rRT-PCR ASSAY MODIFICATION FOR AFRICAN HORSE SICKNESS VIRUS DIAGNOSIS (Morales *et al.*, 2025)

Date: 11/03/2026	Document code: GL-LCV-18	*Rev. 02*

1. PURPOSE

The purpose of this method is the detection of African horse sickness virus (AHSV) by real-time reverse transcription polymerase chain reaction (rRT-PCR) assay, including the South African variants previously undetected by the Agüero 2008 method, with the β-actin as internal control.

2. SCOPE

The method is applied to specifically detect AHSV RNA in clinical samples from equines, specifically EDTA blood or tissue homogenates, as well as 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

Optic sealing film for real time PCR

Reagents (see description in point 4. Reagents)

rRT-PCR Kit

Primers

Probes

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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

NOTE: several one step real-time RT-PCR kits are commercially available, which can be used depending upon local/case-specific requirements and equipment available.

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Probe (P) and Primers Forward (F) / Reverse (R) for AHSV detection, (Morales *et al.,* 2025).

	Oligo name	Oligo sequence (5´- 3´)	Target
Probe	AHS-P-A2024	5´-FAM-GCT AGC RGC YTA CCA CTA-MGB-3´	**AHSV segment 7**
Primer forward	AHS-F1-A2008	5´-CCA GTA GGC CAG ATC AAC AG-3´	
Primer reverse	AHS-R-A2024	5´-CTA ATG AAA GCG GYG ACC GT-3´	

Probe (P) and Primers Forward (F) / Reverse (R) for β-actin detection, (Toussaint JF *et al*., 2007)

	Oligo name	Oligo sequence (5´- 3´)
Probe	ACT-1081-HEX*	5´-HEX-TCG CTG TCC AC CTT CCA GCA GAT GT–BHQ1-3´
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 T-3´

*Same sequence described in the publication, but fluorescent reporter and quencher of fluorescence were modified and the probe was termed ACT-1081-HEX.

Controls

- Extraction controls.

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

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

- Amplification controls.

  -*Amplification Positive Controls (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.

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5. METHOD

General Aspects

- The assay generates an amplicon of 83 bp of the AHSV segment-7 genome region.
- AHS probe is labelled at the 5´ end with 6-FAM reporter and at the 3´end with the MGB quencher.
- Each PCR is carried out in a final volume of 20 µl.
- RNA sample is added to each reaction tube containing the AHS primers and heat denatured.
- One step RT-PCR master mix including the AHS probe as well as primers/probe for internal control is added to reaction tubes after denaturation.
- Commercial kits to extract viral nucleic acid are widely available. RNA extraction from blood and tissue samples can be performed following manufacturer´s instructions.

Protocol

a. Thaw all reagents and store on ice.
b. Maintain an RNAse and DNAse free work environment.
c. After each reagent mixture, shake thoroughly and spin down.
d. 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 2,86 µM) is added to each well. The plate is held on ice.
e. Add 2 µl of RNA sample, positive and/or negative controls of extraction (EPC, ENC) or amplification (AMP, ANC) to each reaction well containing the primers mix.

Table 1. Primers and sample. Volumes and concentrations

Reagent*	µl/well	Working concentration	Final reaction mix concentration
Primer Forward AHS-1F-A2008	2,5	8 µM	1 µM
Primer Reverse AHS-R-2024	2,5	8 µM	1 µM
Sample (RNA)	2		
Total Volume	**7**		

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

f. Heat denaturation at 95°C for 5 minutes, followed by rapid cooling on ice for further 5 minutes.

g. 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. β-actin primers/probe should be included in a final concentration of 0,05 µM

Table 2. Complete RT-PCR mix. Volumes and concentrations

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**		

h. 13 µl of complete RT-PCR mix are distributed in each well on the PCR plate containing denatured primer mix + sample RNA (7 µl). Total volume is 20 µl and final AHS primers concentration is 1 µM.

i. The plate is placed in a real time thermal cycler with the following programme:

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	95°C	15 sec	40x

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Primer annealing/extension	55°C*	30 sec

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

6. ASSAY VALIDATION

Test validation

To have a valid assay after completion of thermocycling program:

- Positive controls (EPC, APC) should produce amplification curves. Positive controls Ct values should be in the range of the previously assigned Ct value ±2. If not, the assay should be repeated.
- 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, a new PCR test could be performed using the same extracted nucleic acid, otherwise the sample should be retested from the nucleic acid extraction.
- Inhibition Control (IC) should produce amplification curves in the range of the previously assigned Ct value. For each single well, if the IC result is negative but the AHS sample result is positive, the sample result will be accepted. If the IC result is negative and the AHS sample result is also negative, the sample should be retested and if inhibition is observed again, 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 the sample result is not valid and the assay must be repeated.

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