4.A1.B.8. Recommended Standard Operating Procedure for The Viral Hemorrhagic Septicemia Virus Real-time RT-PCR

1. **Purpose/Scope**

   The purpose of this Standard Operating Procedure (SOP) is to describe the reagents and materials required for real time reverse transcription polymerase chain reaction (rRT-PCR) detection of Viral Hemorrhagic Septicemia Virus (VHSV) RNA from tissue samples or virus isolates. The assay was developed and optimized for the detection of all VHSV genotypes by the Technical University of Denmark (Jonstrup et al. 2013) and evaluated for purpose by the USDA-NVSL (Warg et al. 2014a; Warg et al. 2014b).

2. **Definitions**

   a. Cycle threshold (Ct) – The cycle number at which the fluorescence passes a determined threshold.

   b. Positive extraction control (PEC) – Successful performance of this control (meaning that the control Cycle threshold (Ct) value falls within a pre-defined range) indicates that the nucleic acid extraction process was performed properly. The PEC may be infectious virus, inactivated virus or an artificial construct.

   c. Negative extraction control (NEC) – Successful performance of this control (Ct value undetermined or zero) indicates that neither non-specific reactions nor contamination occurred during the extraction process. The PEC may be negative tissue homogenate, cell culture media, or nuclease-free water.

   d. Positive amplification control (PAC) – The PAC typically consists of different concentrations of the target nucleic acid. Successful performance of this control (Ct value falls within pre-defined ranges) set indicates that the PCR reaction was properly performed and all components of master mix are working properly. The PAC may be RNA previously extracted and evaluated from infectious virus, inactivated virus or an artificial RNA construct.

   e. No template control (NTC) – Successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR master mix setup and template addition phase of the protocol. Nuclease-free water serves as the NTC, as the NTC does not contain template.

3. **Safety Precautions**
    a. Personnel
        i. Personnel will have read, understood, and agreed to abide by the contents of this protocol.
        ii. Personnel performing the test must demonstrate proficiency prior to conducting the test and on an ongoing basis.
        iii. Personnel will have completed appropriate safety training such as use of biological safety cabinet (BSC) operation, autoclave safety, and be familiar with Safety Data Sheets (SDS) associated with chemicals and reagents required to perform this SOP.
        iv. Personnel will observe standard laboratory safety procedures, including using appropriate personal protective equipment (PPE): lab coats, safety glasses, and disposable gloves.
    b. Sample, Equipment, and Material Handling
        i. Personnel must also be aware of the calibration, maintenance, and proper use of instruments included in this protocol.
        ii. All equipment used is properly calibrated and maintained within the specified requirements and following individual laboratory policy.
        iii. Do not use components past their expiration date and do not mix components from kits with different lot numbers.
        iv. Personnel performing the protocol will be aware of the potential hazards associated with the procedures performed. Specific hazards include biological hazard, chemical hazard, and the ergonomic hazard associated with repetitive motions.
            1. All procedures conform to Biosafety level 2 guidelines. Handle all samples and reagents as if capable of transmitting disease.
            2. Throughout protocol, ensure use of proper disinfectants, such as 10% bleach.

4. **Equipment and Materials Required**
    a. Powder-free disposable gloves
    b. Lab coat
    c. Vortex mixer
    d. Biohazard waste disposal receptacles
    e. Ultra-low freezer set at ≤ -60°C
    f. Non frost-free (manual defrost) freezer set at -20°C ± 10°C
    g. Refrigerator set at 4°C ± 3°C
    h. Ice buckets
    i. qPCR detection system (such as ABI 7500 or BioRad CFX)
    j. Microcentrifuge tube rack

k. Pipettes: 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL, 8 or 12 channel pipettes
l. Aerosol filtered RNase & DNase free pipette tips: 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL
m. Repeat pipette (optional)
n. 500 µL repeat pipette tips (optional)
o. Sterile polypropylene conical tubes
p. Sterile polypropylene 1.5 mL tubes
q. Absorbent wipes
r. Autoclave bags
s. Appropriate 96-well reaction plates (such as ABI MicroAmp™ Optical 96-well Reaction Plate Cat No. 4316813 or USA Scientific Cat No. 1402-9800)
t. Appropriate optical adhesive film (such as ABI MicroAmp™ Optical Adhesive Film Cat No. 4360854 or BioRad Cat No. 1814030)
u. Plate sealer (optional)
v. Plate centrifuge
w. Ice
x. RNA-free water
y. VHSV forward primer: 5'-AAA-CTC-GCA-GGA-TGT-GTG-CGT-CC-3'
z. VHSV reverse primer: 5'-TCT-GCG-ATC-TCA-GTC-AGG-ATG-AA-3'
aa. Probe: 5'-(6-FAM)-TAG-AGG-GCC-TTG-GTG-ATC-TTC-TG-(BHQ-1)-3'
bb. Qiagen QuantiTect Probe RT-PCR Kit (Cat No. 204443)
cc. Extracted RNA and appropriate controls (PEC, NEC, NTC, PAC)
dd. TE buffer 1X (Tris EDTA pH 8.0)*

*Primers and probe should be ordered from Invitrogen, Integrated DNA Technologies Inc. (IDT) or equivalent. They are reconstituted with TE buffer, and diluted to working concentrations with water. Primers and probe are aliquoted into smaller volumes to minimize freeze-thaw cycles and minimize the probe exposure to light.

5. Specimens

a. Specimen RNA should be extracted using the MagMAX™ Viral RNA Isolation Kit (ThermoFisher Cat No. AM1836) following the manufacturer's instructions. Appropriate specimens include:
    i. RNA extracted directly from fresh (or frozen ≤ -60°C and thawed immediately prior to extraction) kidney, spleen, heart, or encephalon tissue.
    ii. RNA extracted from heart, kidney, encephalon and spleen preserved in RNAlater®.
    iii. RNA extracted from cell cultures or supernatants from cell cultures suspected as being infected with VHSV.

6. Procedure

For this procedure, it is critical to have separate preparation areas and equipment for nucleic acid extraction, RNA transfer, amplified nucleic acids, and "clean" procedures such as master mix preparation. Never introduce amplified cDNA or sample RNA into the clean area. Designate a single biological safety cabinet (BSC) or PCR workstation for this "clean" work only. Similarly, designate a set of calibrated pipettes, tips, RNase-free water, tubes, ice buckets, and racks for "clean" use only to be used only for the preparation of clean reagents and to never leave the area. In addition, designate a -20°C non frost-free (manual defrost) freezer for storage of "clean" reagents. The extraction procedure requires the dedication of a separate BSC, set of calibrated pipettes, equipment and reagents. Use and assign a third BSC or PCR workstation and calibrated pipettes for transfer of RNA to amplification tubes or plates. Powder-free latex/nitrile or equivalent gloves must be worn throughout the procedure and must be changed frequently. Always wear fresh gloves when working with "clean" reagents. RNA is very labile and easily degraded by RNases. Supplies such as pipette tips and micro-centrifuge tubes as well as all reagents should be certified RNase-free. Always change gloves after working with sample RNA or amplified DNA. Wear protective eyewear, gloves, and lab coats as some reagents used are toxic.

a. Fill out plate map (for example see Appendix 1, VHSV rRT-PCR worksheet) and determine the total number of reactions that you will need to prepare master mix for. Each plate must have a NTC, PAC set, NEC, and a PEC. Include 1 extra reaction for every 15 reactions when calculating reagent volumes to account for reagent loss associated with pipetting. Fill out the worksheet for reagent volumes and lot numbers (Appendix 1).
b. Thaw 2X QuantiTect Probe RT-PCR Master Mix (if stored at -20°C), primer and probe solutions, and RNase-free water. Mix the individual solutions and place them on ice. QuantiTect RT Mix (enzyme mix) should be taken from -20°C immediately before use, always kept on ice, and returned to storage at -20°C immediately after use.
c. Determine the number of reactions (at minimum in duplicate) and prepare reaction mix according to Table.

Reagent	**Stock concentration**	**Volume per reaction (µL)**
Water	NA	2.125
2X master mix	2X	12.500
Forward primer	10 µM	2.250
Reverse primer	10 µM	2.250
Probe	10 µM	0.625
Enzyme mix	10X	0.250
Total volume		20.000

d. Mix the reaction components thoroughly and dispense 20 µL into 96-well reaction plate.
e. In the area dedicated for RNA transfer, add 5 µL template RNA, controls, or nuclease-free water to the appropriate individual PCR wells containing the reaction mix. Load samples first, add control templates last. Cover the plate with plate sealing film ensuring a good seal on the plate before moving the plate outside of the BSC or PCR workstation.
f. Briefly centrifuge the plate, ensuring the reaction mix is pulled down to the bottom of the wells and air bubbles are eliminated.
g. Set up and run the VHSV real-time PCR program with the following parameters:
    i. Run mode: Standard 7500 (if applicable)
    ii. Reaction volume: 25 µL
    iii. Reporter dye/ quencher: FAM™/ BHQ-1 (select appropriate channel)
    iv. Real time program outline below:

Step #, # of cycles: purpose	**Temperature**	**Time**
Step 1, 1 cycle: RT step	50°C	30 min
Step 2, 1 cycle: activation & denaturation	95°C	15 min
Step 3, 40 cycles:		
denaturation	94°C	15 sec
annealing and amplification*	60°C	40 sec
polish	72°C	20 sec

*collection of fluorescence

h. Place the PCR plate in the real-time cycler, and start the cycling program.
i. Once the program is completed, use appropriate methods to export, save and archive all data.

7. Data analysis

a. Refer to the user guide for your real-time PCR instrument for instructions on how to analyze the data, using the recommendations below.
b. Use the Auto Ct setting. The Auto setting minimizes subjectivity when setting the threshold. Review baseline and threshold settings.
c. If the baseline was set too low (high background), use the auto threshold and manual baseline settings with a default of 3 to 15 cycle range.

8. Acceptance Criteria

a. Verify that all cycles were completed and the run is valid before analyzing the test sample results.
b. All PCR and extraction controls must fall within the acceptable positive or negative Ct range in order for the test to be considered valid.

i. The NEC and NTC have an undetermined value.
ii. The PEC is established by the testing lab. Laboratories aim for an RNA sample with a Ct range of 25-30.
iii. A PAC should contain several RNA samples representing a low, mid and high range of RNA concentrations across the linear range of the assay's performance.

c. If the NEC yields a Ct value, the extraction and run are not valid and should be repeated. Evaluate the reagents, equipment and procedure for introduction of contaminating nucleic acid.
d. If the PEC yields an undetermined Ct value, all samples tested on the plate or run must be re-extracted and these RNA samples must be re-tested for a valid test result.

9. Interpretation of Results

a. Once the real time program is completed, analyze the results with auto baseline and auto Ct features. The threshold should fall in the exponential phase of the amplification plot.
b. Check the component plot for samples and controls.
c. If a sample has no Ct value, the sample is reported as **NEGATIVE** or not detected.
d. If a sample has a Ct value less than 37 with a sigmoidal curve, the test result is reported as **POSITIVE**. Presumptive positive samples should then be confirmed using a second detection methods
e. If a sample has a Ct value 37-40 with a sigmoidal curve, the sample is **SUSPECT**. In this case, the original sample must be re-extracted and both RNA samples must be tested (in at least duplicate reactions) on the same run.
    i. If a sample had an initial result with a Ct value 37-40 with a sigmoidal curve and the second test yields a Ct value and a sigmoidal curve, the sample is considered **POSITIVE**. Presumptive positives should then be confirmed using a second detection methods
    ii. If a sample had an initial result with a Ct value 37-40 with a sigmoidal curve and the second test yields no Ct value, the sample is **SUSPECT**. Suspected positives should then be analyzed using an alternative detection method.
f. If a sample has a non-sigmoidal curve that crosses the threshold, check the component values. If the FAM component has a straight line, with no upward curves the sample is reported as **NEGATIVE**.
g. Any samples that test **POSITIVE** or **SUSPECT** by the rRT-PCR (qPCR) assay initiates follow-up confirmatory testing as appropriate (conventional RT-PCR with sequence analysis of PCR amplicon or viral isolation; see chapter 2.2.7. in Section 1). If follow up confirmation testing fails to support a positive or suspect rRT-PCR result, the sample is reported as **SUSPECT**.

10. Associated Documentation

a. Jonstrup SP, Kahns S, et al. (2013) Development and validation of a novel Taqman based real-time RT-PCR assay suitable for demonstrating freedom from viral haemorrhagic septicaemia virus. Journal of Fish Diseases 36: 9−23.
b. Warg, J.V., T. Clement, et al. (2014a). Detection and surveillance of viral hemorrhagic septicemia virus using real-time RT-PCR. I. Initial comparison of four protocols. Diseases of Aquatic Organisms 111(1): 1-13.
c. Warg, J.V., T. Clement, et al. (2014b). Detection and surveillance of viral hemorrhagic septicemia virus using real-time RT-PCR. II. Diagnostic evaluation of two protocols. Diseases of Aquatic Organisms 111(1): 15-22.
d. Product insert for Qiagen QuantiTect Probe RT-PCR kit (Cat No. 204443).
e. VHSV rRT-PCR Worksheet (Appendix 1).

11. Revision History

New document June 2020

12. Appendices

a. Appendix 1 – VHSV rRT-PCR Worksheet

VHSV rRT-PCR Worksheet

Case Number_____ **Date_____**

Master Mix

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 25 µL)	Volume for ____samples
d-H_2O*		-	-	2.125 µL	
2X QuantiTect Probe RT-PCR Master Mix		1X	2X	12.50 µL	
Forward Primer		900 nM	10 µM	2.250 µL	
Reverse Primer		900 nM	10 µM	2.250 µL	
Probe		250 nM	10 µM	0.625 µL	
QuantiTect RT mix		1X	10X	0.250 µL	
RNA template		-	-	5 µL	-

*Add nuclease free water to Master Mix first, Quantitect RT mix last.

Primer/ Probe

Forward Primer	Reverse Primer
5'-AAA-CTC-GCA-GGA-TGT-GTG-CGT-CC-3'	5'-TCT-GCG-ATC-TCA-GTC-AGG-ATG-AA-3'
Probe	
5'-(6-FAM)-TAG-AGG-GCC-TTG-GTG-ATC-TTC-TG-(BHQ-1)-3'	

Control Information

POSITIVE CONTROLS		NEGATIVE CONTROLS	
PEC	PAC	NEC	NTC

Amplification (Thermocycle Process)

Date & Time	Program #	NOTES

Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

This chapter was prepared for the Blue Book in 2020 by Isaac Standish, Janet V. Warg, Nicholas B. D. Phelps, Jan Lovy, Rodman G. Getchell, Gavin Glenney, Mohamed Faisal