or DNA ExitusPlus

6. REAGENTS AND PRODUCTS

6.1. One-step RT-PCR kit

RNA Ultrasense Enzyme mix (Invitrogen* One-Step Quantitative RT-PCR system) stored at < -16°C
RNA Ultrasense 5x reaction mix (Invitrogen* One Step Quantitative RT-PCR system) stored at < -16°C
Platinum Taq DNA polymerase (Invitrogen*) stored at < -16°C
*Invitrogen by Thermo Fisher Scientific
(It is also possible to use another RT-PCR kit)

Ultrapure, DEPC-treated, RNase-/DNase-free H_2O
Tris EDTA (TE) Buffer Solution pH 8.0

6.2. Primers and probes

The master stocks are at 100 µM, aliquoted and stored at < -16°C

Primer/probe	Sequence (5'- 3')	Reference
pf_SVA	5'-AGAATTTGGAAGCCATGCTCT-3'	1
pr_SVA	5'-GAGCCAACATAGARACAGATTGC-3'	1
tp_SVA	5'-FAM/TTCAAACCAGGAACACTACTCGAGA/BHQ1-3'	1
pf_IC-ACTB	5'-CAGCACAATGAAGATCAAGATCATC-3	2
pr_IC-ACTB	5'-CGGACTCATCGTACTCCTGCTT-3'	2
tp_IC-ACTB	5'-HEX/TCGCTGTCCACCTTCCAGCAGATGT/BHQ1-3'	2
pf_EC-EXTR	5'-GACGTTTGTAATGTCCGCTC-3'	3
pr_EC-EXTR	5'-CCAGTTGCTACCGATTTTACATA-3'	3
tp_EC-EXTR	5-'RED610/TTTGTACCACCTCCCACCGACCATC/BHQ2-3'	4*

*Probe sequence adapted from published sequence

The primer and probe working stocks are aliquoted and stored at < -16°C
When a new batch of primers or probes is purchased, the solutions from the new batches are compared with the old batches by undertaking rtRT-PCR for minimum 3 parallel assays.

Primer/probe	Working concentration	Dilution of stock solution (100 µM) to working solution
pf_SVA	10 µM	Add 9 volumes of TE buffer to 1 volume stock solution
pr_SVA	10 µM	Add 9 volumes of TE buffer to 1 volume stock solution
tp_SVA	5 µM	Add 19 volumes of TE buffer to 1 volume stock solution
pf_IC-ACTB	5 µM	Add 19 volumes of TE buffer to 1 volume stock solution

pr_IC-ACTB	5 µM	Add 19 volumes of TE buffer to 1 volume stock solution
tp_IC-ACTB	2 µM	Add 49 volumes of TE buffer to 1 volume stock solution
pf_EC-EXTR	10 µM	Add 9 volumes of TE buffer to 1 volume stock solution
pr_EC-EXTR	10 µM	Add 9 volumes of TE buffer to 1 volume stock solution
tp_EC-EXTR	2 µM	Add 49 volumes of TE buffer to 1 volume stock solution

7. PREPARATION OF SAMPLES

- Extract the viral RNA from liquid samples by silica membrane binding using the Nucleospin RNA virus kit from Macherey-Nagel or use an equivalent RNA extraction method.
Viral RNA from tissue samples is obtained by a mechanical homogenization/lysis step with a tissue lyser, or an equivalent method, and a guanidinium thiocyanate-phenol-chloroform procedure. The RNA present in the upper phase is recovered by silica membrane binding similarly to liquid samples. Viral RNA is eluted in 100 µl of DEPC-H2O.
- The EC-EXTR is added to the lysis buffer.
- Always include at least one sample of ultrapure water in the extraction as a negative extraction control (NEC).
- Always include a positive control (PPC).

Recommendations:
- Follow the usual recommendations to avoid contamination (aliquot the samples; aliquot the reagents; separate the workstations; use filter tips; wear gloves and replace them as needed).
- Work with sterile, single-use and RNase and DNase-free consumables.

- In case the lay-out of the building allows, the preparation of the positive amplification controls, the nucleic acid extraction, the preparation of primer and probe/enzyme mixes and the rtRT-PCR amplification are ideally performed in 4 separate rooms to avoid cross-contamination. Each room must have its gloves, pipettes, microtube holders etc. that should never be exchanged between rooms. Gloves must be worn during all manipulations and removed when leaving the room. Prior to each manipulation, the work zone of the laminar flow cabinets, the shafts of the pipettes, and the inside of the centrifuges must be cleaned with DNA remover. or DNA ExitusPlus. Microtubes/plates must be kept closed whenever possible and always centrifuged briefly before being opened.

8. PROCEDURE

8.1. Preparation of the primer mix and PCR mix

Prepare the primer mix depending on the number of samples in MSC A according to the table below. Include 3 controls (NEC, NPC, PPC) per plate.
Put 3.43 µl primer mix per well in a plate for denaturation.

Primer mix

Reagents	Working concentration [µM]	Final concentration [µM]	Volume/reaction [µl]
pf_SVA	10.0	1.0	2.6
pr_SVA	10.0	1.0	2.6
pf_IC-ACTB	5.0	0.1	0.53
pr_IC-ACTB	5.0	0.1	0.53

pf_EC-EXTR	10.0	0.15	0.39
pr_EC-EXTR	10.0	0.15	0.39

Prepare the PCR mix depending on the number of samples in MSC A according to the table below. Include 3 controls (NEC, NPC, PPC) per plate.
Take a LightCycler plate and add 10µl PCR mix per well.

PCR mix

Reagents	Working concentration [x, U/µl or µM]	Final concentration [x, U/µl or µM]	volume/reaction [µl]	volume/reaction x 1.10 [µl]
DEPC-water	/	/	0.95	1.05
RNA Ultrasense Reaction Mix	5	1	4	4.4
RNA Ultrasense Enzyme Mix	20	1	1	1.1
Platinum Taq DNA Polymerase	4	0.16	0.8	0.88
Probe SVA	5.0	0.375	1.5	1.65
Probe IC-ACTB	2.0	0.075	0.75	0.825
Probe EC-EXTR	2.0	0.1	1	1.1

In MSC B, add 8 µl of RNA sample to the primer mix in each respective well.
Cover the plate with cap strips and centrifuge for 1 minute at 1,000 rpm.
Place the plate in the LightCycler.

8.2. Denaturation and PCR

Denature for 3 minutes at 95°C followed by cooling for 2 minutes at 40°C
Then remove the plate from the LightCycler.
In MSC B, add 10 µl of the denatured RNA sample/primer mix to each well of the PCR plate containing the PCR mix. Cover the plate with LightCycler sealing foil, centrifuge 1 minute at 1,000 rpm and place the PCR plate in the LightCycler.

Select the following program:

Steps	Temperature	Time	Number
Reverse transcription (RT)	55°C	15 min	1x
RT inactivation and DNA polymerase activation	95°C	2 min	1x
Denaturation	95°C	10 sec	45x
Hybridisation, elongation and reading	60°C	30 sec	
Cooling	40°C	2 min	1x

9. EXPRESSION OF RESULTS

9.1. Reading

Fluorescence is read, during each cycle, at the end of the elongation step. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. This threshold is set manually or automatically (by the instrument's software).

9.2. Test validation

The test is validated if:
- The NEC has an undetermined (UNDET) Ct value for SVA and IC and the NPC has an UNDET Ct value for SVA, IC and EC.
- The Ct values for the PPC fulfil the validation criteria for SVA, IC and EC. It is advised to monitor the PPC using Shewhart control charts.

It is important to check the appearance of the amplification curve for each target.

The result for each sample is validated if:
- The Ct obtained for the sample obtained with the ACTB RT-PCR and the EC RT-PCR is less than 40 for each reaction.
If the Ct for ACTB and EC is equal to or greater than 40, or UNDET, dilute the total RNA in RNase- and DNase-free water (1:5 dilution) and repeat the SVA, ACTB and EC reactions using this dilution.
- If the Ct for ACTB and EC is again equal to or greater than 40, or again UNDET, repeat the extraction of viral RNA
- If the Ct for ACTB and EC is again equal to or greater than 40, or UNDET, the sample will be considered as unusable (presence of RT-PCR inhibitors; lysed or putrefied sample, etc.) as the result is “not interpretable”.

Note: For samples with low cell counts, e.g. serum or a viral suspension from infected cell culture, the Ct values for the ACTB can be greater than 40 or undetermined.

A negative result may be validated when the Cp value for IC and EC are less than 35. If this is not the case, there is a risk for a false negative result that may be due to an extraction or RT-qPCR problem. When the result is not interpretable, the test must be repeated starting from extraction. If the repetition gives again a result “not interpretable”, there is no clear result for this sample.

9.3. Expression of results

- The sample is considered negative when the Ct obtained with the SVD RT-PCR is UNDET. The result is reported as “Senecavirus A undetected”.
- The sample is considered positive when a Ct <40 is obtained with a characteristic amplification curve (sigmoidal). The result is reported as “detection of the Senecavirus A genome”.
- The result can be considered questionable if a Ct is obtained with a non-characteristic curve. In this case, the test should be repeated to define the sample's infection status or else compared with other tests undertaken with the sample.
- The result can be considered questionable if a Ct ≥40 is obtained. In this case, the test should be repeated to define the sample's infection status.
- When 2 questionable results are obtained, the final result is “not interpretable”. There is no clear result for this sample