GUIDELINE: ENZYME IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTIBODIES AGAINST *ORBIVIRUS*		
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Index

1. PURPOSE 2

2. SCOPE 2

3. REFERENCES 2

4. TEST PRINCIPLE 3

5. SAFETY PRECAUTIONS 5

6. GENERAL REQUIREMENTS FOR TEST'S PERFORMANCE 5

6.1. Protocol 5

6.2. Samples 6

6.3. Traceability 7

6.4. Precautions 7

6.5. Quality control 8

6.6. Proficiency test 8

7. ASSAY VALIDATION 9

7.1. Plate validation 9

7.2. Interpretation of sample results 9

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1. PURPOSE

The purpose is to provide general guidance to the best implementation and quality control of the Enzyme Immunosorbent Assay (ELISA) for the detection of antibodies against different *Orbivirus* (African horse sickness -AHS- virus and Bluetongue -BT- virus) using commercial validated diagnostic kits.

These guidelines take into account applying principles and rules under the accreditation scope of ISO/IEC 17025, *General Requirements for the competence of testing and calibration laboratories* of the International Organization for Standardization.

2. SCOPE

To detect the presence of antibodies against different *Orbivirus* (AHS virus and BT virus) in serum samples of animal species of *Equidae*, in case of AHS virus, and ruminants for BT virus.

The scope for each ELISA kit will be described in a specific protocol.

3. REFERENCES

ISO/IEC 17025:2017 General Requirements for the competence of testing and calibration laboratories. International Organization for Standardization.

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OIE. Chapter Biosafety and biosecurity: standard for managing biological risk in the veterinary laboratory and animal facilities. Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. English version in force at date.

OIE. Chapter Quality Management in Veterinary testing Laboratories. In: Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. English version in force at date.

OIE. Chapter African horse sickness (Infection with African horse sickness virus). In: Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. English version in force at date.

OIE. Chapter: Bluetongue (Infection with Bluetongue virus). In: Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. English version in force at date.

OIE Chapter Development and optimization of antibody detection assays. In: Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. English version in force at date.

4. TEST PRINCIPLE

The exploitation of the ELISA has been increased through continued development of specifically produced reagents, for example, monoclonal and polyclonal antibodies and peptide antigens coupled with the improvement and expansion of commercial products such as enzyme-linked conjugates, substrates and chromogens, plastics technology and design of microwell plates, instrumentation advances and robotics. However, the principle of the ELISA remains the same.

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The main reason for this success is test flexibility, whereby reagents can be used in different combinations. A key feature of the flexibility of ELISA is that more than one system can be used to measure the same thing.

The principle of the ELISA test is the use of a conjugate and a substrate to detect the inmuno-complex antigen (viral protein) and specific antibodies. Subsequently, a microplate reader (spectrophotometre) detects color development.

For antibodies against Orbivirus there are several ELISA systems described, mainly competitive/blocking (c/b), double recognition (dr) and indirect (i). All of them detect antibodies against the structural viral protein VP7 and are not able to discriminate between antibodies from vaccinated or infected animals.

The competitive/blocking (c/b) as well as the double recognition (dr) ELISA assay are designed to detect virus specific antibodies in serum samples from animals of any specie, preventing the problem of species specificity experienced occasionally using indirect ELISA.

Reagents are supplied by the manufacturers in kit format that includes specific reagents and test control for the correct performance.

ELISA assay is a qualitative test. Optical Density (ODs) and other parameters such as Blocking or S/M percentages are not reported to the customer but could be taken into account at internal level in the quality control.

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5. SAFETY PRECAUTIONS

- The ELISA assay shall be carried out following Biosafety, Biocontainment and Bioprotection guidance, and rules established according to international and national regulations (see references) considering the target pathogen. The EURL, according to the recommendation of these guidances, has classified BTV and AHSV in the group risk 3 under the animal health point of view.
- According to OIE Manual, there is no evidence that humans become infected with any field strain of AHS or BT virus either through contact with naturally or experimentally infected animals or by virus manipulation in laboratories.
- The ELISA reagents have not a specific biological risk.
- Serum samples could come from infected animal during the viremia phase. Although, Orbivirus particles have hemagglutination activity that allows them to adhere to circulating erythrocytes, when the viremia is very intense, there can be infective Orbivirus particles also in the serum sample. Therefore, the risk assessment of the serum samples must be carried out and safe procedure have to be stablished.
- The remaining kit reagents, once finished, must be separated between biological and chemical reagents and dispose according to environmental rules for waste management

6. GENERAL REQUIREMENTS FOR TEST'S PERFORMANCE

6.1. Protocol

- The manufacturer's kit instructions shall be followed as the basis for test implementation.

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- The laboratory will keep records of the current instruction edition in force and, with this purpose, will revise systematically the booklet inserted in each purchased kit to ensure that always the information of latest edition is used.
- Obsolete instruction editions shall be archived for the time period determined by the corresponding Accreditation Body.
- The laboratory shall made available a Standard Operating Procedure (SOP) that, taking as the basis manufacturer's kit instructions, adapts operations to the laboratory quality system established according to ISO 17025, as well as to every aspect that might be relevant for a correct assay performance (e.g. quality control of assay, interpretation of results, actions to take in case of doubtful results, etc).
- According to ISO 17025 requirements, reagents and equipment must be used under control (i.e. reagents after the expiring date stablished is strongly not recommended).
- All reagents and samples must be allowed to come to room temperature before use.
- Mix reagents by gentle inverting or swirling.
- Use a separate pipette tip for each sample and control.
- Do not expose the substrate solution to strong light or any oxidizing agents.
- Do use clean and clear out bucket to dispense the reagents and do not pour unused reagents back into containers in order to prevent contamination of kit components.
- Use distilled or deionized water for preparation of reagents that require it (as wash solution).
- Mix the content of the plate after the addition of the samples using a microplate shaker.

6.2. Samples

- Sample's biological properties should be guaranteed by applying correct sampling, preservation and transport procedures until arrival at the laboratory. Any relevant issue regarding to the conservation of the samples during the transport must be registered in the laboratory.

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- The laboratory should implement a protocol to reject samples in poor conditions to be analyzed (e.g. strongly hemolization, putrefaction).
- The laboratory should implement protocols to prepare samples (p. e. centrifugate at 700g during 10 min.to separate the serum fraction, filtrate using 0,45 µm filters and/or centrifugate at 5000g during 15 min. to eliminate bacterial contamination).
- Once sera are obtained from un-clotted blood samples, they shall be kept at +5ºC (3º - 8ºC) until their assay. For prolonged periods, samples shall be frozen in capped tubes/vials (or similar) at -20 º C or below.

6.3. Traceability

The traceability system in the laboratory shall preserve the correct identity of samples during laboratory operations.

6.4. Precautions

Please, refer to the manufacturer's kit instructions. In particular:

- Reagents and samples must be kept at room temperature for 30 minutes before testing. Frozen samples might need extra time to reach the required temperature.
- Plates and other kit components must be stored at 5 ºC (3º-8ºC). Use clean and sterile material (e.g. pipettes, tips and vessels) in contact to kit reagents. Such practices avoid contamination and preserve kits reagents until expiring date.
- Do not mix reagents from different kit batches.
- Use equipment (pipettes, incubators, spectrophotometer) correctly maintained, and periodically verified and calibrated.

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6.5. Quality control

- The kit includes positive and negative controls that shall be assayed in duplicates for the best reliability. Although the manufacturer's kit instructions usually don´t include repeatability criteria, taken into account that the average of OD obtained for duplicates in controls can be used for the calculation of quantitative result, it is highly recommended to control the variability between replicates. A variation less than 10% between duplicates will be considered as the best performance, although sporadic greater variation (≤ 20%) might be accepted.
- If available, a weak positive serum assayed in duplicate should be included in each test run as sensitivity control. In general, qualitative assessment should be enough; nevertheless, a quantitative assessment could be done too.
- In addition to test repeatability, it is advisable to test a number of samples in duplicate (e.g. at least 2 samples per assay). In general, qualitative assessment should be enough, both replicates positive or both negative. Nevertheless, a quantitative assessment could be done too. In case of samples close to grey zone (inconclusive), a variation of blocking percentage less than 10% between duplicates will be considered as the best performance, although sporadic greater variation (≤ 20%) might be accepted.

6.6. Proficiency test

- The laboratory shall participate regularly in *external proficiency testing schemes*. Participation in such scheme is a requirement for accredited laboratories.
- According to EU rules, National Reference Laboratories for Bluetongue and African horse sickness in each EU Member State, have to participate in the Annual Proficiency Tests organized by the European Union Reference Laboratory (EURL).
- Proficiency testing material has been well characterized and any spare material, once the proficiency testing has been completed, can be useful to demonstrate staff competence, or to regularly check test performance.

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7. ASSAY VALIDATION

It is advisable to develop an excel sheet for calculations that avoid mistakes. A kit specific template is available at the EURL under request.

7.1. Plate validation

- Although an assay could include two or more plates, each assayed plate must be validated individually according to manufacturer's kit instructions.
- When a weak positive serum is used as sensitivity control, it is recommended to include it in each plate and it must give the expected result.
- In case of not acceptance plate validation, it is important to investigate the causes and according to the reasons of the deviation, to take corrective action and then to repeat the assay.

7.2. Interpretation of sample results

- Once the plate has been validated, the formula provided by the manufacturer must be applied in order to know the status of the sample (Positive, Negative or Doubtful)
- Doubtful results should be further investigated by retesting the sample, analyzing using other method if available or by sampling again the animal.
- A protocol to apply when the replicates generate a different qualitative result (NEG or POS) must be available.

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