# ANTIGEN DETECTION ELISA

## 1. PURPOSE AND SCOPE

This test is used as a first-line test during clinical suspicions of foot-and-mouth disease. It enables the detection of foot-and-mouth disease virus antigens in a lesion sample or from an infected cell culture, as well as the identification of the serotype responsible for the virus. It derives from the assay recommended by the World Organisation for Animal Health (WOAH).
If the suspicion involves pigs, this test can also include screening for the swine vesicular disease virus antigen as part of a differential diagnosis. This test can also be used for the diagnosis of vesicular stomatitis in horses.

## 2. NORMATIVE REFERENCES

Analytical method derived from the official WOAH test described in Chapter 3.1.8 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (English version adopted in May 2022).

## 3. DEFINITIONS

Matrix: clarified homogenate from vesicular lesions, fluid from vesicular lesions, or infected cell culture

Analyte: total antigens of foot-and-mouth disease virus

Abbreviations:

ELISA: enzyme-linked immunosorbent assay

OD: optical density

OPD: ortho-phenylenediamine

HRPO: horseradish peroxidase enzyme

## 4. PRINCIPLE OF THE METHOD

It is a sandwich ELISA, which means that any foot-and-mouth disease virus antigens contained in the test sample are “captured” via specific antibodies. This test is able to differentiate between the seven possible serotypes of foot-and-mouth disease virus (O, A, C, SAT1, SAT2, SAT3 and Asia-1).

It relies on the use of pairs of hyper-immune rabbit and guinea-pig sera containing specific antibodies to each of the serotypes of foot-and-mouth disease virus.

The wells of the ELISA plates have been coated with the specific antibodies contained in the hyper-immune rabbit sera.

The test is undertaken by successively adding the test sample, hyper-immune guinea-pig serum, followed by the conjugate (rabbit anti-guinea-pig serum) coupled to the peroxidase enzyme (HRPO). Each step includes incubation and washing. The reaction is revealed by adding an HRPO chromogen substrate and stopped by adding an acid solution.

Colour development indicates the binding of the conjugate to the guinea-pig antibodies and thus the presence of the captured antigen. Specific testing for the seven serotypes in parallel thus supplements the diagnosis by determining the responsible serotype.

The same principle is used for the detection of swine vesicular disease virus or vesicular stomatitis virus with the use of a specific pair of hyper-immune rabbit and guinea-pig sera.

# 5. EQUIPMENT AND MATERIALS

- Class II microbiological safety cabinet (MSC): the use of live FMD virus, a category Ea3 agent, makes it necessary to work in a containment level-3 laboratory complying with the EuFMD standards
- Refrigerated chambers: -21°C (± 5°C) and +5°C (± 3°C)
- Temperature-controlled chamber at +37°C (± 2°C)
- Single-channel and multi-channel micropipettes, suited to the various volumes to be collected
- Sterile tips
- Sterile, flat-bottomed, 96-well plates, for ELISA MaxiSorp
- Microplate sealers
- Microplate shaker
- Plastic reservoirs (drip trays)
- Spectrophotometer (ELISA reader)
- pH meter or pH paper
- Weighing scale (+/- 0.1 g)
- Sterile small standard laboratory equipment: Erlenmeyer flasks with caps, beakers, graduated cylinders, magnetic rods, tubes with caps, Eppendorf tubes, cryotubes, pipettes, etc.
- Vortex
- Magnetic stirrer
- Timer

# 6. PREPARATION OF REAGENTS

## 6.1. CHEMICAL PRODUCTS

- PBS tablets (GIBCO ref.: 18912-014)
- ANIOS SPS 2000 disinfectant
- Tween-20 (Sigma ref.: P1379)
- Skimmed milk powder (BIO-RAD ref.: 170-6404)
- OPD tablets (SigmaFast P9187 (50SET))
- Concentrated sulphuric acid (Sigma ref.: 32050-1)
- Pure water: Aqua B. Braun (Braun Medical. A/S. ref.: 3610531)

## 6.2. PREPARATION OF BUFFERS

PBS: For 1 L of PBS, dissolve two PBS tablets in 1 L of Braun water: mix until the tablets have completely dissolved.

PBS-Tween: For 1 L of solution, add 500 µl of Tween-20 to the prepared PBS (to obtain a final Tween-20 concentration of 0.05%) and mix.

PBS-Tween-milk: Weigh 2 g of skimmed milk powder and add to 40 ml of PBS-Tween (to obtain a final milk concentration of 5%). Mix well until the milk powder is dissolved and the buffer is homogeneous.

OPD solution: For one or two 96-well plates, in a 50 ml tube, add 20 ml of Braun water, a tablet of buffer (in silver foil) and a tablet of $H_2O_2$ (in gold foil). This solution is prepared in two steps: Add the tablet of buffer (in silver foil) to the water one hour prior to use, then add the tablet of $H_2O_2$ (in gold foil) immediately prior to use. The entire preparation process should take place away from light. **Important: This product is carcinogenic. It should be handled with caution. Gloves must be worn.**

1.25 M sulphuric acid solution (stop solution): In a chemical hood, add 33.5 ml of concentrated sulphuric acid to 466.5 ml of pure water (Braun water). **For safety reasons, never add water to acid**. Note the manufacture date on the container and keep the solution at ambient temperature.

## 6.3. BIOLOGICAL REAGENTS

### 6.3.1. Sera used to coat the plates (to capture the antigen)

Prepared hyper-immune rabbit antisera to each of the seven serotypes of foot-and-mouth disease virus (and/or swine vesicular diseases virus and/or vesicular stomatitis virus).
These sera are obtained from the World Reference Laboratory for foot-and-mouth disease (Pirbright Institute).

They are aliquoted and stored at -21°C (± 5°C) until use.

The final dilution for use, diluted in PBS, is pre-determined for each batch.

### 6.3.2. Sera used to detect the captured antigen

Prepared hyper-immune guinea-pig antisera to each of the seven serotypes of foot-and-mouth disease virus (and/or swine vesicular diseases virus and/or vesicular stomatitis virus).

These sera are obtained from the World Reference Laboratory for foot-and-mouth disease (Pirbright Institute).

They are aliquoted and stored at -21°C (± 5°C) until use.

The final dilution for use, diluted in PBS-Tween20 (0.05 v/v)-milk (5% w/v) buffer, is pre-determined for each batch.

### 6.3.3. Other sera

Rabbit anti-guinea-pig serum (against guinea-pig immunoglobulins), conjugated to peroxidase, stored at +5°C (± 3°C). The final dilution for use, diluted in PBS-Tween-milk buffer, is given by the supplier (DAKO France SAS, ref.: P0141).

Goat anti-rabbit serum (against rabbit immunoglobulins), conjugated to peroxidase, stored at +5°C (± 3°C). The final dilution for use, diluted in PBS-Tween-milk buffer, is given by the supplier (DAKO France SAS, ref.: P0448).

### 6.3.4. Antigens

Inactivated control antigens, corresponding to the seven serotypes of foot-and-mouth disease virus, swine vesicular disease virus, or vesicular stomatitis virus (four serotypes). They are obtained from the World Reference Laboratory for foot-and-mouth disease (Pirbright Institute) or from Boehringer-Ingelheim. They are supernatants from infected cell cultures that have been inactivated and then lyophilised or inactivated, purified and concentrated.

Each inactivated antigen is first suspended in sterile distilled water according to the supplier's instructions, aliquoted, and stored at -80°C.

These inactivated antigens have been pre-titrated using ELISA. When they are used, these antigens may be pre-diluted in PBS-Tween before they are placed in column 1 of ELISA plate 1.

# 7. PREPARATION OF SAMPLES

The preparation of lesion samples is described in Procedure **LSA-INS-0899**.

# 8. PROCEDURE

## 8.1. PLATE SENSITISATION WITH THE PREPARED RABBIT SERA

- Dilute the various rabbit antisera to serotypes O, A, C, SAT1, SAT2, SAT3 and Asia-1 (and/or SVD, and/or VS)
- Add 50 µl of each diluted serum to all the wells in a row of an ELISA plate
- Tap the plates to ensure the even distribution of the sera in the wells
- Seal the plates
- Place the plates on a shaker, at a speed of 250 rpm, for one hour at 37°C (± 2°C)
- After incubation, empty the contents of the plates if they will be used immediately
- Complete the **LSA-FSE-1213** file

*Note: Non-emptied plates can be covered with adhesive film and stored at -21°C (± 5°C) for several months (their sensitivity should be checked at least once every six months). Thus, in the event of a suspicion, the plates, which have already been sensitised, can simply be removed from the freezer and used as follows.*

- Wash the plates five times, with PBS buffer at a rate of 250 µl per well.

## 8.2. DISPENSING OF CONTROL ANTIGENS AND TEST SAMPLES

- According to the plate sheet provided in the **LSA-FSE-1055** file, the first plate (control plate) includes various controls. Dispense:
- 50 µl of PBS-Tween to columns 2 and 3 which will be used for diluting the positive control antigens
- 50 µl of PBS-Tween to columns 5, 6 and 7 which will be used for monitoring plate sensitisation
- 50 µl of PBS-Tween to columns 4, 8 and 12 for the $OD_{min}$, necessary to determine the corrected OD.

  Add the control antigens (column 1 of the control plate) and prepare the five-fold dilutions, in accordance with the plate sheet. For example, for the antigen to serotype O:
  - ✓ Add 62.5 µl of antigen type O to well A1
  - ✓ Mix the antigen and transfer 12.5 µl of the mixture to well A2
  - ✓ Mix the antigen and diluent in well A2 and transfer 12.5 µl of the mixture to well A3
  - ✓ Mix the antigen and diluent in well A3 and discard 12.5 µl of the mixture
- Repeat these steps for all of the control antigens
- Dispense the test samples: each sample is added, at a rate of 50 µl per well, to all the wells of two or three columns (in triplicate or duplicate, depending on the amount of available materials). The first sample is placed in the wells of columns 9, 10 and 11 of the control plate.

*Note: If there is more than one test sample, use as many sensitised plates as needed. In this case, the control antigens and sensitisation control are only tested in the control plate for a run of no more than four plates (including the control plate).*

- Seal the plates
- Place the plates on a shaker, at a speed of 250 rpm, for one hour at 37°C (± 2°C)
- After incubation, empty the plates and wash seven times with PBS-Tween at a rate of 250 µl/well.

### 8.3. DISPENSING OF THE PREPARED GUINEA-PIG SERA

- Dilute the various guinea-pig antisera to serotypes O, A, C, SAT1, SAT2, SAT3 and Asia-1 (and/or SVD, and/or VS) in PBS-Tween-milk
- For each serotype, dispense 50 µl of each corresponding diluted serum to the wells containing the control antigens, negative controls and test samples. **Dispense 50 µl of PBS-Tween to the wells of the sensitisation controls** (columns 5, 6 and 7 of the control plate)
- Tap the plates to ensure the even distribution of the sera in the wells
- Seal the plates
- Place the plates on a shaker, at a speed of 250 rpm, for one hour at 37°C (± 2°C)
- After incubation, empty the plates and wash seven times as described in 8.2.

### 8.4. CONJUGATES

- Dilute the (anti-guinea-pig and anti-rabbit) conjugates in PBS-Tween-milk (see Section 6.3.3.)
- Dispense 50 µl of diluted anti-guinea-pig serum to all the wells containing the control antigens, negative controls and test samples, as well as **50 µl of diluted anti-rabbit serum to the wells of the sensitisation control columns (columns 5, 6 and 7 of the control plate)**
- Tap the plates to ensure the even distribution of the sera in the wells
- Seal the plates
- Place the plates on a shaker, at a speed of 250 rpm, for one hour at 37°C (± 2°C)
- After incubation, empty the plates and wash seven times as described in 8.2.

### 8.5. CHROMOGEN SUBSTRATE

- Prepare the OPD solution extemporaneously according to the supplier's instructions (as described in 6.2)
- Dispense 100 µl of the OPD solution to each well
- Cover the plates with aluminium foil to maintain in the dark, at ambient temperature (22°C ± 3°C)
- After 15 minutes, stop the reaction by adding 100 µl of 1.25M sulphuric acid to each well
- Measure OD at 492 nm

## 9. EXPRESSION OF RESULTS

### 9.1. CALCULATIONS

Use the configured Excel spreadsheet (**LSA-FSE-1055**) to perform automatic calculations after copying and pasting the raw OD values obtained.

For each row (detection of a specific serotype):

- The raw OD values from the three control-antigen wells are used separately (e.g. for serotype O: A1, A2 and A3) = $OD_{high/serotype,}$ $OD_{medium/serotype}$ and $OD_{low/serotype}$
- Calculate the mean raw OD value for the triplicates of each sample (e.g. for the first sample with regard to serotype O: mean OD for wells A5, A6 and A7) = $OD_{sample/serotype}$
- Calculate the mean raw OD value for the negative controls (mean OD value for wells 4, 8 and 12) = $OD_{min/serotype}$
- Calculate the corrected OD values for the control antigens and the samples by subtracting the $OD_{min/serotype}$ value from the raw OD value
- Check the OD values of the sensitisation controls for each serotype (>1.400).

### 9.2. TEST VALIDATION

For each specific serotype, the test is validated if:

- The corrected OD values from the first control-antigen dilution are ≥ **2.000**. The other dilutions must be greater than or equal to 0.1 **(≥ 0.1)**
- The OD of the sensitisation controls is >1.400

If these criteria are not met, the manager or deputy manager of the NRL can decide to carry out new tests to confirm the observed result.

### 9.3. EXPRESSION OF RESULTS

For a sample:

- **Corrected $OD_{sample/serotype}$ value ≥ 0.1**: positive sample for the virus of the corresponding serotype
- **Corrected $OD_{sample/serotype}$ value < 0.1**: negative sample for the virus of the corresponding serotype
- **Corrected $OD_{sample/serotype}$ value between 0.1 and 0.2**: the result should be confirmed by other direct analytical techniques undertaken at the same time, or by a new sandwich ELISA either directly with the initial sample or after amplification in cell culture

**NOTE:** For the same sample, the corrected $OD_{sample/serotype}$ value may be ≥ 0.1 for several serotypes. In this case, after consultation with and upon recommendation of the technical manager, the sample may be considered positive for the serotype with the highest OD.