rRT-PCR FOR AFRICAN HORSE SICKNESS DETECTION METHOD (Agüero *et al.* 2008)		
Date: 22/06/2021	Document code: GL-LCV-07	*Rev. 02*

1. PURPOSE

The purpose of this method is to rapidly detect the specific presence of African horse sickness virus RNA by a real time reverse transcription polymerase chain reaction assay (rRT-PCR) including the β-actin internal control.

Currently this method is one of those included in the OIE Chapter 2.5.1 of the Manual of Diagnostic Test and Vaccines for the Terrestrial Animals 2019 as a recommended nucleic acid detection test to detect African horse sickness virus (AHSV).

The method described here has been fully validated according to OIE requirements and endorsed by the OIE Biological Standard Commission in September 2015.

2. SCOPE

The method is applied to specifically detect AHSV RNA in equine samples, such as EDTA-blood or tissue homogenates, and in inoculated cell culture.

3. MATERIALS AND EQUPMENTS

Material

Disposable hand gloves

Microcentrifuge tubes (1,5 - 2 ml), PCR clean, RNase free

Centrifuge tubes (12 ml)

96-well PCR plate

Pippete filter tips, sterile (10µl, 100-200µl and 1000µl)

PCR water

Optical sealing film for real time PCR

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Reagents (see description in point 4. Reagents)

rRT-PCR kit

Primers

Probe

Equipment

Adjustable micropipettes (full range)

Laminar flow cabinet

Ice or Labtop Cooler

Real time Thermal cycler

Refrigerator (-20ºC)

Micro-centrifuge (up to 15000 rpm)

Vortex mixer

4. REAGENTS

Amplification kit

AGPATH-ID ONE-STEP RT-PCR KIT (for fast rRT-PCR)	
Components:	RT-PCR Buffer
	RT-PCR Enzyme Mix
	Nuclease-free water

RRT-PCR AgPath-ID™ One-Step RT-PCR (Thermo Fisher Scientific) Kit manual.

Primers and Probe:

Agüero et al; 2008 (AHSV detection)

Primer Forward:	AHS-F1	5´-CCA GTA GGC CAG ATC AAC AG- 3´
Primer Reverse:	AHS-R1	5´-CTA ATG AAA GCG GTG ACC GT- 3´
Probe:	AHS-PR1	5´-FAM-GCT AGC AGC CTA CCA CTA MGB-3´

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Toussaint JF et al.; 2007 (β-actin detection)

Primer Forward:	ACT-1005-F	5´-CAG CAC AAT GAA GAT CAA GAT CAT C-3´
Primer Reverse:	ACT-1135-R	5´-CGG ACT CAT CGT ACT CCT GCT T3´
Probe:	ACT-1081-HEX*	5´-HEX-TCG CTG TCC AC CTT CCA GCA GAT GT–BHQ1-3

*Same sequence described in the publication, but fluorescent reporter and quencher of fluorescence were modified

Controls

- Extraction controls.

  -*Extraction Positive Control* (EPC) should contain the target virus

  -*Extraction Negative Control* (ENC): Water or buffer is normally used

- Amplification controls.

  -*Amplification Positive Control (APC):* PCR mix prepared with the same volume and concentration of reagents as the PCR mix being used to test specimens, but with the addition of target nucleic acid

  -*Amplification Negative Control (ANC):* PCR mix being used to test specimens, but with the addition of a volume of sterile nuclease free water or sterile nuclease free buffer instead of extracted template nucleic acid.

5. METHOD

General Aspects

The assay amplifies a RNA fragment of 83 base pairs (bp) of the AHSV VP7 genome region.

The PCR is carried out in a final volume of 20 µl.

RNA sample is added to each reaction tube containing the primers mix and heat denaturized.

One step RT-PCR master mix including Taqman probe is added to reaction tubes after denaturation.

Protocol

- Commercial kits to extract viral nucleic acid are widely available. RNA extraction from blood and tissue samples can be performed following manufacturer´s instructions.

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- Several one step real-time RT-PCR kits are commercially available, which can be used depending upon local/case-specific requirements and equipment available.
- Thaw all reagents and store in ice, except the enzyme mix.
- Maintain an RNAse and DNAse free work environment.
- Mix all individual reagents thoroughly and spin down.
- A test plate layout should be designed and loaded into the real time PCR machine software. Using the layout as a guide 2.5 µl of each primer working stock 8 µM (final concentration 1 µM) is added to each well that will contain RNA samples, positive (EPC, APC) and/or negative (ENC, ANC) controls. The plate is held on ice.

  Note: PCR plates can be replaced with tubes or strips as appropriate.

Reagent	µl/sample	Working concentration	Final reaction mix concentration
Primer AHS-F1	2,5	8 µM	1 µM
Primer AHS-R1	2,5	8 µM	1 µM
Sample (RNA)	2		
Total Volume	**7**		

- Heat denaturation at 95°C for 5 minutes, followed by rapid cooling on ice for further 5 minutes.
- An appropriate volume of real time one-step complete RT-PCR mix for the number of samples to be tested is prepared following manufacturer´s instructions.
- AHS probe should be included in a final concentration of 0,25 µM (0,1µl of probe working stock 50 µM per sample).
- β-actin primers/probe should be included in a final concentration of 0,05 µM (0,1µl of probe working stock 10 µM per sample).
- 13 µl of complete RT-PCR mix is distributed in each well on the PCR plate containing the denatured primers and RNA.

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Reagent (AgPATH-ID One Step RT-PCR kit)	µl/sample	Working concentration	Final reaction mix concentration
RT-PCR Buffer	10	2x	
RT-PCR Enzyme	0,8	25x	
AHS-PR1	0,1	50 µM	0,25 µM
ACT-1005-F	0,1	10 µM	0,05 µM
ACT-1135-R	0,1	10 µM	0,05 µM
ACT -1081-HEX	0,1	10 µM	0,05 µM
PCR water	1,8		
Denatured mix (Sample RNA + primers)	7		20
Total Volume	**20**		

The plate is placed in a real time thermal cycler programmed with the following profile:

Step	Cycle step	Temperature	Time	Nº cycles
Reverse transcription		48°C	10 min	1x
"Hot start"		95°C	10 min	1x
PCR cycle	DNA denaturation Primer annealing/extension	97°C 55°C*	2 sec 30 sec	40x

* Fluorescence data are acquired at the end of the 55°C step.

6. ASSAY VALIDATION

Test validation

The PCR assay will be valid if after completion of thermocycling program:

- All positive controls (EPC, APC) should produce amplification curves. Positive control Ct values should be in the range of the previously assigned Ct value. If not, the assay should be repeated.
- All negative controls (ENC, ANC) should not produce any amplification curve and the finding of PCR amplification curves in any negative control means that the assay should be repeated. If only ANC showed amplification, the new PCR test could be performed using the same extracted nucleic acid, otherwise the sample should be retested from the nucleic acid extraction.

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- Inhibition Control (IC) should produce amplification curves in the range of the previously assigned Ct value. For each single well, if IC result is negative but the AHS sample result is positive, the sample result will be accepted. In case of the IC result is negative and AHS sample result is also negative, the sample should be re tested and if inhibition is newly observed a new sample should be requested.

Sample results interpretation

- The sample result is **POSITIVE** when a typical amplification curve is obtained and the Ct value (cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold) is lower or equal to the defined Ct threshold (35) within 40 PCR cycles (Ct≤35).
- The sample result is **INCONCLUSIVE** when a typical amplification curve is obtained and the Ct value is higher to the defined Ct threshold (35) within 40 PCR cycles (Ct>35).
- The sample result is **NEGATIVE** when a horizontal amplification curve is obtained and does not cross the threshold line within 40 PCR cycles (No Ct).

If atypical amplification curves are obtained result will not be valid and the assay must be repeated.

7. REFERENCES

Agüero M., Gómez-Tejedor C., Cubillo MA., Rubio C., Romero E. & Jiménez-Clavero A. (2008). Real time fluorogenic reverse transcription polymerase chain reaction assay for detection of African horse sickness virus. J. Vet. Diagn. Invest., 20, 325–328.

Toussaint J.F., C. Sailleau, E. Breard , S. Zientara, K. De Clercq. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. Journal of Virological Methods 140 (2007) 115–123

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