STANDARD OPERATING PROCEDURE

EEEV and WEEV rtRT-PCR (adapted from Lambert et al., 2003)

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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 EURL PT can be used for this essay.

This SOP describes the real-time RT-PCR technique using the TaqMan method, derived from the publication of Amy J. Lambert et al.(Lambert AJ, Martin DA, Lanciotti RS. Detection of North American eastern and western equine encephalitis viruses by nucleic acid amplification assays. Journal of Clinical Microbiology (2003); Vol. 41(1), p. 379–385) and quoted in The OIE in the Manual on Diagnostic Tests and Vaccines for Terrestrial Animal, 2019 edition (English version) of the Office International des Epizooties (OIE), Chapter 3.5.5.

This protocol is suitable for the amplification and detection of EEEV strains belonging to the North American group and WEEV RNA genome

Eastern equine encephalomyelitis virus (EEEV): EEEV virus is a member of the genus *Alphavirus*, family *Togaviridae*. Two variants of EEEV have been described, Central/South American and North American, the latter being more pathogenic. The common range for EEEV is Gulf and Atlantic coastal regions of the USA, but sporadic outbreaks in interior regions (usually) east of the Mississippi River occur annually. EEEV may cause severe disease in humans, horses, and some birds. EEEV is considered a Select Agent in the United States.
Western equine encephalomyelitis virus (WEEV): WEEV is also a member of the genus *Alphavirus*, family *Togaviridae*. All aspects of WEE virus are very similar to EEE virus, except clinical disease in horses is often mild and fatalities are rare. WEEV is generally confined to the western half of the U.S. WEEV is not classified as a Select Agent, however, humans may become infected.
Select Agent: Those agents and toxins that have the potential to pose a severe threat to public health and safety, to animal and plant health, or to animal and plant products. The list of Select Agents and toxins are listed in 9 CFR, Parts 121.3 and 121.4.

1. SAFETY

EEEV and WEEV are assigned to Biosafety Level 3 (BSL3). Both viruses are human pathogens which could cause potentially severe or life-threatening illness. Users have to follow the rules applicable to the handling of infectious materials and waste: work in a secured level 3 biosafety laboratory is mandatory when infectious EEEV or WEEV is manipulated (before sample lysis). Process samples that may potentially contain live agents in an approved biological safety cabinet with HEPA filtration. All surfaces and equipment that come in contact with infected materials must be disinfected with an appropriate

disinfectant and virucide (i.e. Anios for example). All contaminated instruments, containers and fluids must be autoclaved before reuse or disposal.
Work on RNA samples can be carried out in conventional laboratories.
A lab coat must be worn at all times while in the laboratory. Gloves must be worn throughout the PCR procedure, both for the protection of the person performing the task (potential pathogens in samples and hazardous chemical use), and for the integrity of the test (to prevent RNase contamination of the samples and cross-contamination between samples).

Specimens may include neurologic tissue (brain and/or spinal cord) and/or internal organ tissues from a wide range of mammalian or avian species.

2. COLLECTION OF SAMPLES

Tissue material to be examined: whole blood collected in tubes with anticoagulant (EDTA) and neurological tissue (brain and/or spinal cord) for horses

Transport of samples: samples must be transported under negative cold (≤ -16°C).

3. MATERIALS, EQUIPMENTS AND REAGENTS

3.1 MATERIALS AND EQUIPMENTS

- Real time PCR system and software
- Gloves
- Plasticware:
  • 96-well plates or tubes appropriate for real-time PCR (Optical tube and cap strips (Applied Biosystems, reference MicroAmp® 8-Cap Strip N8010535 and MicroAmp® Fast 8-Tube Strip, 0.1 ml 4358293)
  • Reagent reservoirs or tubes for preparing master mix.
  • Nuclease-free pipettors and tips, reagent reservoirs or tubes for preparing master Mix.
  •Optical tube and cap strips (Applied Biosystems)
- Ice bucket and ice
- Class II microbiological safety cabinet
- Laboratory benchtop centrifuge
- Laboratory benchtop microcentrifuge
- Vortex

3.2 CHEMICALS AND REAGENTS

Sequences (5'-3') and Nucleotides position:

EEEV (target):

- Primer Forward: EEE Fwd 5 '- ACACCGCACCCTGATTTTACA - 3' 9391-9411
- Primer Reverse: EEE Rev 5' - CTTCCAAGTGACCTGGTCGTC - 3' 9459-9439
- Probe: EEE probe 5' – Fam - TGCACCCGGACCATCCGACCT – Tamra - 3' 9414-9434

Length of amplicon = 69bp.

WEEV (target):

- Primer Forward: WEE Fwd 5'-CTGAAAGTCGGCCTGCGTAT-3' 10,248-10,267
- Primer Reverse: WEE Rev 5'-CGCCATTGACGAACGTATCC-3' 10,314-10,295
- Probe: WEE probe 5' – Fam – ATACGGCAATACCACCGCGCACC - Tamra - 3' 10,271-10,293

Length of amplicon = 67bp.

ß-Actine (control) (from Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. J Virol Methods (2007);140(1-2):115-23)**:**

- Primer Forward: ACTBFwd 5'-CAGCACAATGAAGATCAAGATCATC-3' 966-991
- Primer Reverse: ACTBrev1096 5'-CGGACTCATCGTACTCCTGCTT-3' 1096-1121
- Probe: ACTB 5'-VIC-TCGCTGTCCACCTTCCAGCAGATGT-TAMRA-3' 1042-1067

Length of amplicon = 156bp

AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystem,4387424)

- Primers 100µM
- Probe 10µM
- H_2O DEPC or RNAse free
- Standard EEEV or WEEV RNAs 10^6 copy/µL

4. PROCEDURE

4.1 RNA EXTRACTION KIT :

Used kit: QIAamp RNA viral kit, QIAGEN, reference 52906
This kit is mentioned as used by the EURL for equine diseases other than African horse sickness. Any other RNA extraction kit can be used as long as it has been previously validated by the user laboratory.

4.2. TEST RELIABILITY

Keep RNAs and reagents in ice until tubes are placed in the thermocycler.

Used kit: AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystem,4387424)
Any other RNA extraction kit can be used as long as it has been previously validated by the user laboratory.

Prepare a standard range: prepare 10-fold serial dilutions of WEEV or EEEV standard RNAs, 10^6 to 10 copies/ µL: 2µL of the previous dilution + 18µL RNase free water.

Recommendations:

- Do not store the standard range. For each PCR run, prepare a new one, because some RNAs are lost at every defrosting and standard RNAs quantity is modified.

- Comply with the standard procedures recommended to avoid contamination (prepare aliquots of samples; prepare aliquots of reagents; separate workstations; use filter tips; wear protective gloves).
- Work with sterile RNase-free consumables.
- Add negative extraction controls to ensure the absence of inter-sample contamination. A volume of water will be used that is equal to the volume recommended for the sample, and these negative extraction controls will be treated as samples thereafter.

4.2. METHOD STEPS

Prepare a mix for each RT-PCR (number of RT-PCR reactions + 1 or 2 extra reactions):
Both enzyme reactions are carried out separately in two tubes (two-step RT-qPCR).

Mix for 1 tube:	*Volume:*
- H_2O DEPC or RNAse free:	5.1 µL
- Buffer 2X:	12.5 µL
- RT-PCR Mix 25X:	1 µL
- Primers EEEV or WEEV Fwd 100µM:	0.1 µL
- Primers EEEV or WEEV Rev 100µM:	0.1 µL
- Probe EEEV or WEEV 10µM:	0.5 µL
- Primers ACTB Fwd 100µM:	0.1 µL
- Primers ACTB Rev 100µM:	0.1 µL
- Probe ACTB 10µM:	0.5 µL

Distribute 20µL of mix into each PCR tube.

Add 5µL of sample RNA or of the standard (1 tube per dilution), or add 5µL RNAse free water in the tube corresponding to the no template control (NTC). Complete the program on the AB7300 (or Step One Plus) thermocycler and the reaction volume as follows:
10 min at 45°C for reverse transcription;
10min at 95°C to activate DNA polymerase and to inactivate reverse transcriptase;
45 cycles of 15 s at 95°C (denaturation phase) and 1min at 60°C (annealing and elongation steps, acquisition of results during this latter stage)
Hold at 4°C.
Volume = 25 µL
Detector manager = FAM-TAMRA and VIC-TAMRA.

5. VALIDATION AND INTERPRETATION OF RESULTS

Check the Ct of the sample.
A positive sample will produce a Ct value.

5.1. TEST VALIDATION

Check the NTC and negative extraction controls all have an undetermined Ct value (UNDET) with the EEEV/WEEV RT PCR and the cellular gene RT PCR.
Check whether the Ct of the standard control corresponds to the expected value and check that you have a PCR efficiency close to 100% by checking the standard curve range and correlation coefficient (R^2 should be close to 1).

5.2. INTERPRETATION

Data analysis should be undertaken with a software provided by the RT-PCR system implemented in the lab.

When the sample result is validated:

- The sample is considered negative when the Ct obtained with EEEV/WEEV RT PCR is undetermined (UNDET, >45).
- The sample is considered positive when the Ct obtained with EEEV/WEEV RT PCR is less than or equal to 40.
- The sample is considered doubtful when the Ct obtained with EEEV/WEEV RT PCR is over 40 cycles.