rRT-PCR ASSAYs FOR TYPING AFRICAN HORSE SICKNESS VIRUS (Villalba, 2024)

Date: 13/04/2026	Document code: GL-LCV-16	*Rev. 02*

DOCUMENT CHANGE HISTORY

Revision	Date	
01	December 2024	Not applicable
02	April 2026	Pag. 3. Correction in the sequences of some primers and probes (AHS-2F, AHS-9P, AHS-9R, AHS-3F, AHS-3R, AHS-7P, AHS-7F and AHS-7R)

1. PURPOSE

The purpose of this method is to rapidly provide African horse sickness virus (AHSV) serotype identification by three triplex real time reverse transcription polymerase chain reaction (rRT-PCR) assays targeted to the segment-2 (VP2) of the virus genome.

2. SCOPE

The methods are applied to specifically detect AHSV RNA in clinical samples from equines, specifically EDTA blood or tissue homogenates, as well as inoculated cell culture. These methods should be applied on samples or viral suspension previously confirmed as positive to AHSV genome by a rRT-PCR serogroup specific (first step of the AHS diagnosis).

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

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Optic sealing film for real time PCR

Reagents (see description in point 4. Reagents)

rRT-PCR Kit

Primers

Probes

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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Primers Forward (F) / Reverse (R) and Probes (P) (Duran et al. 2022; Villalba et al; 2024)

Triplex 1, 6, 8:

AHSV serotype	Oligo name	Oligo sequence (5´- 3´)
AHSV-1	AHS-1P	FAM-AAT GTC TTA GAT CGT CAA CT-MGB
	AHS-1F	GCA AGC GCT GGC ACT TG
	AHS-1R	TTC GAA CTC ATT CCT TAC ATC AAC A
AHSV-6	AHS-6P	VIC-GTC ATC ACC GTA AGC G-MGB
	AHS-6F	AGC CAG GGC TTC TTT GCA
	AHS-6R	CTC ATG TTC AAC CCA CTG TAC ATT AA
AHSV-8	AHS-8P	NED-ACG TGA TTC TTT TCC C-MGB
	AHS-8F	GAA ATT ATC AGC GGA CTG ACT AAG AA
	AHS-8R	AAA CAT CTA CCT TTT GCG AAT CTT G

Triplex 2, 4, 9:

AHSV serotype	Oligo name	Oligo sequence (5´- 3´)
AHSV-2	AHS-2P	Cy5-TGA AGG TGC TTA CCC GAT CTT TCC ACA-BBQ
	AHS-2F	CGG AAA CYA TGT ATT GCC AA
	AHS-2R	TTG TCR TCC TGA TCA ACC CTA A
AHSV-4	AHS-4P	VIC-TAT CGG RAT TTA TGT ACA ATG AG-MGB
	AHS-4F	TGA GGT GGA ACA CGA YAT GTC
	AHS-4R	GAT ATG CCC CCT CAC AYC TGA
AHSV-9	AHS-9P	FAM-TTC ACA TTT CGT TTG TTT-MGB
	AHS-9F	TAC TGT GTC GGT GAG GGA TTT T
	AHS-9R	GGC ACG ACC GGA TAT GA

Triplex 3, 5, 7:

AHSV serotype	Oligo name	Oligo sequence (5´- 3´)
AHSV-3	AHS-3P	FAM-AGA GTT GAG GTT GCG GGA-MGB
	AHS-3F	AAT TAT TAC AGC GGA RAA TGC AGT T
	AHS-3R	TCG CAC CCC ACT CAT AAC C
AHSV-5	AHS-5P	NED -TGT TGA RAT GCT GAG GC-MGB
	AHS-5F	GAA GAG ACA GGC GAT TCA AAT GA
	AHS-5R	AAA GCC ACC CTT TTT GGT ACA AA
AHSV-7	AHS-7P	VIC-CGA TAT CTG AGT TCA TGT ATG AA-MGB
	AHS-7F	GAG TGG GGC GCA ACA AAT C
	AHS-7R	CCG GAT ATG CAC CCT CAC AT

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Controls

- Extraction controls.

  -*Extraction Positive Controls* (EPC) should contain the target viruses (one for each serotype)

  -*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 (one for each serotype)

  -*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 nine single RT-PCRs have been optimized in three triplex assays by combining the following serotypes: Serotypes 2, 4 and 9 // Serotypes 3, 5 and 7 // Serotypes 1, 6 and 8
- The assays amplify RNA fragments of 62 to 133 bp of the AHSV segment-2 genome region depending on the serotype.
- Probes are labelled at their 5´ extreme with 6-FAM, VIC, NED or Cy5 reporter dyes; and at their 3´extreme with Black Hole Quencher (BHQ) MGB or BBQ
- Each triplex 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 denatured.
- One step RT-PCR master mix including the probes 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.

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Protocol (For each triplex rRT-PCR assay should be used the corresponding primers and probes and positive controls):

a. Thaw all reagents and store on ice.

b. Maintain an RNAse and DNAse free work environment.

c. After each reagent mixture al reagents, 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, 0.25 µl of each primer working stock 20 µM (final concentration 0.25 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 (forward and reverse for each triplex reaction, total 6 primers).

Table 1. Primers and sample. Volumes and concentrations

Reagent*	µl/well	Working concentration	Final reaction mix concentration
Primer Forward-A	0.25	20 µM	0.25 µM
Primer Reverse-A	0.25	20 µM	0.25 µM
Primer Forward-B	0.25	20 µM	0.25 µM
Primer Reverse-B	0.25	20 µM	0.25 µM
Primer Reverse-C	0.25	20 µM	0.25 µM
Primer Reverse-C	0.25	20 µM	0.25 µM
RNase free water	3.5		
Sample (RNA)	2		
Total Volume	**7**		

*A, B or C stands for the corresponding serotype included in the triplex RT-PCR assay (1, 6, 8 or 2, 4, 9 or 3, 5, 7)

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

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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. Probes should be included in a final concentration of 0.0625µM

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

Reagent* (AgPATH-ID One StepRT-PCR kit)	µl/well	Working concentration	Final reaction mix concentration
RT-PCR Buffer	10	2x	
RT-PCR Enzyme	0.8	25x	
Probe-A	0.025	50 µM	0.0625 µM
Probe-B	0.025	50 µM	0.0625 µM
Probe-C	0.025	50 µM	0.0625 µM
RNase free water	2.125		
Total Volume	**13**		

*A, B or C stands for the corresponding serotype included in the triplex RT-PCR assay (1, 6, 8 or 2, 4, 9 or 3, 5, 7)

h. 13 µl of complete RT-PCR mix (including probes) is distributed in each well on the PCR plate containing denatured primer mix + sample RNA (7 µl). Total volume 20 µl

i. 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	95°C	15 sec	40x
	Primer annealing/extension	55°C*	30 sec	

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

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6. ASSAY VALIDATION

Test validation

The PCR assays will be valid if after completion of thermocycling program. Each RT-PCR included in the triplex assay should be individually evaluated:

- 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, the new PCR test could be performed using the same extracted nucleic acid, otherwise the sample should be retested from the nucleic acid extraction.

Sample results interpretation (for each serotype included in the triplex assay):

- 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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7. REFERENCES

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