STANDARD OPERATING PROCEDURE	
Detection of WNV RNA genome by WNV real-time RT-PCR (adapted from Linke et al., 2007)	
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This SOP is a method used at the EURL, all RT-PCR based methods including commercial kits validated and used successfully in the EURL PT can be used for this essay

This standard operating procedure (SOP) is used for the molecular diagnosis of West Nile virus infection by RT-PCR, in case of clinical suspicion of West Nile (WN) infection in horses.

This SOP describes the real-time (rt) RT-PCR technique using the TaqMan method, derived from the publication of S. Linke et al. (Linke S, Ellerbrok H, Niedrig M, Nitsche A, Pauli G. Detection of West Nile virus lineages 1 and 2 by real-time PCR. J Virol Methods. 2007 Dec;146(1-2):355-8.)
This protocol is suitable for the amplification and detection of WNV strains belonging to lineages 1 and 2. rtRTPCR methods
West Nile virus (WNV): WNV is a member of the genus *Flavivirus*, family *Flaviridae*. WNV is one of the most widely distributed arbovirus and WNV outbreaks have been described in Europe (Mediterranean and Eastern parts), Asia (India), Africa (Maghreb and South Africa in particular), the Middle-East and America (US and Canada). WNV may cause severe disease in humans, horses, and some bird species.

1. SAFETY

WNV is assigned to Biosafety Level 3 (BSL3). WNV is a human pathogen which can cause 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 WNV 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 into 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.

Wear a lab coat and gloves. Gloves must be worn throughout the RT-PCR procedure, both for the protection of the person performing the task (potential pathogens in samples and use of hazardous chemicals), and for the integrity of the test (to prevent RNAse contamination of the samples and cross-contamination between samples).

Work on only one sample at a time when processing samples.

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
- Plasticwares:
  - 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)
  - Nuclease-free reagent reservoirs or tubes for preparing the master mix.
- Micropipets and RNAse-free DNAse-free filter tips, adapted to micropipets
- Ice bucket and ice
- Class2 biosafety cabinet
- Laboratory benchtop centrifuge
- Laboratory benchtop microcentrifuge
- Vortex

3.2 CHEMICALS AND REAGENTS

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

- Primer Forward: WNproC Fwd 5'-CCTGTGTGAGCTGACAAACTTAGT-3' 10 -33
- Primer Reverse: WNproC Rev 5'-GCGTTTTAGCATATTGACAGCC-3' 132-153
- Probe: WNproC probe 5' - Fam - CCTGGTTTCTTAGACATCGAGATCT - Tamra - 3'

Length of the amplicon = 144bp.

ß-Actin (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: ACTBRev 5'-CGGACTCATCGTACTCCTGCTT-3' 1096-1121
- Probe: ACTB 5'-VIC-TCGCTGTCCACCTTCCAGCAGATGT-TAMRA-3' 1042-1067

Length of the amplicon = 156bp

Reagents

- AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystem, 4387424).
Storage temperature: < - 16°C

- Primers 100µM. Storage temperature: < - 16 °C
- Probe 10µM. Storage temperature: < - 16 °C
- DEPC or RNAse-free water. Storage temperature: < - 16 °C
- Standard WNV RNA at 10^7 copy/µL. Storage temperature: < - 76 °C

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). 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.

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

Recommendations:

- Do not store the standard range. For each RT-PCR run, prepare a new standard range.
- Comply with the standard procedures recommended to avoid contamination (prepare aliquots of samples; prepare aliquots of reagents; use separate workstations; use filter tips; wear protective gloves).
- Work with sterile RNase-free consumables.
- Add negative extraction and RT-PCR controls to ensure the absence of inter-sample contamination. These negative controls will be treated as samples thereafter. For RT-PCR controls, water will be used instead of sample RNA.

4.3. METHOD STEPS

4.3.1 PREPARATION OF THE RT-PCR MIX

WNV and ß-Actin RNA amplifications are carried out in the same tube (duplex rtRT-PCR).
Prepare a master mix for each RT-PCR reaction (number of RT-PCR reactions + 1 or 2 extra reactions).

Reagent:	*Volume for 1 tube:*
H2O DEPC or RNAse free:	5.1 µL
Buffer 2X:	12.5 µL
RT-PCR Mix 25X:	1 µL
Primer WNProC Fwd 100µM:	0.1 µL
Primer WNProC Rev 100µM:	0.1 µL
WNProC Probe 10µM:	0.5 µL
Primer ACTB Fwd 100µM:	0.1 µL

Primer ACTB Rev 100µM:	0.1 µL
Probe ACTB 10µM:	0.5 µL

Distribute 20µL of master mix into each PCR tube.

4.3.2 ADDITION OF NUCLEIC ACID

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 or RT-PCR control). Plug the tubes.

4.2.3 PCR RUN (

Complete the program on on the Real time 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;
40 cycles of 15s 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 managers = 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 should have an undetermined Ct value (UNDET) with the WNV RT-PCR and the ß-Actin RT-PCR.
Check whether the Ct of the standard control corresponds to the expected value :
Check that you have a PCR efficiency close to 100% by checking the standard curve slope (the slope S should be comprised between -4,00 and -3,32) and correlation coefficient (R^2 should be close to 1).

5.2. INTERPRETATION

If the test has been validated and if the Ct with the ß-Actin RT-PCR is correct (cf table below):

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