# STANDARD OPERATING PROCEDURE

## VEEV rtRT-PCR (adapted from Rodriguez et al., 2016)

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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 the real-time RT-PCR technique using the TaqMan method, derived from the publication of Vina-Rodriguez, A., et al., A Quantitative Real-Time RT-PCR Assay for the Detection of Venezuelan equine encephalitis virus Utilizing a Universal Alphavirus Control RNA. Biomed Res Int, 2016. 2016: p. 8543204. 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.

**Venezuelan equine encephalomyelitis virus (VEEV):** VEEV is a member of the genus *Alphavirus*, family *Togaviridae*. The VEE viruses comprise six subtypes with enzootic and epizootic antigenic variants. The enzootic variants and subtypes can produce clinical diseases in humans whereas the epizootic are associated with epizootics in equids but also with epidemics in humans. The foci of distribution of the enzootic variants of VEE viruses can be found in tropical wet forests of the Americas. The epizootic VEE viruses originally have been found in the northern and western South America but have now spread to other countries including Mexico and the USA (Texas).
**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

VEEV is assigned to Biosafety Level 3 (BSL3). The virus is a human pathogen that can cause potentially severe or life-threatening illness. Users have to follow the rules applicable to the handling of infectious materials and waste: Handling of infectious VEEV in a secured level 3 biosafety laboratory is mandatory (before sample lysis). samples that may potentially contain live agents must be processed 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:

**VEEV (target):**

- Primer Forward: VEE Fwd 5 '- TCCATGCTAATGCYAGAGCGTTTTCGCA - 3' 151-178
- Primer Reverse: VEE Rev 5' - TGGCGCACTTCCAATGTCHAGGAT - 3' 248- 225
- Probe: VEE probe 5' – Fam - TGATCGARACGGAGGTRGAMCCATCC – Tamra - 3' 193-218

Length of amplicon = 98bp.

Reagents

- QuantiTect Probe RT-PCR Kit (Qiagen, 204443)
- Primers 100µM, Storage temperature: < - 16 °C
- Probe 100µM, Storage temperature: < - 16 °C
- $H_2O$ DEPC or RNAse free

- Standard VEEV RNA

# 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: QuantiTect Probe RT-PCR Kit (Qiagen, 204443). 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 VEEV standard RNAs: 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 step 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.

## 4.3. METHOD STEPS

Prepare mastermix for each RT-PCR

| VEEV-Mix-FAM | Volume |
|---|---|
| VEEV-Fwd primer (100µM) | 20µl |
| VEEV-Rev primer (100µM) | 20µl |
| VEEV probe (100µM) | 2µl |
| 0,1 X TE (pH 8,0) | 158µl |

Prepare a mix for each RT-PCR (number of RT-PCR reactions + 1 or 2 extra reactions):

| *Mix for 1 tube:* | *Volume:* |
|---|---|
| -$H_2O$ DEPC or RNAse free: | 5,25 µL |
| -2X QuantiTect-Probe RT-PCR Mastermix : | 12,5 µL |
| -Reverse Transkriptase Mix (RT-Mix): | 0,25 µl |

-VEEV-Mix-FAM: 2 µL

The RNA isolation control using the β actin control is carried out according to SOP EEEV and WEEV RT-PCR (adapted from Lambert et al., 2003)

**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 Real-time thermocycler and the reaction volume as follows:
30 min at 50°C for reverse transcription;
15min at 95°C to activate DNA polymerase and to inactivate reverse transcriptase;
45 cycles of 15 s at 95°C (denaturation phase), 30 s at 55°C (annealing phase) and 30s at 72°C (elongation step, acquisition of results during the annealing stage)
Hold at 4°C.
Volume = 25 µL
Detected fluorophores = FAM-TAMRA

# 5. VALIDATION AND INTERPRETATION OF RESULTS

Check the Ct of the sample.
A positive sample will produce a low Ct value (see below).

## 5.1. TEST VALIDATION

Check the NTC and negative extraction controls, all have an undetermined Ct value (N/A) with the VEEV RT PCR and the IC control RT PCR.
Ensure that the Ct of the standard control corresponds to the expected value
Checking the standard curve to ensure that the PCR efficiency is close to 100% with a correlation coefficient ($R^2$) 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 test is validated:

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