Real-time RT-PCR to detect the genome of Equine Arteritis Virus

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This SOP is an OIE-based method used at the EURL, all OIE-RT-PCR based methods validated and used successfully in the PT can be used for this essay.

This SOP describes a quantitative reverse transcription (RT) polymerase chain reaction (PCR) assay to detect the presence of EAV in equine semen.

1 SAMPLE COLLECTION

This SOP can be used to detect the presence or absence of the EAV genome in **equine semen**.

The semen sample must be part of a full collection of sperm rich, do not use pre-ejaculation samples since they are often negatives for EAV and are not considered valid specimens.

Semen should be delivered on ice as soon as possible after collection (no longer than 48h) and transported to the laboratory under refrigerated conditions. An insulated styrofoam shipping container with frozen ice packs usually suffices for overnight delivery.

2 TECHNIQUE

The table below presents the different stages of its analytical process:

Analytical process	Protocol
Preparation of sample	Centrifuge the semen at 1900 rpm for 10 minutes at 4°C
RNA extraction	RNA extraction is performed from 150 µl of sample to analyze (Following the RNA extraction kit instructions)
Preparation of reagents and mix PCR	Prepare the RT-qPCR mix as described in chapter 2.2.1
Amplification	Add 22.5 µl of RT-QPCR mix 1 or mix 2 into two different tubs Add 2.5 µl of extracted RNA in each mix Run amplification as described in chapter 2.2.2
Analysis of results	Analysis of the amplification signals Express the results as: Detected Not detected Not conclusive

2.1 RNA EXTRACTION KIT :

Used kit: QIAamp RNA viral kit, QIAGEN, reference 52906

This kit is mentioned as it is used by the EURL for equine diseases other than African horse sickness for African horses. Any other RNA extraction kit can be used as long as it has been previously validated by the user laboratory.

2.2 AMPLIFICATION KIT :

Used kit: Quantitect virus, QIAGEN, reference 211035

This kit is mentioned as it is used by the EURL for equine diseases other than African horse sickness for African horses. Any other RT-qPCR kit can be used as long as it has been previously validated by the user laboratory.

2.2.1 RT-qPCR Mix preparation:

This preparation mix is done when the used kit is the Quantitect virus from Qiagen :

	Volume per reaction
H_2O	14.75 µl
Quantitect virus master mix 5 X	5 µl
Forward primer 10µM	1 µl
Reverse primer 10µM	1 µl
Probe 10µM	0.5 µl
Quantitect virus RT mix	0.25µl
RNA	2.5µl

Two different RT-qPCR mixes are done:

2.2.1.1 Mix 1:

Mix 1 is prepared to detect EAV genome using primers and probe by amplifying a portion of EAV ORF7 sequence:

Primer/Probe	Sequence	Concentration
EAV_ORF7_F ORF7 forward primer	GGC GAC AGC CTA CAA GCT ACA	10 µM
EAV_ORF7_R ORF7 reverse primer	CGG CAT CTG CAG TGA GTG A	10 µM
EAV_ORF7_Pr ORF7 Probe	[6FAM]-TTG CGG ACC CGC ATC TGA CCA A-[TAM]	10 µM

Primers and probe are described by Balasuriya et al, 2002 (*Detection of equine arteritis virus by real-time TaqMan reverse transcription-PCR assay. Balasuriya UB, Leutenegger CM, Topol JB, McCollum WH, Timoney PJ, MacLachlan NJ. J Virol Methods. 2002 Mar;101(1-2):21-8*)

2.2.1.2 Mix 2:

Mix 2 is prepared to amplify a sequence portion of β–Actine using primers and probe described in the chart above to ensure the absence of PCR inhibitors in the extracted RNA obtained from the sample to analyze:

Primer/Probe	Sequence	Concentration
βACT_F β–Actine forward primer	CAGCACAATGAAGATCAAGATCATC	10 µM
βACT_R β–Actine reverse primer	CGGACTCATCGTACTCCTGCTT	10 µM
βACT_Pr β–Actine probe	[6FAM]TCGCTGTCCACCTTCCAGCAGATGT[TAM]	10 µM

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2.2.2 RT-qPCR program:

Temperature	Time	
50°C :	20 min.	
95°C :	5 min.	
95°C :	15 sec.	45 cycles
60°C :	45 sec.	

Measure fluorescence at the end of each cycle.

3 PREPARATION OF CONTROLS:

3.1 POSITIVE CONTROL SEMEN:

In the absence of a characterized reference material, a positive control sample may be produced to be used as a positive control of the process. The production below is described as an example:

- Mix an EAV negative equine semen and an EAV positive supernatant issued from virus isolation on cell culture test. The dilution of the EAV supernatant in the negative semen is depending on the amount of EAV particles and should be done to get a Ct value ranged from 25 to 35.
- Once diluted, aliquote the positive control at least 150µl per tubs.
- Freeze the positive control aliquots at ≤ - 16°C.

3.2 NEGATIVE CONTROL SEMEN:

In the absence of a characterized reference material, a mix of negative equine semen can be used as a negative control of the process.

- Aliquot the negative control (at least 150µl par tubs).
- Freeze the negative control aliquots at ≤ - 16°C.

4 PREPARATION OF THE SAMPLES TO BE ANALYZED

RNA extraction is performed in accordance with the used RNA extraction kit instructions from 150 µl of semen that has been previously centrifuged at 1900 rpm for 10 minutes at 4°C.

5 VALIDATION AND INTERPRETATION OF RESULTS

5.1 VALIDATION OF THE ANALYTICAL PROCESS

The validation of the analysis is carried out by observing the EAV and β–Actin fluorescence curves generated from the control samples.

When the fluorescence values are plotted on a logarithmic scale, the operator fixes

- the baseline, which must be positioned as low as possible without including the background noise but high enough to cross the non-characteristic part of the fluorescence curves of the positive controls obtained during the first cycles,

- the threshold line (= minimum fluorescence limit to be reached for a fluorescence signal emitted to be significantly greater than the background noise signal represented by the baseline) which must be crossed the middle of the curves of fluorescence of positive controls during the exponential increase phase of amplification.

For each control, the operator observes the presence or absence of an amplification signal and checks the consistency of this signal in order to validate or invalidate the controls (absence of a characteristic amplification curve for the negative controls and presence of a characteristic amplification curve for the positive controls and internal controls).

The expected results for the positive and negative samples of the process are detailed in the chart below:

	EAV RT-qPCR		**β–Actin RT-qPCR**	
	Amplification signal	**Result**	**Amplification signal**	**Result**
Positive control	Presence	Detected	Presence	Detected
Negative control	Absence	Not detected	Presence	Detected

If the obtained results of positive and negative control are in accordance with those expected (chart above), the operator can proceed to the analysis of the presence or absence of an amplification signal and checks the consistency of this signal for all the samples.

If the series of analysis is not validated, and depending on the anomaly observed, all or part of the analytical process has to be redone.

5.2 INTERPRETATION OF RESULTS

For a semen sample, the result can be expressed as follows:

- “Detected” when the signal corresponds to a characteristic amplification curve for EAV and β-Actin amplification,

- “Not detect” in the absence of amplification signal for EAV and a characteristic amplification curve β-Actin amplification,

- “Uninterpretable or not conclusive” when one or more signal analysis criteria do not match the expected characteristics or in the absence of amplification signal for β-Actin amplification.

6 STORAGE CONDITIONS AND ELIMINATION OF SAMPLES

Store products and consumables in keeping with suppliers’ recommendations.

Keep all semen samples in a refrigerated unit at (5 ± 3)°C until the test is done (not more than 24h after receipt) or in a freezer at - 16°C once the test is done.

RNA extractions and PCR products may be kept in a freezer at ≤ - 60°C and at ≤ - 16°C, respectively.