



NATIONAL CENTRE FOR ANIMAL HEALTH
NATIONAL VETERINARY LABORATORY
STANDARD OPERATING PROCEDURE



SOP No.	SoPs Title	Page No.
<i>NCAH/LSU/SERO 01</i>	<i>SOP for Serum Sample Collection</i>	<i>2</i>
<i>NCAH/LSU/SERO 02</i>	<i>SOP for ELISA for Brucella abortus</i>	<i>5</i>
<i>NCAH/LSU /SERO 03</i>	<i>SOP for ELISA for Avian Leukosis Complex</i>	<i>9</i>
<i>NCAH/LSU /SERO 04</i>	<i>SOP for ELISA for M.paratuberculosis</i>	<i>12</i>
<i>NCAH/LSU /SERO 05</i>	<i>SOP for NSP ELISA for FMD</i>	<i>15</i>
<i>NCAH/LSU /SERO 06</i>	<i>SOP for ELISA for Mycoplasma</i>	<i>23</i>
<i>NCAH/LSU/SERO 07</i>	<i>SOP for ELISA for LPBE FMD</i>	<i>29</i>
<i>NCAH/LSU/SERO 08</i>	<i>SOP for ELISA for NCD</i>	<i>43</i>
<i>NCAH/LSU/SERO 09</i>	<i>SOP for Sandwich ELISA for FMD</i>	<i>50</i>
<i>NCAH/LSU/SERO 10</i>	<i>SOP for ELISA for Rabies</i>	<i>55</i>
<i>NCAH/LSU/SERO 11</i>	<i>SOP for RBT for Brucella</i>	<i>62</i>
<i>NCAH/LSU/SERO 12</i>	<i>SOP for SAT for Salmonella</i>	<i>65</i>
<i>NCAH/LSU/SERO 13</i>	<i>SOP for SAT for Mycoplasma</i>	<i>70</i>
<i>NCAH/LSU/SERO 14</i>	<i>SOP for ELISA for IBD</i>	<i>75</i>
<i>NCAH/LSU/SERO 15</i>	<i>SOP for ELISA for IBR</i>	<i>78</i>
<i>NCAH/LSU/SERO 16</i>	<i>SOP for ELISA for EIA</i>	<i>81</i>
<i>NCAH/LSU/SERO 17</i>	<i>SOP for ELISA for CSFV antibody</i>	<i>84</i>
<i>NCAH/LSU/SERO 18</i>	<i>SOP for ELISA for CSFV antigen</i>	<i>88</i>
<i>NCAH/LSU/SERO 19</i>	<i>SOP for HA/HI for NCD</i>	<i>92</i>
<i>NCAH/LSU/SERO 20</i>	<i>SOP for AGID for NCD</i>	<i>97</i>
<i>NCAH/LSU/SERO 21</i>	<i>SOP for ELISA for CBPP</i>	<i>101</i>
<i>NCAH/LSU/SERO 22</i>	<i>SOP for ELISA for BVD</i>	<i>107</i>
<i>NCAH/LSU/SERO 23</i>	<i>SOP for ELISA for Leptospira hardjo</i>	<i>114</i>
<i>NCAH/LSU/SERO 24</i>	<i>SOP for ELISA for Peste Petits Ruminant</i>	<i>121</i>
<i>NCAH/LSU/SERO 25</i>	<i>SOP for ELISA for Porcine Circovirus 2</i>	<i>126</i>
<i>NCAH/LSU/SERO 26</i>	<i>SOP for ELISA for Japanese Encephalitis</i>	<i>131</i>
<i>NCAH/LSU/SERO 27</i>	<i>SOP for ELISA for PRRS</i>	<i>135</i>



**NATIONAL CENTRE FOR ANIMAL HEALTH
NATIONAL VETERINARY LABORATORY
STANDARD OPERATING PROCEDURE**



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SOP No: NCAH/LSU/SERO 01

Title: SOP on serum sample collection

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Prepared by: Serology

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Application/Distribution: NCAH/NVH/RVLs/DVLs



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NATIONAL VETERINARY LABORATORY
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1. Scope

This SOP outlines the preferred methods for collecting blood serum from different animals based on their anatomy.

2. Objective

To guide the laboratory technicians in collecting, processing and preserving serum samples for serological tests

3. Equipment and Consumables

- a. Plain vacutainer without EDTA (Red cap) / Plain Vacutiner with gel coated (yellow cap)
- b. Razor blade / hair clipper
- c. Alcohol swab
- d. Glass slides, 3 x 1 inch with frosted edge
- e. Scissors
- f. Vacutainers needle & adapter,
- g. Serum vial,
- h. Cotton etc.
- i. Needle & syringe
- j. Swabs with VTM

4. Procedure

Blood Serum

For the sites for collection of blood samples in different animals, refer hematology SOP

- a. As described earlier the blood can be collected in sterile vial without anticoagulant or Vacutiner without anticoagulant.
- b. This is kept in standing position without disturbing until the clear serous fluid develops or forms on top or side.
- c. This fluid should be collected in sterile serum vial and should be stored at low temperature or preserved in refrigerator until all the necessary tests are done.
- d. If clear serous fluid does not develop, the serum has to be collected after centrifuging.
- e. This is kept in slanting position without disturbing until the clear serous fluid develops or forms on top or side.
- f. This fluid should be collected in sterile serum vial and should be stored at low temperature or preserved in refrigerator until all the necessary tests are done.
- g. If clear serous fluid does not develop within 24 hours, the blood should be kept in the incubator for few hours or the clots can be also broken down with thin clean stick and should keep in slanting position till the serous fluid develops.
- h. To obtain serum, blood collected into a clean plastic or glass container is allowed to clot at room temperature, normally in several hours. Serum can then be removed after clot retraction and centrifugation.
- i. Serological tests should be performed as soon as possible; otherwise, serum



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NATIONAL VETERINARY LABORATORY
STANDARD OPERATING PROCEDURE**



samples can be stored deep-frozen.

Storage and dispatch of samples

Blood should be kept chilled, not frozen, while being held prior to dispatch and during transport to the laboratory. Blood should be submitted to the laboratory chilled in insulated containers. Avoid contact between the blood samples and ice bricks. Do not chill blood films or expose them to formalin vapour.

Blood film can be submitted in the same container provided they are securely packed and well wrapped for insulation against chilling

5. Safety

- a. When samples are taken from live animals, care should be taken to avoid injury or distress to the animal or danger to the operator and attendants.
- b. It may be necessary to use mechanical restraint, tranquilization or anesthesia.
- c. Whenever handling biological material, from either live or dead animals, the risk of zoonotic disease should be kept in mind and precautions taken to avoid human infection.

6. Waste Disposal

All the waste generated during sample collection should be disposed appropriately based on zoonotic and non-zoonotic nature of the cases.

7. References

- 7.1 Preventive Veterinary Medicine Record (RAGACOVAS)
- 7.2 USAID PREDICT. Marcela Uhart, University of California, Davis (2016) Livestock Sampling Methods: Cattle, Sheep, Goats, Camels, and Swine
- 7.3 Veterinary Clinical Pathology (Ganti A. Sastry)
Maxine M. Benjamin. (2005). Outline of Veterinary Clinical Pathology. Kalyani publishers, New Delhi. P 8-9



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SOP No: NCAH/LSU/SERO 02

Title: SOP on *ELISA* test for *Brucella abortus*

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Laboratory service unit

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1. SCOPE

This ELISA test is designed for the detection of antibodies against *Brucella abortus* in bovine serum and plasma samples. It is intended for the diagnosis and sero-surveillance of brucellosis in cattle at the district and national levels.

2. PRINCIPLES

Microtiter plates are coated with inactivated antigen. Dilutions of the samples to be tested are incubated in the wells of these plates. Any antibody specific for *B. abortus* binds to the antigen in the wells and forms an antigen/antibody complex on the plate well surface. Unbound material is removed from the wells by washing. A peroxidase-labelled anti-ruminant IgG Conjugate is added, which binds to the ruminant antibody complex with the *B. abortus* antigen. Unbound conjugate is removed by washing and TMB substrate added to the wells. The degree of color that develops (Optical density) measured at 450nm) is directly proportional to the amount of antibody specific for *B. abortus* present in the sample. The result is obtained by comparing the optical density (OD) that develops in wells containing the samples with the OD from wells containing the Positive Control.

3. APPLICATION

The IDEXX Brucellosis Serum X2 test kit provides a rapid, simple, sensitive and specific method for detecting antibodies against *Brucella abortus* in individual serum and in pools of up to 10 individual serum samples from ruminants.

4. OBJECTIVE

To describe the procedure for detecting antibodies against *Brucella abortus* in serum samples from ruminants.

5. APPARATUS/TEST KIT/REAGENTS

IDEXX Brucellosis Serum X2

- 5.1 *B. abortus* antigen coated plate
- 5.2 Positive control
- 5.3 Negative control
- 5.4 Conjugate
- 5.5 TMB Substrate N12
- 5.6 Stop Solution N.3
- 5.7 Wash Concentration (10X). **Dilute the wash Concentrate (10X) 1:10 with distilled water.**

6. TEST PROCEDURE (OR Follow the Product Leaflet)

All the reagents must be allowed to come to 18-26 °C before use. Mix reagents by gently inverting or swirling.

- 6.1 Obtain coated plate and record the sample position.
- 6.2 Dispense 90µL wash solution into each well
- 6.3 Dispense 10 µL of the un diluted positive control into duplicate wells
- 6.4 Dispense 10 µL of the un diluted Negative control into duplicate wells



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NATIONAL VETERINARY LABORATORY
STANDARD OPERATING PROCEDURE**



- 6.5 Dispense 10 μ L of the un diluted serum samples or pools of up to 10 serum samples in to appropriate wells
- 6.6 Mix the content of the micro wells by gently tapping the plate or use micro plate shaker.
- 6.7 **Short Incubation**-Cover the plate and incubate for 60 minutes at 37°C. The plate should be tightly sealed or incubated in a humid chamber using plate covers.
- 6.8 **Overnight incubation**- Cover the plate and incubate overnight for 14-18 hours at 2-8°C. The plate should be tightly sealed or incubated in a humid chamber using plate covers
- 6.9 Remove the solution and wash each well with 300 μ L of wash solution 3 times. Avoid the plate drying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
- 6.10 Dispense 100 μ L conjugate in to each well.
- 6.11 Incubate for 60 minutes at 37 °C. The plate should be tightly sealed or incubate in a humid chamber using plate covers.
- 6.12 Repeat step 9
- 6.13 Dispense 100 μ L of TMB substrate N.12 into each well.
- 6.14 Incubate at 18-26°C for 15 minutes.
- 6.15 Dispense 100 μ L of stop solution N.3 into each well.
- 6.16 Read the results at a wavelength of 450 nm.

7. RESULT INTERPRETATION

Sample A (450) – NC

$$\text{SP}\% = 100 \frac{\text{Sample A (450) – NC}}{\text{PC - NC}}$$

Negative - SP % < 80

Positive - SP % \geq 80

8. WASTE DISPOSAL

Treat it as the infectious material and dispose accordingly.

9. RISK ASSESSMENT

- 9.1 Handle all reagents and samples as bio-hazardous material.
- 9.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- 9.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontaminated with bleach or other strong oxidizing agent before disposal
- 9.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
- 9.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be compromised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.



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NATIONAL VETERINARY LABORATORY
STANDARD OPERATING PROCEDURE**



9.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.

9.7 Do not use this kit after the expiration date.

9.8 Never pipette by mouth. Allow all reagents to come to room temperature before starting.

10. TROUBLESHOOTING

NA

11. REFERENCES

IDEXX Brucellosis Serum X 2 kit manual 06-04130-14



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SOP No: NCAH/LSU/SERO 03

Title: SOP on ELISA for Avian Leukosis Complex Ag

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Application/Distribution: NCAH



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1. SCOPE

This ELISA test is used for the detection of Avian Leukosis Complex (ALC) antigen in poultry samples, such as serum or plasma. It is intended for surveillance, early diagnosis, and monitoring of ALC infection in flocks to support disease control and management programs in poultry farms in Bhutan.

2. TEST PRINCIPLES

This assay is designed to detect p27, an antigen common to all subgroup of ALV including endogenous viruses. The recommended sample types are light albumin and cloacal samples. While serum has been validated for use on the ALV-AG test, it is not a recommended sample for the detection of exogenous virus because of potential interference from endogenous sequence. A microtitration format has been developed in which anti-p27 antibody is coated onto 96-well plates.

3. OBJECTIVE

IDEXX ALV AG is an enzyme immunoassay from IDEXX for the detection of avian leukosis virus Antigen p27 in chicken serum, Cloacal or albumin samples.

4. APPARATUS/TEST KIT/REAGENTS

IDEXX ALV Ag

- 4.1 Anti-27 Antibody coated plate
- 4.2 Positive control
- 4.3 Negative control
- 4.4 Conjugate
- 4.5 Sample Diluent
- 4.6 TMB Substrate N12
- 4.7 Stop Solution N.3
- 4.8 Albumin Wash Concentration (20X). **Dilute the wash Concentrate (10X) 1:20 with distilled water.**

5. PROCEDURE

A. Preparation of samples

- Albumin- collect light albumin and add directly to the plate without prior dilution. Freeze/thaw the sample to help reduce the viscosity.
- Cloacal swabs – place cloacal swab into 1mL culture media or sample diluents and freeze. Prior to testing, bring the sample to 18-26°C and allow coarse material to settle. Pipette 100µL of the supernatant directly onto the ELISA plate.
- Serum – for general detection of p27, the sample is added directly to the well prior dilution. Testing of serum samples for exogenous derived p27 is not recommended because of interference from endogenous virus.

All the reagents must be allowed to come to 18-26°C before use. Mix reagents by gently inverting or swirling.

- 5.1 Obtain coated plate and record the sample position.
- 5.2 Dispense 10 µL of the un diluted Positive control into duplicate wells



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STANDARD OPERATING PROCEDURE**



- 5.3 Dispense 10 μ L of the un diluted Negative control into duplicate wells
- 5.4 Dispense 10 μ L of samples in to appropriate wells. Samples may be tested in duplicate but a single well is acceptable. None of the samples are diluted for testing.
- 5.5 Cover the plate and incubate for 60 minutes at 18 - 26°C.
- 5.6 Wash each well with 350 μ L of distilled water 3-5 times. Aspirate completely.
- 5.7 Dispense 100 μ L conjugate in to each well.
- 5.8 Incubate for 60 minutes at 18 - 26°C.
- 5.9 Repeat step 6.
- 5.10 Dispense 100 μ L of TMB substrate into each well.
- 5.11 Incubate at 18 - 26°C for 15 minutes.
- 5.12 Dispense 100 μ L of stop solution into each well.
- 5.13 Read the results at a wavelength of 650 nm.

6. RESULT INTERPRETATION and REPORTING

$$\text{SP ratio} = \frac{\text{Sample mean} - \text{NC}}{\text{PC-NC}}$$

Negative - SP ratio \leq 0.20

Positive - SP ratio \geq 0.20

7. WASTE DISPOSAL

Treat the samples and the reagents as infectious agent and dispose properly.

8. RISK ASSESSMENT

- Samples may contain infectious agents (ALV)
- Risk through contact or aerosols
- Stop solution (acid) and reagents may irritate skin/eyes
- Injury from pipette tips or spills
- Electrical equipment hazard
- Cross-contamination and wrong pipetting

9. TROUBLESHOOTING

NA

10. REFERENCES

Avian Leukosis Complex Virus Ag test kit 06-01154-15 manual



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SOP No: NCAH/LSU/SERO 04

Title: SOP on Ab ELISA for Mycobacterium Paratuberculosis

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Application/Distribution: NCAH



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1. SCOPE

This ELISA test is intended for the detection of antibodies against *Mycobacterium paratuberculosis* in ruminant serum or plasma samples. It is used for the diagnosis, surveillance, and monitoring of Johne's disease in cattle, sheep, and goats, supporting herd health management and control programs in Bhutan.

2. TEST PRINCIPLES

Microplates are coated with MAP Antigen. Samples to be tested are pre-incubated with *Mycobacterium phlei* extract in order to bind unspecific Antibodies. Then samples are transferred and incubated in the wells of coated microplate. Upon incubation of the samples in the coated wells, MAP specific Antibodies form a Antibody-Antigen immune-complex.

3. OBJECTIVE

Paratuberculosis Screening is IDEXX enzyme immunoassay for the detection of antibodies directed against *Mycobacterium avium* spp paratuberculosis (MAP) in bovine individual serum, plasma and milk samples, and in individual serum and plasma samples from small ruminants.

4. APPARATUS/TEST KIT/REAGENTS

IDEXX Paratuberculosis Screening

- 4.1 MAP Antigen coated plate
- 4.2 Positive control
- 4.3 Negative control
- 4.4 Conjugate Concentrate
- 4.5 Dilution Buffer N.1
- 4.6 Dilution Buffer N.12
- 4.7 TBM Substrate N.9
- 4.8 Stop Solution N.3
- 4.9 Albumin Wash Concentration (20X). **Dilute the wash Concentrate (10X) 1:20 with distilled water.**

5. PROCEDURE

A. Preparation of samples

- Samples and Controls are pre-diluted and pre-incubated on an uncoated predilution microplate. Milk samples: defatted milk (obtained after centrifugation) as well as a whole milk sample can be implemented.

All the reagents must be allowed to come to 18-26oC before use. Mix reagents by gently inverting or swirling.

- 5.1 Obtain coated plate and record the sample position.
- 5.2 Dispense 10 μ L of the un diluted Positive control into duplicate wells
- 5.3 Dispense 10 μ L of the un diluted Negative control into duplicate wells
- 5.4 Dispense 10 μ L of samples in to appropriate wells. Samples may be tested in duplicate but a single well is acceptable. None of the samples are diluted for testing.
- 5.5 Cover the plate and incubate for 60 minutes at 18 - 26°C.



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- 5.6 Wash each well with 350 μ L of distilled water 3-5 times. Aspirate completely.
- 5.7 Dispense 100 μ L conjugate in to each well.
- 5.8 Incubate for 60 minutes at at 18 - 26°C.
- 5.9 Repeat step 6
- 5.10 Dispense 100 μ L of TMB substrate into each well.
- 5.11 Incubate at 18 - 26°C for 15 minutes.
- 5.12 Dispense 100 μ L of stop solution N.3 into each well.
- 5.13 Read the results at a wavelength of 650 nm.

6. RESULT INTERPRETATION

$$\text{SP ratio} = \frac{\text{Sample mean} - \text{NC}}{\text{PC-NC}}$$

Negative - SP ratio \leq 0.20

Positive - SP ration \geq 0.20

7. WASTE DISPOSAL

Consider all the specimens and reagents as infectious material and dispose it properly.

8. RISK ASSESSMENT

NA

9. TROUBLESHOOTING

NA

10. REFERENCES

Mycobacterium paratuberculosis Ab test int 06-07130-23 manual



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<i>SOP No:</i> NCAH/LSU/SERO 05
<i>Title:</i> SOP on ELISA for detection of NSP antibodies against FMD virus
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<i>Prepared by:</i> Serology Laboratory service unit National center for Animal Health
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<i>Application/Distribution:</i> NCAH



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1. SCOPE

This ELISA test is designed for the detection of non-structural protein (NSP) antibodies against Foot-and-Mouth Disease (FMD) virus in the serum of susceptible animals. It is intended for differentiating infected from vaccinated animals (DIVA), supporting surveillance, outbreak investigation, and control programs in Bhutan's livestock population.

2. PRINCIPLES

2.1 The PrioCHECK® FMDV NS is a blocking ELISA. The Test Plates are coated with 3ABC specific monoclonal antibody (mAb), followed by incubation with antigen (3ABC protein). Consequently, Test Plates of the kit contain FMDV NS antigen captured by the coated mAb. The test is performed by dispensing the test samples to the wells of a Test Plate. After incubation the plate is washed and the Conjugate is added. FMDV NS specific antibodies, directed against the non- structural proteins, that may be present in the test sample will bind to the 3ABC protein and will block the binding of the mAb-HRPO. After incubation, the plate is washed and the Chromogen (TMB) Substrate is dispensed. After incubation at room temperature ($22\pm 3^{\circ}\text{C}$) the color development is stopped. Color development measured optically at a wavelength of 450 nm shows the presence of antibodies directed against Foot and Mouth Disease Virus. The PrioCHECK® FMDV NS is a single dilution test. Serum samples are tested in a 1:5 dilution.

2.2 PD-FMD DIVA Kit: Sero-conversion against NSPs (3AB3) is observed since 10- 14 days after FMD virus infection. Whereas if the animal is not exposed to FMD virus infection but vaccinated with inactivated purified polyvalent FMD vaccine, no anti-NSP immune response is elicited in host's body. This differential induction of anti-NSP antibody is exploited in DIVA ELISA to discriminate between infected and vaccinated animals. In this DIVA test reactivity of anti-3AB3 antibodies present in the serum of an infected animal (bovine species only) is assessed against purified recombinant 3AB3 (~38 kD) NSP in an indirect ELISA format. A sample producing OD value more than the fixed cut-off ratio $\{(\text{test serum sample mean OD}/\text{positive control serum mean OD}) \times 100 \text{ i.e., percent positivity value or PP value} > 40\%$ } is qualitatively diagnosed as positive for FMD infection. The present DIVA kit as described here is a recombinant 3AB3 NSP based indirect ELISA kit designed, developed and evaluated using in-house produced and standardized reagents at the central FMD laboratory, Project Directorate on FMD, Mukteswar. This DIVA kit is very much in toe with the OIE approved test as a companion test for inactivated vaccines.

3. APPLICATION

This test is done to differentiate between vaccine antibody and infection antibody. It is also done to monitor the sero-conversion against NSPs (3ABC and 3AB3) specific monoclonal antibody.

4. OBJECTIVE

To describe the procedure for conducting the ELISA for NSP antibodies against FMD virus in serum.



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STANDARD OPERATING PROCEDURE**



5. APPARATUS/TEST KIT/REAGENTS AND BUFFER

- 5.1 Negative Control (Ready-to-use) - One vial contains 0.6 ml Negative Control.
- 5.2 Chromogen (TMB) Substrate (Ready-to-use) One vial contains 60 ml Chromogen (TMB) Substrate.
- 5.3 Stop Solution (Ready-to-use) - One vial contains 60 ml Stop Solution.
- 5.4 Package Insert, 10 plate sealers
- 5.5 Certificate of Analysis

Kit Components (Prionics NS ELISA)

- a. Five Test Plates are delivered in bags which contain a desiccant sachet.
- b. Conjugate (30x) (30x concentrated, dilute before use). One vial contains 2.5 ml Conjugate.
- c. Dilution Buffer (2x) (2x concentrated, dilute before use). One vial contains 60 ml Dilution Buffer.
- d. Additive (lyophilized) (Reconstitute and dilute before use). Five vials, each contains 2.5 ml lyophilized Additive. Shelf life of reconstituted additive: until expiry date at - 20°C.
- e. Two vials, each contains 10 ml Demineralized Water.
- f. Washing Fluid (200x) (200x concentrated, dilute before use). One vial contains 60 ml washing Fluid.
- g. Positive Control (Ready-to-use) - One vial contains 0.6 ml Positive Control.
- h. Weak Positive Control (Ready-to-use) - One vial contains 0.6 ml Weak Positive Control.
- i. Negative Control (Ready-to-use) - One vial contains 0.6 ml Negative Control.
- j. Chromogen (TMB) Substrate (Ready-to-use) One vial contains 60 ml Chromogen (TMB) Substrate.
- k. Stop Solution (Ready-to-use) - One vial contains 60 ml Stop Solution.
- l. Package Insert, 10 plate sealers
- m. Certificate of Analysis

PD-FMD DIVA Kits contents

- a. 96-well Nunc Maxisorp ELISA plates (Cat. No.442404): 10 plates
- b. Freeze dried recombinant 3AB3 protein (~38 kD): Ten vials (Store at -200C)
- c. Each vial is to be dissolved in 1ml of Carbonate-Bicarbonate coating buffer, pH 9.5 and then added to 4.5 ml of additional coating buffer. This reconstituted vial is sufficient for coating one 96-well immunoplates. After reconstitution the vial is to be used immediately without storing further.
- d. Freeze-dried positive and negative control sera: One vial each (Store at - 200C)
- e. Each vial is to be dissolved in 160 µl of distilled water and then distributed into single use aliquots for storage at -200C, if to be consumed in more than a month's time or else store the entire reconstituted vial at 40C. Working dilution of 1:20 in diluent buffer (as appears in the protocol) for control serum is recommended.
- ii. *E. coli* lysate: One vial (Store at -200C)
 - a. Each vial of *E. coli* lysate is to be reconstituted with 70 µl of PBS and stored at -200C.



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- b. Lapin Anti Bovine-HRP conjugate (DAKO; Cat. No.P0159): 35 µl (Store at 40C)
- c. Skimmed milk powder (Merck; Cat. No.1.15363.0500): 8 gram
- d. Chicken serum {Sera Laboratories International Ltd. (SLI); Cat. No. S-606-HSL}: 25 mL (Store at -200C)
- e. Salts for PBS, pH 7.4
- f. Tween-20: 5mL
- g. Carbonate-Bicarbonate coating buffer (Sigma; Cat.No.C3041): 1 capsule (for preparing 100 mL of buffer)
- h. Phosphate-Citrate substrate buffer (Sigma; Cat.No.P4809): 1 tablet (for preparing 100 mL of buffer)
- i. OPD (Sigma; Cat.No.P1526-100G): 50 mg (Store at -200C)
- j. 30 % H₂O₂ (Merck; Cat.No.1.07210.0250): 50 µl
- k. 1M H₂SO₄: 70mL

Additional Material required

- i. Plate Reader e.g. Multiscan EX or equivalent. The reader has to have an appropriate filter set to read the plates at 450 nm.
- ii. Plate washer e.g. Tecan EIA Tray Washer or equivalent.

6. PROCEDURE

Prio CHECK FMDV NS ELISA

SOLUTIONS TO BE MADE IN ADVANCE Dilution buffer working solution

- Dilute Dilution Buffer (2x) (Component 3) 1/2 in demineralized water; e.g. for one Test Plate prepare 24 ml (add 12 ml Dilution Buffer (2x) to 12 ml demineralized water). Can be stored at 5±3°C for up to 24 hours.
- Equilibrate the vial to 22±3°C and reconstitute 1 the Additive (Component 4) with 2.5 ml Demineralized Water (Component 5). Can be stored at -20oC until expiry date.
- Dilute reconstituted additive 1/10 in dilution buffer working solution; e.g. for one Test Plate prepare 24 ml (add 2.4 ml reconstituted additive to 21.6 ml dilution buffer working solution). Unused ELISA buffer can be stored at 5±3°C for up to 24 hours.
- Conjugate dilution
- Dilute Conjugate (30x) (Component 2) 1/30 in ELISA buffer; e.g. for one plate prepare 12 ml (add 400 µl Conjugate (30x) to 11.6 ml ELISA buffer). The diluted conjugate must be prepared just before use.
- Dilute Washing Fluid (200x) (Component 6) 1/200 in demineralized water. The amount of Washing Fluid is sufficient to prepare a final volume of 12 liters washing solution. Stability of washing solution: 1 week stored at 22±3°C.
- Reconstitution of the lyophilized Additive should be performed as follows:
- Equilibrate the vial to 22±3°C.
 - ✓ With the vial in an upright position, tap the vial gently against the worktop to ensure that the content is on the bottom of the vial.
 - ✓ Carefully open the vial.
 - ✓ Add the specified amount of Demineralized Water (Component 5).



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- ✓ Replace the stopper on the vial and allow the lyophilized material to dissolve.
- ✓ Gently agitate the vial so that any remaining dry material will be dissolved.
- ✓ Allow the material to stand at least for 15 minutes at $22\pm 3^{\circ}\text{C}$ before use.
- ✓ Mix gently and intermittently (formation of foam should be avoided).

b. DAY 1 (incubation with test serum)

- Dispense 80 μl ELISA buffer to all wells of the Test Plate (Component 1).
- Dispense 20 μl of Negative Control (Component 9) to wells A1 and B1.
- Dispense 20 μl of Weak Positive Control (Component 8) to wells C1 and D1.
- Dispense 20 μl of Positive Control (Component 7) to wells E1 and F1.
- Dispense 20 μl of test samples to the remaining wells.
- Seal the Test Plate using the enclosed plate sealers.
- Shake the Test Plate gently.
- Incubate overnight (16–18 hours) at $22\pm 3^{\circ}\text{C}$.

c. DAY 2 incubation with conjugate

- Empty the Test Plate after the incubation period and wash the plate 6 times with 200 to 300 μl washing solution. Tap the plate firmly after the last washing step.
- Dispense 100 μl of diluted conjugate to all wells.
- Seal the Test Plate using the enclosed plate sealers.
- Incubate 60 ± 5 minutes at $22\pm 3^{\circ}\text{C}$.

d. Incubation with chromogen (TMB) substrate

- Empty the Test Plate after the incubation period and wash the plate 6 times with 200 to 300 μl washing solution. Tap the plate firmly after the last washing step.
- Dispense 100 μl of Chromogen (TMB) Substrate (Component 10) to all wells.
- Incubate 20 minutes at $22\pm 3^{\circ}\text{C}$.
- Add 100 μl of Stop Solution (Component 11) to all wells.
- Mix the content of the wells of the Test Plate prior to measuring.
- Note: Start the addition of Stop Solution 20 minutes after the first well was filled with Chromogen (TMB) Substrate. Add the Stop Solution in the same order and at the same pace as the Chromogen (TMB) Substrate was dispensed

e. PD-FMD DIVA ELISA

- I. Dissolve the freeze-dried recombinant protein in the vial with 1 ml of coating buffer and add another 4.5 ml of coating buffer to it (One vial is sufficient for coating one 96-well ELISA plates). The reconstituted protein vial should be used for coating immediately without storing. Reconstitute the freeze-dried vials of positive (one-month postinfection serum) and negative (one month post vaccination serum) control serum with distilled water as appears on the sticker and store at -20°C after reconstitution in single use aliquots. Dissolve the content of freeze-dried vial carrying *E. coli* lysate in 70 μl of PBS and store at -20°C after use.
- II. Coat 96-well polystyrene (Nunc Maxisorp) Immuno plates with the diluted recombinant protein @ 50 μl per well (~50 ng of purified recombinant protein per well). Tap the plate gently from all sides and incubate (refrigerate) the plate at 4°C for overnight.
- III. Remove the plates from the refrigerator and thaw them at 37°C for 15 minutes.



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- IV. In a low protein binding Perspex plate dilute the test and the supplied negative and positive control sera @ 1:20 in diluent buffer. Only serum from bovine host is compatible with this test. Prepare a total volume of 220 µl of diluted serum so that 100 µl of the mixture can be transferred to the coated ELISA plates in duplicate. On a coated ELISA plate, a total of 45 test sera samples, one positive and one negative control serum and two background controls can be accommodated. For background controls only 100 µl of diluent buffer is dispensed without any serum.
- V. Give three continuous washes (no hold time) with wash buffer.
- VI. Transfer 100 µl of the serum and diluent buffer mixture from Perspex plate to the ELISA plate in duplicate wells. Incubate for 1 hour at 37°C and tap the plate gently from all sides at every 15-minute intervals or incubate for 30 minutes in a plate shaker at 37°C with 20-30 rpm.
- VII. Give three washes of 3-minute soak period each.
- VIII. Dispense anti-bovine-HRP conjugate diluted in the diluent buffer (1:2000) @ 50 µl per well. Incubate for 1 hour at 37°C and tap the plate gently from all sides at every 15-minute intervals or incubate for 30 minutes in a plate shaker at 37°C with 20-30 rpm. 6ml of diluted conjugate solution is sufficient for an ELISA plate.
- IX. Give three washes of 5-minute soak period each.
- X. Add freshly prepared substrate solution @ 50 µl per well and incubate for 15 minutes at 37°C without shaking. Then stop the color reaction by adding 1M H₂SO₄ @ 50 µl per well.
- XI. Measure the optical density values at wave length of 492 nm (Reference 620 nm).

7. RESULT INTERPRETATION

a. The PrioCHECK FMDV NS ELISA

- Measure the optical density (OD) of the wells 450 nm within 15 minutes after color development has been stopped.
- Calculate the mean OD₄₅₀ value of wells A1 and B1 (Negative Control = OD₄₅₀ max).
- The percentage inhibition (PI) of the Controls and the test sera are calculated according to the formula below.
- The OD₄₅₀ values of all samples are expressed as

Percentage Inhibition (PI) relative to the OD₄₅₀ max.

$$\left| \begin{array}{c} \text{OD}_{450} \text{ test sample} \\ \text{PI} = 100 - \frac{\text{-----}}{\text{OD}_{450} \text{ max}} \end{array} \right| \times 100$$

If the OD₄₅₀ of a test sample is higher than the OD₄₅₀ max, the Percent Inhibition can be interpreted as 0%. **Interpretation of the percent inhibition**



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b. PD-FMD DIVA

- To reduce inter-run variation due to differences in absolute absorbance between runs, final results for each test serum needs to be expressed as the PP value $\{(\text{test serum sample mean OD} / \text{positive control serum mean OD}) \times 100 \text{ i.e., percent positivity value or PP value } \geq 40\%$ }, calculated by dividing the reaction of the test serum by that of the positive control serum and then multiplying with 100. The results should be interpreted based on the following cut-off zones.

1. Precaution /Limitation of Procedure

If the mean OD450 of the Negative Control is below 1.000 possibly the Chromogen (TMB) Substrate is too cold.

In that case warm the solution to $22 \pm 3^\circ\text{C}$ or incubate up to 30 minutes. If the mean OD450 of the Negative Control is above 2.000 a shorter incubation period with Chromogen (TMB) Substrate is recommended.

2. Reporting

2.1. The PrioCHECK FMDV NS ELISA

PI = $< 50\%$ (negative)

Antibodies against the NS protein of FMDV are absent in the test sample.

PI = 50% (positive)

Antibodies against the NS protein of FMDV are present in the test sample.

Quality Control

2.2. PrioCHECK FMDV NS ELISA

- The OD450 max (mean OD450 of the Negative Control) must be > 1.000 .
- The mean percentage inhibition of the Weak Positive Control must be $> 50\%$.
- The mean percentage inhibition of the Positive Control must be $> 70\%$.
- Not meeting any of these criteria is reason to discard the results of that specific Test Plate.

2.3. PD-FMD DIVA - the test is to be considered valid provided the mean absorbance of the positive control wells is not less than 0.8. Likewise, the plate has to be rejected if the mean absorbance of the supplied negative control serum is > 0.3 . The O.D. in back ground control wells should be less than 0.1.

2.4. PD-FMD ELISA

- 3AB3 NSP reactivity positive: If PP value is more than 40%
- 3AB3 NSP reactivity negative: If PP value is less than 40%

8. WASTE DISPOSAL

All sample leftover materials are disinfected with 2% Virkon solution and other infectious materials are autoclaved.

9. RISK ASSESSMENT

9.1 Handle all reagents and samples as bio-hazardous material.



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- 9.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- 9.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontaminated with bleach or other strong oxidizing agent before disposal
- 9.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
- 9.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be compromised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
- 9.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
- 9.7 Do not use this kit after the expiration date.
- 9.8 Never pipette by mouth. Allow all reagents to come to room temperature before starting!

10. TROUBLESHOOTING

NA

11. REFERENCES

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<i>SOP No:</i> NCAH/LSU/SERO 06
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<i>Application/Distribution:</i> NCAH



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1. SCOPE

This ELISA test is intended for the detection of antibodies against *Mycoplasma* species in the serum or plasma of livestock. It is used for the diagnosis, surveillance, and monitoring of mycoplasmosis, supporting herd health management and control programs in Bhutan.

2. PRINCIPLES

The assay is designed to measure antibody bonded to antigen coated plates. The principle of the test is as follows: Serum obtained from chickens or turkeys exposed to MG or MS antigens contain specific MG or MS antibodies. Serum, diluted in Dilution Buffer, is added to an antigen coated plate. Specific antibody in the serum forms an antibody-antigen complex with the antigen bound to the plate. After washing the plate, an affinity purified goat anti-chicken IgG (H+L) peroxidase conjugate is added to each well. The antibody-antigen complex remaining from the previous step binds with the conjugate. After a brief incubation period, the unbound conjugate is removed by a second wash step. Substrate, which contains a chromagen (ABTS), is added to each well. Chromagen color change (from clear to green- blue) occurs in the presence of the peroxidase enzyme. The relative intensity of color developed in 15 minutes (compared to controls) is directly proportional to the level of antibody in the serum. After the substrate has incubated, Stop Solution is added to each well to terminate the reaction and the plate is read using an ELISA plate reader at 405-410 nm.

3. APPLICATION

The kit is used to diagnose *Mycoplasma* infection in chicken and turkey.

4. OBJECTIVE

The Synbiotics' ProFLOK® MG/MS Combo ELISA kit is a presumptive *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) screening test for the detection of MG and MS antibodies in chicken and turkey serum.

5. APPARATUS/TEST KIT/REAGENTS

- 5.1 1 antigen coated plate (MGIMS)
- 5.2 10 □1 Positive Control Serum (MGIMS)
- 5.3 10 □1 Normal Control Serum (MGIMS)
- 5.4 100 □1 Goat anti-Chicken 19G (H+L) Peroxidase Conjugate Solution (MGIMS)
- 5.5 40 mL Dilution Buffer Plus
- 5.6 10 mL ABTS-Hydrogen Peroxide Substrate Solution
- 5.7 2.5 mL 5X Stop Solution, 5% SDS (dilute [1:5] with laboratory grade water)
- 5.8 20 mL 20X Wash Solution (dilute [1:20] with laboratory grade water)
- 5.9 High precision pipette (i.e. 1-20 microliter pipette)
- 5.10 0.2 mL, 1.0 mL, and 5.0 mL pipettes
- 5.11 8 or 12 channel pipette (or transplating device) and pipette tips
- 5.12 2 graduated cylinders (50 mL)
- 5.13 1 mL or 5 mL borosilicate glass test tubes
- 5.14 Uncoated low binding 96 well test plates (i.e. Nunc catalog #269620)
- 5.15 Laboratory grade (distilled or R.O.) water



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- 5.16 96 well plate reading spectrophotometer with 405-410 nm filter
- 5.17 Plate washing apparatus
- 5.18 Waste container with bleach or another oxidizing agent

6. PREPARATION OF REAGENTS AND SOLUTIONS

Dilute serum samples using Dilution Buffer in a clean, uncoated 96 well microtiter plate. Frozen serum samples should be completely thawed and thoroughly mixed before diluting. Set up samples and controls as shown in Figure 1.

A	1 (+)	2 (-)	3 (+)	4 (1)	5 (2)	6 (3)	7 (4)	8 (5)	9 (6)	10 (7)	11 (8)	12 (9)
B	10	11	12	13	14	15	16	17	18	19	20	21
C	22	23	24	25	26	27	28	29	30	31	32	33
D	34	35	36	37	38	39	40	41	42	43	44	45
E	46	47	48	49	50	51	52	53	54	55	56	57
F	58	59	60	61	62	63	64	65	66	67	68	69
G	70	71	72	73	74	75	76	77	78	79	80	81
H	82	83	84	85	86	87	88	89	90	-	+	-

- 6.1 Add 300 μ l, Dilution Buffer to each well of an uncoated 96 well microtiter plate. This plate is referred to as the serum dilution plate.
- 6.2 Add 6 μ l unknown serum per well as per Figure 1 (producing a 1:50 dilution). Start with well A4 and end with well H9 (moving left to right, row by row of wells). For example, wells 1 through 30 contain the diluted sera of flock 1 and wells 31-60 contain the diluted sera of flock 2, etc.
- 6.3 Add 6 μ l of Normal Control Serum (producing a 1:50 dilution) to wells A2, H10, and H12.
- 6.4 Aspirate and remove any liquid in dilution plate wells A1, A3, and H11.
- 6.5 Allow all diluted serums to equilibrate in Dilution Buffer for 5 minutes before transferring to an antigen coated ELISA plate.
- 6.6 Diluted serum should be tested within 24 hours. This dilution format provides adequate quantities of diluted serum samples to conduct three additional ProFLOK® ELISA tests using the same serum dilution plate.

A. Preparation of Positive Control

A Positive Control Serum for each agent has been provided with this kit. Dilute the appropriate volume of the appropriate Positive Control Serum with Dilution Buffer (1:50) in a clean, glass test tube. For example, dilute 6 μ l. of positive control serum in 300 μ l. Dilution Buffer. Mix well. 150 μ l, of diluted Positive Control is needed per ELISA plate.

B. Preparation of Conjugate Solution

The horseradish peroxidase conjugated anti-chicken IgG (H+L) is supplied in HRP



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stabilizer. Dilute 100 μ l stock conjugate in 10 mL Dilution Buffer (1:100 dilutions). Mix well. This 10 mL preparation will supply sufficient conjugate for one 96 well ELISA plate.

C. Preparation of 1X Wash Solution

Dilute 20 mL concentrated Wash Solution in 380 mL laboratory grade (distilled or R.O.) water (1:20). Mix well. Approximately 400 mL Wash Solution is needed for each 96 well ELISA plate.

D. Preparation of the Substrate Solution

The Substrate Solution is ready to use. Each plate will require approximately 10 mL substrate solution. For best results, the substrate solution must be equilibrated to room temperature before use.

E. Preparation of 1X Stop Solution

Dilute 2.5 mL concentrated Stop Solution in 10 mL laboratory grade (distilled or R.O.) water (1:5). Mix well. Approximately 12.5 mL Stop Solution is needed for each 96 well ELISA plate.

F. TEST PROCEDURE

- Remove an antigen coated test plate from the protective bag and label according to the dilution plate identification.
- Add 50 μ l, Dilution Buffer to all wells on the test plate.
- Add 50 μ l, diluted Positive Control Serum to wells A1, A3, and H11. Discard pipette tip.
- Using an 8 or 12 channel pipette transfer 50 μ l/well of each of the diluted serum samples and Normal Control Serum samples from the dilution plate to the corresponding wells of the coated test plate (yields a 1: 100 dilutions. Discard pipette tips after each row of sample is transferred of samples to the ELISA plate should be done as quickly as possible.
- Incubate plate for 30 minutes at room temperature.
- Tap out liquid from each well into an appropriate vessel containing bleach or other decontamination agent.
- Using an 8 or 12 channel pipette (or comparable automatic washing device), fill each well with approximately 300 μ l, Wash Solution. Allow to soak in wells for 3 minutes; then discard contents into an appropriate waste container (waste container should contain bleach solution). Tap inverted plate to ensure that all residual liquid is removed.
- Repeat wash procedure 2 more times.
- Using an 8 or 12 channel pipette (or transplanting device) dispense 100 μ l, diluted conjugate (prepared as described above) into each assay well. Discard pipette tips.
- Incubate for 30 minutes at room temperature. Wash as in steps above.
- Using an 8 or 12 channel pipette (or transplanting device) dispense 100 μ l, diluted Substrate Solution (prepared as described above) into each test well. Discard pipette tips.



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- Incubate 15 minutes at room temperature.
- Using an 8 or 12 channel pipette (or transplanting device) dispense 100 μ l, diluted Stop Solution (prepared as described above) into each test well.
- Allow bubbles to dissipate before reading plate.
- Read the plate using an ELISA plate reader set at 405-410 nm. Be sure to blank the reader as directed.

9 RESULT INTERPRETATION

- 9.1 Calculate the average Positive Control Serum absorbance (Optical Density [O.D.]) using the absorbance values of wells A1, A3, and H11. Calculate the Normal Control Serum absorbance using values obtained from wells A2, H10, and H12. Record both averages.
- 9.2 Subtract the average normal control absorbance from the average positive absorbance. The difference is the Corrected Positive Control
- 9.3 Calculate a sample to positive (Sp) ratio by subtracting the average normal control absorbance from each sample absorbance. The difference is divided by the corrected positive control. Use the following equation format:

(SAMPLE ABSORBANCE - AVERAGE NORMAL CONTROL ABSORBANCE)

SP =

CORRECTED POSITIVE CONTROL ABSORBANCE

An ELISA titer for MGIMS can be calculated by the following suggested equation:

$$\text{LOG}_{10} \text{ TITER} = (1.464 \times \text{LOG}_{10} \text{ Sp}) + 3.197$$

$$\text{TITER} = \text{ANTILOG of LOG}_{10} \text{ TITER}$$

Example:

- Example Positive Control Absorbance: 0.585, 0.610, 0.590
Average = $(0.585 + 0.610 + 0.590) / 3 = 0.595$
- Example Normal Controls: 0.078, 0.067, 0.057
Average = $(0.078 + 0.067 + 0.057) / 3 = 0.067$
 $(0.595) - (0.067) = 0.528$
- Example Sp value calculation: Absorbance of sample = 0.560
 $(0.560) - (0.067) / 0.528 = 0.934$
- Example of Calculation of titer using the Sp from above:
 $\text{Log Titer} = 1.464 \times (\text{Log}_{10} 0.934) + 3.197$
Titer = ANTILOG 3.15
Titer = 1413

10 WASTE DISPOSAL

Consider as infectious materials and dispose the samples and kits properly after the test.

11 RISK ASSESSMENT

- 11.1 Handle all reagents and samples as bio-hazardous material.
- 11.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- 11.3 Wash solution, control sera, test plates, field samples and all other test kit reagents



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- should be properly decontaminated with bleach or other strong oxidizing agent before disposal
- 11.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
 - 11.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be compromised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
 - 11.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
 - 11.7 Do not use this kit after the expiration date.
 - 11.8 Never pipette by mouth. Allow all reagents to come to room temperature before starting.

12 TROUBLESHOOTING

NA

13 REFERENCES

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<i>Application/Distribution: NCAH</i>



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1. SCOPE

The Liquid Phase Blocking ELISA (LPB-ELISA) for Foot-and-Mouth Disease is used to detect and quantify specific antibodies against FMD virus serotypes in serum samples of susceptible animals such as cattle, sheep, goats, and pigs. The test is widely applied in sero-surveillance, post-vaccination monitoring, and epidemiological studies to assess herd immunity and exposure status. It is particularly valuable for differentiating antibody responses to different FMD virus serotypes and for supporting disease control and eradication programs at district and national levels.

2. TEST PRINCIPLES

2.1 PD-FMD

A serial two-fold dilutions of test serum are mixed with equal volume of a constant dose of viral inactivated antigens in a liquid medium and allowed to react overnight at 4°C. The antigen-antibody reaction is carried out in a suspension (or liquid medium), and the antigen is blocked by the homologous antibodies, if present, in the test serum for subsequent detection by guinea pig serum. Next day, the antigens which are not completely blocked by the antibodies in the test serum are trapped to the wells of the ELISA plates by the pre-coated type-specific rabbit antibodies. Antigen and background controls are added containing antigen without serum and blocking buffer respectively as described in plate layout. Subsequently, the presence of antigen is traced by adding pre-titrated guinea pig (type-specific) serum and anti-guinea pig-HRPO conjugate and substrate reaction is followed in a standard ELISA procedure. Color reaction is stopped and measured in terms of optical density (OD) at 492 nm wavelength with reference to 620nm. OD of reference antigen controls are used for the calculation of 50% inhibition in OD for calculation of the titers.

2.2 BDSL, WRL

The test is based upon specific blocking of the FMDV antigen in liquid phase by antibodies in the test serum sample. Rabbit antisera specific for the different serotypes of FMDV are passively adsorbed to polystyrene microwells. After the test serum is allowed to react with the specific FMDV antigen, the test serum/antigen mixture is then transferred to an ELISA plate coated with FMDV trapping antibodies. The presence of antibodies to FMDV in the serum sample will result in the formation of immune complexes and consequently reduce the amount of free antigen trapped by the immobilized rabbit antisera. In turn, fewer guinea pig anti-FMDV detecting antibodies will react in the next incubation step. After the addition of enzyme-labelled (HRP) anti-guinea pig Ig conjugate and substrate/chromogen solution, a reduction in colour development will be observed when compared to controls containing free antigen only.

3. APPLICATION

The kit is used for serotyping of FMD in animals.

4. OBJECTIVE



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To describe the procedures for serotyping of FMD in animals.

5. APPARATUS/TEST KIT/REAGENTS

- 5.1 96-well Nunc Maxisorp ELISA plates (cat. No. 442404)
- 5.2 Freeze dried Anti-FMDV coating serum: Type specific anti-146S FMDV serum, raised in rabbits.
- 5.3 Freeze dried Anti-FMDV tracing serum: type specific anti-146S FMV serum, raised in guinea pig.
- 5.4 Freeze dried inactivated Type O, A, and Asia1 antigens.
- 5.5 Anti-guinea pig conjugate: Rabbit/ goat anti-guinea pig immunoglobulin HRPO conjugate (DAKO)
- 5.6 Chromogen: OPD (Orthophenylenediamine dihydrochloride, Sigma)
- 5.7 Hydrogen peroxide (H₂O₂)
- 5.8 Phosphate buffer saline vials (Sigma)
- 5.9 Substrate buffer capsules
- 5.10 Coating buffer tablets
- 5.11 Tween-20

Materials needed but not provided:

- Precision pipettes: multi-channel pipettes variable range from 50 to 200µl & Single channel pipettes variable range from 1 to 20µl, 20 to 100µl, 50 to 200µl and 200 to 1000µl along with disposable tips.
- Distilled water
- H₂SO₄
- Wash bottle fitted with Immunowasher
- 1 container: 1 to 2 liters
- ELISA plate reader, 492 nm filter.
- Photometer: Multiscan type Micro pate ELISA reader with an interference filter of 492nm and reference filter of 620nm.
- Refrigerator: range of +2°C to +6°C
- Freezer: range of -15 to -20°C.
- Incubator: warm wall incubator maintained at +35°C to 37°C.
- pH meter: with accuracy of 0.01pH units or good quality pH strips with varying range from 2 to 10 can be used.
- Glassware/ plastic ware: flasks (50-5000ml), graduated cylinders (10-2000ml), graduated pipettes (1-100ml).
- Weighing balance

6. PROCEDURE

6.1 PD-FMD

Coating: Coat the individual plates with type-specific (O, A, Asia1) rabbit (dilute the serum as specified in the serum vial) serum in coating buffer (Carbonate-Bicarbonate buffer, pH 9.6). Dispense 50µl of diluted serum to each wells of ELISA plate. Tap gently for even distribution of coating solution to wells and cover with lid. Incubate at 37°C for 1 hr. followed by incubation at 4°C overnight.



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Preparation of Ag-Ab mixture: Prepare two-fold dilutions (4 dilutions starting from 1:16) of serum samples in a low binding Perspex plate with PBS containing 0.05% Tween-20. Distribute 75 μ l each dilution of the serum sample to four separate (labeled O, A, and Asia1) Perspex plates. Dilute the antigen as specified in the antigen batch provided. Add equal volume of diluted antigen (75 μ l) to all the distributed serum samples. For antigen control, add equal volume of PBS-Tween 20 (0.05%) to the already diluted antigen. The dilution of each antigen and antibody in this step will be strictly made with PBS containing tween-20 (not the PBS containing 0.1% tween-20 as used for washing of the plate). Incubate the diluted antigen and antibody mixture at 4°C overnight without disturbance.

Washing: Wash the plate by adding washing buffer using immune-wash and discard the contents of wells by abrupt downward hand motion. Repeat the washing 3 times with 5 minutes of hold period between each wash. Slap the inverted microplate 3-4 times onto a dust free absorbent pad to remove all residual contents in the wells.

Dispensing of antigen-antibody (Ag-Ab) mixture: Dispense 50 μ l of the overnight incubated Ag-Ab mixture in duplicate to the ELISA plates as instructed in the plate lay out. Incubate the plates at 37°C for 1 hr.

Wash the plate as in the step 3.

Addition of tracing antibodies: Dilute the guinea pig serum, as specified in the serum vials, in blocking buffer. Add the 50 μ l of diluted tracing serum to each type-specific plate. Incubate at 37°C for 1 hr. Wash the plate as in the step 2.

Conjugate: Add the anti-guinea pig-HRPO conjugate diluted in blocking buffer to all the wells. Incubate at 37°C for 1 hr. Wash the plate as in step 2.

Substrate reaction: Add freshly prepared substrate solution to the plate and keep in the incubator at 37°C for 15 minutes.

Stop the color reaction by adding 50 μ l of stopping solution. Read the plate at 492 nm.

6.2 BDSL, WRL

Coating of Microplates

- Gently agitate the contents of vials of trapping rabbit antibody stock (FMDV serotypes O, A, C, SAT 1, SAT 2, SAT 3 and/or Asia 1).
- Prepare a 1:1000 working dilution of trapping antibody stock in coating buffer in a volume sufficient for the number of plates required (5 ml per plate plus an additional 1 ml, e.g. 6 μ l of rabbit antibody stock in 6 ml of coating buffer) and agitate gently to ensure uniform dispersion.
- For the serotype(s) being tested, immediately dispense 50 μ l volumes of the working dilution of the respective trapping antibody into all 96 wells of appropriately labelled polystyrene microplate(s) (**NUNC Maxisorp**). Make sure that all the plates are aligned correctly (with the letters on the left hand side), as the ELISA reader will only accept the plates in one position. Tap the sides of the microplates to ensure that the trapping antibody is evenly distributed over



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the bottom of each well.

- Cover or seal the microplates and incubate at +1 to 8°C.
- Return the remainder of the aliquot of the FMDV serotype O, A, C, SAT 1, SAT 2, and SAT 3 and/or Asia 1 trapping rabbit antibody stock to 1 to 8°C.

Test and Control Serum Incubation (Liquid phase)

SCREENING ASSAY

- Agitate the test and control sera gently to ensure homogeneity.
- Test and control sera will be added to the wells of the **polypropylene** U-bottom microplate at a dilution of 1/16.
- First, prepare a 1/16 dilution of each control and test sera in suitable dilution tubes (e.g. for each control serum (C++, C+ and C-, add 15 µl of an undiluted control bovine serum to 225 µl of Diluent Buffer A; for each test serum, add 10 µl of undiluted serum to 150 µl of Diluent Buffer A). Again, gently agitate these dilutions to ensure homogeneity. **These volumes are sufficient for 1 microplate; volumes will need to be increased according to the number of microplates being run in the assay.**
- Return the undiluted test sera and the remainder of the undiluted control sera stocks to +1 to 8°C for future use.
- According to the **Plate Layout #1 (Appendix 1)** add 50 µl volumes of prediluted test and control sera to the wells of a **polypropylene** U-bottom microplate. Add 50 µl of Diluent Buffer A to the antigen control (Ca) wells.
- Record details and position of test sera on the **ELISA Data Sheet (Appendix 3)**.

TITRATION ASSAY

Two-fold dilution range (for evaluation of positive sera)

- First prepare a 1/16 dilution of the control sera as in 5.2.1. Next, prepare a 1/8 dilution of test sera in suitable dilution tubes or microplates (e.g. for each test serum add 20 µl of undiluted serum to 140 µl of Diluent Buffer A). **These volumes are sufficient for 1 microplate; volumes will need to be increased according to the number of microplates being run in the assay.** For each run and for each FMDV serotype the C++ control should be additionally titrated in the same manner as a test serum.
- Dispense 50 µl of Diluent Buffer A to all columns 3-12 for each **polypropylene** U- bottom microplate. According to **Plate Layout #2 (Appendix 2)**, add 50 µl volumes of the prediluted control sera to the appropriate wells. Add 50 µl of Diluent Buffer A to the antigen control (Ca) wells.
- According to the **Plate Layout #2**, add 50 µl volumes of prediluted test serum and the C++ control to the appropriate wells of row A or E, columns 3-12. Carefully mix the 100 µl contents in the well by filling and emptying the pipette tip several times, taking care not to introduce air bubbles. This will result in a 100 µl volume of a 1/16 test serum dilution. Transfer 50 µl of this dilution to the next row (A to B) and carefully mix the contents of row B. This will result in a doubling dilution. Transfer 50 µl from row B into the next row (B to C) and repeat the mixing procedure. Transfer 50 µl from row C to row D, mix carefully and finally discard 50 µl of this dilution. This will result in a test



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NATIONAL VETERINARY LABORATORY,
STANDARD OPERATING PROCEDURE



sample dilution series from 1/16 to 1/128 in 50 µl volumes. For the next test sample, repeat the above procedure for rows E through H using new pipette tips.

- Record details and position of test sera on the **ELISA Data Sheet (Appendix 4)**.

Five-fold dilution range (for evaluation of post-vaccinal sera)

- Firstly, prepare a 1/16 dilution of the control sera as in 5.2.1 but for each control serum, add
 - 20 µl of an undiluted control serum to 300 µl of Diluent Buffer A. According to **Plate Layout #2 (Appendix 2)**, add 60 µl volumes of the prediluted control sera to the appropriate wells of the **polypropylene** microplates. Add 60 µl of Diluent Buffer A to the antigen control (Ca) wells.
 - Secondly, prepare a 1/5 dilution of each test serum (and the C++ control) by adding 20 µl of undiluted serum to 80 µl of Diluent Buffer A.
 - Next, add 60 µl of Diluent Buffer A to all columns 3-12 in each **polypropylene** microplate. According to **Plate Layout #2 (Appendix 2)**, add 15 µl volumes of 1/5 diluted test serum (and the C++ control) to the appropriate wells of rows A or E, columns 3-12. This will result in a five-fold dilution. Carefully mix the 75 µl contents in the well by filling and emptying the pipette tip several times, taking care not to introduce air bubbles. This will result in a 75 µl volume of a 1/25 test serum dilution. Transfer 15 µl of this dilution from row A to B and carefully mix the contents of row B. Transfer 15 µl of this dilution from row B to C and repeat the mixing procedure. Transfer 15 µl of this dilution from row C to D, mix carefully and finally discard 15 µl of the dilution from row D. This will result in a test sample dilution series from 1/25 to 1/3125 in 60 µl volumes. For the next test sample, repeat the above procedure for rows E through H using new pipette tips.
- Record details and position of test sera on the **ELISA Data Sheet (Appendix 4)**.

Addition of FMDV Antigen

- Prepare a working dilution of the FMDV antigen (FMDV serotype O, A, C, SAT 1, SAT 2, SAT 3 and/or Asia 1) in Diluent Buffer A in a volume sufficient for the microplates used (5 ml per plate plus an additional 1 ml for two-fold dilution range plates or 6 ml per plate plus an additional 1 ml for five-fold dilution range plates).

Suggested working dilutions:

O1 Manisa	n	1:x
A22 IRQ 24/64	n	1:x
C PHI 7/84	n	1:x
SAT 1 (105)	n	1:x
SAT 2 Eritrea	n	1:x
SAT 3 (309)	n	1:x
Asia 1 Shamir	n	1:x

- For evaluation of positive sera, add 50 µl of the antigen working dilution to all 96 wells of the respective **polypropylene** U-bottom microplate. All wells



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NATIONAL VETERINARY LABORATORY,
STANDARD OPERATING PROCEDURE**



should now contain 100 µl total of a serum dilution and antigen. This will result in a final serum dilution of 1/32 to 1/256 in the titration assay.

- For evaluation of post-vaccinal sera, add 60 µl of the antigen working dilution to all 96 wells of the respective **polypropylene** U-bottom microplate. All wells should now contain 120 µl total of a serum dilution and antigen. This will result in a final serum dilution of 1/50 to 1/6250 in the titration assay.
- Briefly place the microplates on an orbital shaker (or tap the sides of the microplates) to ensure thorough mixing.
- Incubate the **polypropylene** microplates at +1 to 8°C overnight. Do not stack any more than four test plates in order to prevent thermal gradient formation. Seal the top plate or cover with an empty plate.
- **Return the remainder of the master stock antigens to -90 to -50°C for further use.**

Transfer of Serum/Antigen Mixture to the ELISA Plate

- By inverting the microplate and using an abrupt downward hand motion, discharge the contents of all antibody coated microplates (**NUNC Maxisorp**) into a sink or other reservoir and slap the inverted microplates onto a lint-free absorbent towel to remove all residual contents. Using the Handiwash or equivalent, fill all 96 wells of all microplates with wash buffer. After filling, again discharge the contents of the microplates and slap the inverted microplates onto a lint-free absorbent towel to remove all residual contents. Repeat with two more wash cycles of filling and emptying.
- After three complete wash cycles and ensuring that no residual contents are left in the microplates, immediately transfer 50 µl volumes of the serum/antigen mixture from the **polypropylene** U-bottom microplates to the appropriate wells of the different polystyrene microplates (**Nunc Maxisorp**) according to the same plate layout as being used for the "liquid phase".
- Cover or seal the microplates and place on an orbital shaker housed in a '+37°C' warm incubator or hot room and incubate at 35 to 39°C for 1 hour with continuous shaking.

Preparation of Diluent Buffer B

- M Phosphate Buffered Saline, pH 7.4.+/- 0.20 plus 0.05% Tween 20 plus 5% (w/v) Skimmed Milk Powder
- On the day of testing, calculate the amount of Diluent Buffer **B** required and prepare by adding skimmed milk powder to a final concentration of 5% (w/v) to the required volume of Diluent Buffer **A**.
- For example: to prepare 100 ml Diluent Buffer **B** add 5 gm of skimmed milk powder to
- 100 ml of Diluent Buffer **A**.
- Adjust the pH to pH 7.4 +/- 0.20 using 0.1 M NaOH.

Addition of Detecting Antibody

- Immediately before the end of the serum/antigen mixture incubation for the first plate, prepare a 1:100 working dilution from the homologous detecting antibody



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NATIONAL VETERINARY LABORATORY,
STANDARD OPERATING PROCEDURE**



stock (anti- FMDV serotype O, A, C, SAT 1, SAT 2, SAT 3 and/or Asia 1) in Diluent Buffer **B** in a volume sufficient for the plates being used (5 ml of working dilution per plate plus an additional 1 ml, e.g. 60 µl of guinea pig antiserum stock in 6 ml Diluent Buffer **B**).

- Return the remainder of the detecting guinea pig antibody stocks to +1 to 8°C.
- After 1 hour of incubation, remove the microplates from the incubator and wash the microplates with wash buffer as described previously.
- For each serotype, immediately after washing, add 50 µl volumes of the working dilution of the detecting antibody (FMDV serotype O, A, C, SAT 1, SAT 2, SAT 3 and/or Asia 1) into all 96 wells of the respective microplates.
- Tap the sides of the microplates to ensure that the working dilution is evenly distributed over the bottom of each well.
- Cover or seal the microplates and place on an orbital plate shaker housed in a '+37°C' warm incubator or hot room at 35 to 39°C for 1 hour with continuous shaking.

Addition of Conjugate

- Immediately before the end of the detecting antibody incubation, prepare a 1:200 working dilution of the conjugate in Diluent Buffer **B** in a volume sufficient for all microplates (5 ml of working dilution per plate plus an additional 1 ml, e.g. 30 µl of conjugate stock in 6 ml of Diluent Buffer **B**). Both the **conjugate stock** and its working dilution should be handled with care; agitation should be gentle but thorough.
- Return the remainder of the **conjugate stock** to -30 to -5°C.
- After 1 hour of incubation, remove microplates from the incubator and wash with wash buffer as described previously. Immediately after washing, add 50 µl volumes of the working dilution of conjugate into all 96 wells of each microplate. Tap the sides of the microplates to ensure that the conjugate working dilution is evenly distributed over the bottom of each well.
- Cover or seal the microplates and incubate for 1 hour at +35 to 39°C with continuous shaking.

Addition of Substrate/Chromogen and Stopping Solutions

- Immediately before the end of the conjugate incubation, prepare the substrate/chromogen solution in a volume sufficient for the number of plates being run (e.g. for 1 plate, dilute 30 µl of substrate stock (H₂O₂) in 6 ml of chromogen stock solution (OPD)). This represents
- 3.3 mM OPD and 4.4 mM H₂O₂. This final substrate/chromogen solution should be colourless and stored in the dark. If coloured, it should be discarded.
- A clean microplate (not coated with trapping antibody) will be used as the "blinking plate" for the photometric reading (this plate can be washed later and reused for blinking procedures in future tests).
- After 1 hour of conjugate incubation, wash the microplates as described previously. Ensure that all 96 wells of each microplate have been completely flooded with wash buffer to eliminate unreacted conjugate.
- Immediately after washing, add 50 µl volumes of the substrate/chromogen solution to the wells of the microplates, starting with the first column of the



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NATIONAL VETERINARY LABORATORY,
STANDARD OPERATING PROCEDURE**



"blinking plate" followed by all 96 wells of the microplates in the test run. Immediately begin timing after filling the first wells and incubate at ambient temperature for 15 minutes without plate shaking.

- After 15 minutes of substrate/chromogen incubation, immediately add 50 µl volumes of the stopping solution (1.25 M sulphuric acid) starting with the first column of the "blinking plate" followed by all 96 wells of the microplates in the test run. Briefly place the microplates on the shaker (or tap the sides of the microplates) to ensure even mixing. All wells should now contain 50 µl of substrate/chromogen solution plus 50 µl of stopping solution.

Measurement of Substrate Development

- Place the "blinking plate" in the carriage of the photometer and initiate the blinking sequence.
- Place the first microplate of the test run in the carriage of the photometer and initiate the reading sequence. Repeat for each microplate.

7. RESULT INTERPRETATION and REPORTING

7.1 PD-FMD

- Performance of a test can be determined from positive reaction of the known antigens (approximately 1.0) employed in the test and a clear background reaction. The interpretation of the result should be done on the basis of the corrected OD value (OD of test well OD of background well).
- Percent reactivity against each serum dilution is calculated as follows:

$$\text{Mean OD of test wells} \\ \text{\% Reactivity} = \frac{\text{Mean OD of test wells}}{\text{Mean OD of antigen control wells}} \times 100$$

- Titer of serum sample is expressed as the reciprocal of the serum dilution giving 50% optical density (OD) as compared to the antigen control or in other words, reciprocal of the serum dilution which inhibits 50% of the guinea pig serum binding to the homologous virus.

7.2 BDSL, WRL

Data Expression

Microplate readings will be used in two types of data analysis:

A) Percent Inhibition (PI) values which are used for Quality Assurance (QA) acceptance. These PI values are calculated as follows:

$$\text{PI} = 100 - \frac{(\text{Replicate OD of control} \times 100)}{\text{Median OD of Ca}}$$

B) Percentage Inhibition (PI) values which are used for acceptance of replicate values for test sera and diagnostic interpretation. These PI values are calculated as follows:



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NATIONAL VETERINARY LABORATORY,
STANDARD OPERATING PROCEDURE**



$$PI = 100 - \frac{(\text{Replicate OD of test serum} \times 100)}{\text{Median OD of Ca}}$$

Calculation and Acceptance of Control Data

The data expressed in OD values and PI values for the antigen control (Ca) and the data expressed in PI values for the three other controls (C++, C+ and C-) are used to determine whether or not the test has (been) performed within acceptable limits of variability and therefore, whether or not the test sera data may be accepted for any given microplate.

Given that the variation in OD values and PI values of the controls included with the kit should be normally distributed, Upper (UCL) and Lower Control Limits (LCL) for these controls have been established.

Precaution /Limitation of Procedure

- All the glass wares to be used should be clean and sterile.
- Slight drop in pH (less than 7.2) in washing buffer can adversely affect antigen antibody reaction. Ensure correct pH everyday before use.
- Microbial contamination/ precipitation formation of any degree in any of the reagents/ buffer will very much reduce the specificity of the test.
- Mark the plate orientation properly with water proof marker before starting the test to avoid any confusion which may arise later.
- Do not stack plates one over other in incubator.
- Prepare fresh substrate solution every time.
- Do not store prepared buffers more than 1 month.
- Check pH of every buffer every day before starting the test.
- The chromogen (OPD) stock solution and H₂O₂ / OPD solution should be stored in the dark at all times.

Reporting

Diagnostic Interpretation of Test Sera Data

The diagnostic threshold for this assay is set at 50% inhibition (50 PI).

Screening assay

- If both replicate PI values of a test serum fall below 50 PI then that test serum is considered to be negative.
- If either or both replicate PI values of a test serum fall above 50 PI, then that test serum is **tentatively** considered to be positive and should be retested according to the titration assay procedure for both confirmation and estimation of antibody titre.

Two-fold dilution range

- If all replicate PI values of a test serum fall below 50 PI, then that test serum is considered to be negative.
- If one of the two replicate PI values of a test serum dilution of 1/32 fall above 50 PI but all replicate PI values at subsequent dilutions fall below 50 PI, then



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NATIONAL VETERINARY LABORATORY,
STANDARD OPERATING PROCEDURE**



the titer of the test serum is 32 but is considered to be negative.

- If both of the replicate PI values of a test serum dilution of 1/32 fall above 50 PI and all replicate PI values at subsequent dilutions fall below 50 PI, then the titre of the test serum is 45 and is considered to be positive. This is the threshold titer that constitutes positivity.
- The titer of a test serum demonstrating PI values above 50 PI at dilutions greater than 1/32 can be assessed by reference to Table 2.
- An antibody titer of >90 indicates that the animal, at the time of bleeding, was protected against infection from the homologous antigen of the particular FMDV serotype.

Five-fold dilution range

- The titer of the test serum demonstrating replicate PI values above 50 PI can be assessed by reference to Table 3. An antibody titre of = or >112 indicates that the animal, at the time of bleeding, was protected against infection from the homologous antigen of the particular FMDV serotype. The higher the antibody titer, the greater is the confidence of protection.

Quality Control

1. PD-FMD

Performance of a test can be determined from positive reaction of the known antigens (approximately 1.0) employed in the test and a clear background reaction.

2. BDSL, WRL

Calculation and Acceptance of Control Data

- The data expressed in OD values and PI values for the antigen control (Ca) and the data expressed in PI values for the three other controls (C++, C+ and C-) are used to determine whether or not the test has (been) performed within acceptable limits of Variability and therefore, whether or not the test sera data may be accepted for any given microplate.
- Given that the variation in OD values and PI values of the controls included with the kit should be normally distributed, Upper (UCL) and Lower Control Limits (LCL) for these controls have been established.
- The controls should be examined in the following order:

First Level of Microplate Acceptance - Antigen Control (Ca)

- Before calculating PI values, compare the replicate OD values of the Ca control to the UCL and LCL. Both intermediate OD values (i.e. the two values remaining after discarding the lowest and highest values) must fall within these limits. If not, the plate should be rejected.
- Only the two intermediate OD values are used for the calculation of the median Ca OD value and used in subsequent PI calculations.

Second Level of Microplate Acceptance - Antigen Control (Ca), Strong Positive (C++),



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Moderate Positive (C+) and Negative (C-) Control Sera

- For the antigen control (Ca) and for each control serum (C++, C+ and C-), the replicate PI values should be calculated and recorded on the **ELISA DATA SHEET (Appendix 3 or 4)**.

Antigen control (Ca)

- Compare the replicate PI values of the Ca controls to the UCL and LCL for the Ca and use the criteria in Table 1 to accept or reject each **individual** microplate.

Strong Positive, Moderate Positive and Negative Control Sera (C++, C+ and C-)

- Compare the corresponding replicate PI values to the UCL and LCL for the strong positive, moderate positive and negative control sera (C++, C+ and C-) and use the criteria in Table 1 to accept or reject each **individual** microplate.

Table 1. Ca, C++, C+ and C- Control Data

Replicate PI Value		Status
In*	Out**	
4	0	Accept
3	1	Accept
2	2	Reject
1	3	Reject
0	4	Reject

*In Within UCL and LCL

**Out outside UCL and LCL

- Microplate rejected if any one of the Ca, C++, C+ or C- controls fails to meet PI acceptance criteria.

8. WASTE DISPOSAL

Consider it as infectious agent and dispose properly following proper SOP.

9. RISK ASSESSMENT

- Handle all reagents and samples as bio-hazardous material.
- Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly de-contaminated with bleach or other strong oxidizing agent before disposal
- Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
- Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be compromised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.



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9.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.

9.7 Do not use this kit after the expiration date.

9.8 Never pipette by mouth. Allow all reagents to come to room temperature before starting!

10. TROUBLESHOOTING

NA

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1. SCOPE

The ELISA for Newcastle Disease is used to detect and measure antibodies against Newcastle Disease Virus (NDV) in poultry serum samples. It is widely applied for sero-surveillance, evaluation of vaccination programs, and monitoring flock immunity status in chickens and other avian species.

2. PRINCIPLES

Serum obtained from chickens or turkeys exposed to MG or MS antigens contain specific MG or MS antibodies. Serum, diluted in Dilution Buffer, is added to an antigen coated plate. Specific antibody in the serum forms an antibody- antigen complex with the antigen bound to the plate. After washing the plate, an affinity purified goat anti-chicken IgG (H+L) peroxidase conjugate is added to each well. The antibody-antigen complex remaining from the previous step binds with the conjugate. After a brief incubation period, the unbound conjugate is removed by a second wash step. Substrate, which contains a chromagen (ABTS), is added to each well. Chromogen color change (from clear to green- blue) occurs in the presence of the peroxidase enzyme. The relative intensity of color developed in 15 minutes (compared to controls) is directly proportional to the level of antibody in the serum. After the substrate has incubated, Stop Solution is added to each well to terminate the reaction and the plate is read using an ELISA plate reader at 405-410 nm

3. OBJECTIVE

- Assessment of antibody levels in the bird to the NDV group antigen
- To aid in the detection of pre- and post-vaccination NDV antibody levels in chickens.

4. APPARATUS/TEST KIT

- High precision pipette (i.e. 1-20 microliter pipette)
- 0.2 mL, 1.0 mL, and 5.0 mL pipettes
- 8 or 12 channel pipette (or transplating device) and pipette tips
- 2 graduated cylinders (50 mL)
- 1 mL or 5 mL borosilicate glass test tubes
- Uncoated low binding 96 well test plates (i.e. Nunc catalog #269620)
- Laboratory grade (distilled or R.O.) water
- 96 well plate reading spectrophotometer with 405-410 nm filter
- Plate washing apparatus
- Waste container with bleach or other oxidizing agent

5. REAGENTS, SOLUTION AND BUFFER

Reagents Required

- 1 antigen coated plate (*MGIMS*)
- 10 \square l Positive Control Serum (*MGIMS*)
- 10 \square l Normal Control Serum (*MGIMS*)
- 100 \square l. Goat anti-Chicken 19G (H+L) Peroxidase Conjugate Solution (*MGIMS*)
- 40 mL Dilution Buffer Plus
- 10 mL ABTS-Hydrogen Peroxide Substrate Solution



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- g. 2.5 mL 5X Stop Solution, 5% SDS (dilute [1:5] with laboratory grade water)
- h. 20 mL 20X Wash Solution (dilute [1:20] with laboratory grade water)

6. PROCEDURE

Sample Dilution Procedure

- Dilute serum samples using Dilution Buffer in a clean, uncoated 96 well microtiter plate. Frozen serum samples should be completely thawed and thoroughly mixed before diluting. Set up samples and controls as shown in Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	-	+	1	2	3	4	5	6	7	8	9
B	10	11	12	13	14	15	16	17	18	19	20	21
C	22	23	24	25	26	27	28	29	30	31	32	33
D	34	35	36	37	38	39	40	41	42	43	44	45
E	46	47	48	49	50	51	52	53	54	55	56	57
F	58	59	60	61	62	63	64	65	66	67	68	69
G	70	71	72	73	74	75	76	77	78	79	80	81
H	82	83	84	85	86	87	88	89	-	+	-	

Preparation of the serum dilution plate

- Add 300 μ l, Dilution Buffer to each well of an uncoated 96 well microtiter plate. This plate is referred to as the serum dilution plate.
- Add 6 μ l unknown serum per well as per Figure 1 (producing a 1:50 dilution). Start with well A4 and end with well H9 (moving left to right, row by row of wells). For example, wells 1 through 30 contain the diluted sera of flock 1 and wells 31-60 contain the diluted sera of flock 2, etc.
- Add 6 μ l of Normal Control Serum (producing a 1:50 dilution) to wells A2, H10, and H12.
- Aspirate and remove any liquid in dilution plate wells A1, A3, and H11.
- Allow all diluted serums to equilibrate in Dilution Buffer for 5 minutes before transferring to an antigen coated ELISA plate.
- Diluted serum should be tested within 24 hours. This dilution format provides adequate quantities of diluted serum samples to conduct three additional ProFLOK® ELISA tests using the same serum dilution plate.



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Preparation of Positive Control

- A Positive Control Serum for each agent has been provided with this kit. Dilute the appropriate volume of the appropriate Positive Control Serum with Dilution Buffer (1:50) in a clean, glass test tube. For example, dilute 6 μ l of positive control serum in 300 μ l Dilution Buffer. Mix well. 150 μ l of diluted Positive Control is needed per ELISA plate.

Preparation of Conjugate Solution

- The horseradish peroxidase conjugated anti-chicken IgG (H+L) is supplied in HRP stabilizer. Dilute 100 μ l stock conjugate in 10 mL Dilution Buffer (1:100 dilutions). Mix well. This 10 mL preparation will supply sufficient conjugate for one 96 well ELISA plate.

Preparation of 1X Wash Solution

- Dilute 20 mL concentrated Wash Solution in 380 mL laboratory grade (distilled or R.O.) water (1:20). Mix well. Approximately 400 mL Wash Solution is needed for each 96 well ELISA plate.

Preparation of the Substrate Solution

- The Substrate Solution is ready to use. Each plate will require approximately 10 mL substrate solution. For best results, the substrate solution must be equilibrated to room temperature before use.

Preparation of 1X Stop Solution

- Dilute 2.5 mL concentrated Stop Solution in 10 mL laboratory grade (distilled or R.O.) water (1:5). Mix well. Approximately 12.5 mL Stop Solution is needed for each 96 well ELISA plate.

Elisa test procedure

Preparing the test plate

- Remove an antigen coated test plate from the protective bag and label according to the dilution plate identification.
- Add 50 μ l Dilution Buffer to all wells on the test plate.
- Add 50 μ l diluted Positive Control Serum to wells A1, A3, and H11. Discard pipette tip.
- Using an 8 or 12 channel pipette transfer 50 μ l/well of each of the diluted serum samples and Normal Control Serum samples from the dilution plate to the corresponding wells of the coated test plate (yields a 1:100 dilution). Discard pipette tips after each row of sample is transferred of samples to the ELISA plate should be done as quickly as possible.
- Incubate plate for 30 minutes at room temperature.

Wash procedure

- Tap out liquid from each well into an appropriate vessel containing bleach or other decontamination agent.
- Using an 8 or 12 channel pipette (or comparable automatic washing device), fill each well with approximately 300 μ l Wash Solution. Allow to soak in wells for 3 minutes; then discard contents into an appropriate waste container (waste container should contain bleach solution). Tap inverted plate to ensure that all residual liquid is removed.
- Repeat wash procedure 2 more times.



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Addition of anticonjugate IgG peroxidase conjugates, substrate and stop solution

- Using an 8 or 12 channel pipette (or transplating device) dispense 100 μ l, diluted conjugate (prepared as described above) into each assay well. Discard pipette tips.
- Incubate for 30 minutes at room temperature. Wash as in steps above
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 μ l, diluted Substrate Solution (prepared as described above) into each test well. Discard pipette tips.
- Incubate 15 minutes at room temperature.
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 μ l, diluted Stop Solution (prepared as described above) into each test well.
- Allow bubbles to dissipate before reading plate.
- Read the plate using an ELISA plate reader set at 405-410 nm. Be sure to blank the reader as directed.

7. RESULT INTERPRETATION

- 7.1 Calculate the average Positive Control Serum absorbance (Optical Density [O.D.] using the absorbance values of wells A1, A3, and H11. Calculate the Normal Control Serum absorbance using values obtained from wells A2, H10, and H12. Record both averages.
- 7.2 Subtract the average normal control absorbance from the average positive absorbance. The difference is the Corrected Positive Control
- 7.3 Calculate a sample to positive (Sp) ratio by subtracting the average normal control absorbance from each sample absorbance. The difference is divided by the corrected positive control. Use the following equation format:

$$SP = \frac{(\text{SAMPLE ABSORBANCE} - \text{AVERAGE NORMAL CONTROL ABSORBANCE})}{\text{CORRECTED POSITIVE CONTROL ABSORBANCE}}$$

An NDV ELISA titer can be calculated by the following suggested equation:

$$\text{LOG}_{10} \text{ TITER} = (1.464 \times \text{LOG}_{10} \text{ Sp}) + 3.740$$
$$\text{TITER} = \text{ANTILOG OF LOG}_{10} \text{ TITER}$$

Precaution/Limitation of procedure:

- High sensitivity and specificity: keeps assure of accurate test results
- Excellent reproducibility: consistent test results among batches and over time
- Long shelf life at 24 months for easy inventory management

Reporting

- A "0" NDV ELISA titer represents a chicken serum sample that contains an extremely low to insignificant NDV anti- body level compared to the NDV



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ELISA kit positive and normal control sera.

- An NDV ELISA titer value above "0" indicates only that a chicken serum sample contains a significant and ELISA-detectable NDV antibody level compared to the NDV ELISA kit positive and normal control sera. However, these titers do not imply or ensure "protection" nor provide serologic differentiation between a vaccine response and NDV field infection.

Quality Control

- Assay Control Values: Valid NDV ELISA results are obtained when the average optical density (O.D.) value of the Normal Control Serum is less than 0.250 and the Corrected Positive Control (CPC) value range is between 0.250 and 0.900.
- The NDV ELISA titer values obtained represent a comparison of the NDV antibody level within each field chicken serum tested and the NDV ELISA kit positive and normal control sera. Therefore, it is important to first determine that the NDV ELISA positive and normal control sera values obtained are valid as detailed above in the Quality Control section of this pamphlet before ELISA results are interpreted.

8. RISK ASSESSMENT

- 8.1 Handle all reagents and samples as bio-hazardous material.
- 8.2 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- 8.3 Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly de-contaminated with bleach or other strong oxidizing agent before disposal
- 8.4 Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
- 8.5 Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be compromised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
- 8.6 The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques
- 8.7 Never pipette by mouth. Allow all reagents to come to room temperature before starting!
- 8.8 Store all reagents provided in the kit at 2-7°C. Reagents should not be frozen
- 8.9 Storage of 5X Stop Solution at refrigerated temperatures may cause the formation of a white solid. This does not affect product performance. Warm at room temperature or 37°C to dissolve before use

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SOP No: NCAH/LSU/SERO 09

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1. SCOPE

The Sandwich ELISA for Foot-and-Mouth Disease (FMD) is used for the detection of FMD virus antigen in clinical samples such as vesicular fluid, epithelial tissue, and oral or nasal swabs from infected animals. This test is primarily applied for early and rapid diagnosis of active infection, enabling confirmation of the presence of the virus during outbreaks. It is useful in differentiating FMD from other vesicular diseases.

2. PRINCIPLES

Antigen capture sandwich ELISA for virus serotyping is highly sensitive method of virus detection and serotyping in clinical materials. In this test plates are coated with serotype specific rabbit polyclonal serum and viruses present in processed clinical samples are allowed to bind to capturing antibodies. Bound viruses are detected by serotype a specific tracing antibody which increases the specificity of the test. Reaction is developed by tracing antibody specific conjugated antibody and substrate solution.

3. APPLICATION

This test is employed for serotyping O, A Asia I

4. OBJECTIVE

To describe the procedures for serotyping FMD virus to O, A, Asia I

5. APPARATUS/TEST KIT/REAGENTS.

Kit Components

- 5.1 96-well Nunc Maxisorp ELISA plates (cat. No. 442404)
- 5.2 Freeze dried Anti-FMDV coating serum: Type specific anti-146S FMDV serum raised in rabbits.
- 5.3 Freeze dried Anti-FMDV tracing serum: type specific anti-146S FMV serum raised in guinea pig.
- 5.4 Anti-guinea pig conjugate: Rabbit/ goat anti-guinea pig immunoglobulin HRPO conjugate (DAKO)
- 5.5 FMDV inactivated antigens: Sero-type specific inactivated freeze-dried antigens
- 5.6 Chromogen: OPD (Orthophenylenediamine dihydrochloride, Sigma)
- 5.7 Hydrogen peroxide (30% H₂O₂ w/v)
- 5.8 Phosphate buffer saline vials (Sigma)
- 5.9 Substrate buffer capsules
- 5.10 Coating buffer tablets
- 5.11 Tween-20

Materials needed but not provided:

- 5.12 Precision pipettes: multi-channel pipettes variable range from 50 to 200µl & Single channel pipettes variable range from 1 to 20µl, 20 to 100µl, 50 to 200µl and 200 to 1000µl along with disposable tips.
- 5.13 Distilled water
- 5.14 H₂SO₄
- 5.15 Wash bottle fitted with Immuno-washer



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- 5.16 1 container: 1 to 2 liters
- 5.17 ELISA plate reader, 492 nm filter.
- 5.18 Photometer: Multiscan type Micro plate ELISA reader with an interference filter of 492nm and reference filter of 620nm.
- 5.19 Refrigerator: range of +2°C to +6°C
- 5.20 Freezer: range of -15 to -20°C.
- 5.21 Incubator: warm wall incubator maintained at +35°C to 37°C.
- 5.22 pH meter: with accuracy of 0.01pH units or good quality pH strips with varying range from 2 to 10 can be used.
- 5.23 Glassware/ plastic ware: flasks (50-5000ml), graduated cylinders (10-2000ml), graduated pipettes (1-100ml).
- 5.24 Weighing balance

6. TEST PROCEDURE

- 6.1 **Coating of ELISA wells:** Dilute all the 4-coating serum, as suggested, with coating buffer sufficient for the number of plates to be used (2ml of working dilution per serum per plate). Dispense the diluted coating serum in 50µl volumes per well as indicated later in the plate layout. Gently tap the plates to ensure that the liquid has covered the whole well area and incubate at 37°C for 1 hour or 4°C overnight. **Coating serum is diluted as per the batch wise dilution sheet.**
- 6.2 Wash the plate by adding washing buffer using immune-wash and discard the contents of wells by abrupt downward hand motion. Repeat the washing 3 times with 3-5 minutes of hold period between each wash. Slap the inverted microplate 3-4 times onto a dust free absorbent pad to remove all residual contents in the wells.
- 6.3 Dispense 50µl of test sample/ antigen per well according to plate layout. In background wells dispense 50µl of blocking buffer in place of antigen and in positive controls dispense 50µl of respective controls provided. Cover the plate with lid and incubate at 37°C for 1 hour with intermittent gentle shaking followed by washing as described in step 2.
- 6.4 Dilute all the 4-tracing serum, as suggested, with blocking buffer sufficient for the number of plates required. Dispense 50µl volumes per well as indicated in plate layout.
- 6.5 Incubate the plates at 37°C for 1 hour with intermittent gentle shaking followed by washing as described in step 2.
- 6.6 Prepare working dilution of conjugate, as suggested, in blocking buffer in sufficient volume. Dispense 50µl of diluted conjugate to all the wells of ELISA plate and incubate the plates for 1 hour followed by washing as described in step 2.
- 6.7 Prepare substrate solution, as described, and dispense 50µl of substrate solution to each well. Cover the plate with lid and incubate at 37°C for 15 min in dark. Stop the color reaction by adding 50µl of stopping solution to each well.
- 6.8 Measure the optical density of each well at wavelength of 492nm and reference wavelength of 620nm in ELISA reader.

7. RESULT INTERPRETATION



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- 7.1 Performance of a test can be determined from positive reaction of the known antigens employed in the test and a clear background reaction of all the serum. The interpretation of the result should be done on the basis of the corrected OD value (OD of test well OD of background well).
- 7.2 When a test is conducted properly, the background reaction of all the serum provided will lie between 0.02 to 0.08 OD with substrate.
- 7.3 If the reactivity (OD value) of the test antigen with a particular FMDV type serum is ≥ 0.10 (with no heterologous reactivity), then antigen in question can be identified as belonging to that type (OD limit 0-2.5).

8. WASTE DISPOSAL

Consider it as infectious agent and dispose all the materials properly after the test.

9. RISK ASSESSMENT

- 9.1 All the glass wares to be used should be clean and sterile.
- 9.2 Slight drop in pH (less than 7.2) in washing buffer can adversely affect antigen antibody reaction. Ensure correct pH every day before use.
- 9.3 Microbial contamination/ precipitation formation of any degree in any of the reagents/ buffer will very much reduce the specificity of the test.
- 9.4 Mark the plate orientation properly with water proof marker before starting the test to avoid any confusion which may arise later.
- 9.5 Do not stack plates one over other in incubator.
- 9.6 Prepare fresh substrate solution every time.
- 9.7 Do not store prepared buffers more than 1 month.
- 9.8 Check pH of every buffer every day before starting the test.

10. TROUBLESHOOTING

NA

11. REFERENCES

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SOP No: NCAH/LSU/SERO 10

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1. SCOPE

The SERI ELISA for Rabies is used to detect and quantify antibodies against rabies virus in serum samples of animals and, in some cases, humans. It is primarily applied for monitoring post-vaccination immune response, assessing herd or population immunity, and supporting Sero-surveillance programs.

2. PRINCIPLES

The reaction is composed of three steps:

Each serum sample is placed in a well sensitized with inactivated Rabies viral antigens. Antibodies present in the sample bind to the viral antigens coated at the bottom of the well.

After a wash step, Protein A/peroxidase conjugate is added. It fixes to the immunoglobulins (antibodies) previously captured, forming a complex: (Rabies Ag) - (Ab anti-Rabies) - (Protein A/peroxidase).

Excess conjugate is eliminated by a wash step. The enzyme linked to the complex is revealed by addition of a substrate which is transformed into a colored product. After stopping the reaction, the optical densities are measured. The presence or absence of antibodies is determined by using threshold values obtained from the positive control.

3. APPLICATION

The SERI ELISA Synbiotic is used for the detection of Anti-Rabies antibodies in Dog and Cat or Cat individual serum by indirect immunoenzymatic technique.

4. OBJECTIVE

To describe the procedure for the detection of Anti-Rabies antibodies in Dog and Cat or Cat individual serum by indirect immunoenzymatic technique.

5. APPARATUS/TEST KIT

- 5.1 Microplate containing six 16-well strips sensitized with Rabies antigens
- 5.2 Conjugate: Protein A/peroxidase (**CJ**) (10X concentrated)
- 5.3 Buffered peroxidase substrate (**PS**)
- 5.4 Negative control (**N**) (10X concentrated)
- 5.5 Positive control (**P**) (10X concentrated)
- 5.6 Sample diluent (**SD**)
- 5.7 Wash solution (**W**) (10X concentrated)
- 5.8 Conjugate diluent (**CD**)
- 5.9 WHO references serum
- 5.10 Distilled or demineralized water
- 5.11 Adjustable or set pipettes to measure and deliver between 0 to 1000 µl. Measurement deviations must be 10 % for volumes 10 µl and 5 % for all other volumes.
- 5.12 Graduated cylinders (100 ml and 1000 ml).
- 5.13 Manual, automatic or semi-automatic washing device for micro-titration plates.
- 5.14 Microplate reader, fitted with filters for bichromatic reading at 450 and 630 nm. It is also possible to use a monochromatic reader fitted with a 450 nm filter.



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5.15 Incubator at $+37 \pm 3^{\circ}\text{C}$.

6. PROCEDURE

- 6.1 Carefully set up the distribution and identification of controls and samples
- 6.2 Prepare the sera to be tested. Dilutions are performed in the kit sample diluent (SD). Dilutions should be performed as follows: the samples are first pre-diluted at 1:10 in a blank microplate (10 μl of sample in 90 μl of SD)
- 6.3 For serum titration, a set of seven dilutions of the WHO standard serum should be performed either in tubes or in microplates with an initial dilution of 1:10 and 1:100 then 1:25, 1:60, 1:80, 1:170, 1:400 and 1:800.
- 6.4 The preparation of the WHO standard serum range of dilutions must be carried out as follows: 1:100 (not deposit in the test plate): 10 μl WHO 1:10 + 90 μl SD.

WHO dilution	Preparation
1:10	25 μl of WHO + 225 μl of sample
1:25	40 μl of 1:10 dil + 60 μl of SD
1:60	25 μl of 1:10 dil + 125 μl of SD
1:80	20 μl of 1:10 dil + 140 μl of SD
1:170	10 μl of 1:10 dil + 160 μl of SD
1:400	25 μl of 1:100 dil + 75 μl of SD
1:800	20 μl of 1:100 dil + 140 μl of SD

Control distribution

- Controls are not ready-to-use and should be diluted at 1:10.
- Dispense 90 μl of sample diluents, and add 10 μl of the kit negative control into wells A1 and A2, and 10 μl of the positive control to wells B1 and B2.

Distribution of samples and WHO serum dilutions

- Dispense 90 μl of sample diluent, add 10 μl of either 1:10 sample pre-dilution or each WHO serum dilution from 1:10 to 1:800 into the test wells and mix gently
- Strips should always be placed on the frame so that both washer and reader can be used.
- Cover the wells with adhesive film, cut to the necessary length by the number of strips used.
- Mix the plate manually by gentle shaking or by using a plate agitator.
- Incubation of the plate: 1 hour \pm 5 min. at $+37 \pm 3^{\circ}\text{C}$.

	1	2	3	4
A	N 1:10	N 1:10	WHO 1:8000	WHO 1:8000
B	P 1:10	P 1:10	S1 1:100	S1 1:100
C	WHO	WHO 1:100	S2 1:100	S2 1:100
D	WHO	WHO 1:250	S3 1:100	S3 1:100
E	WHO	WHO 1:600	S4 1:100	S4 1:100
F	WHO	WHO 1:800	S5 1:100	S5 1:100
G	WHO	WHO 1:1700	S6 1:100	S6 1:100
H	WHO	WHO 1:4000	S7 1:100	S7 1:100



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Antibody quantification (final dilution)

Over OD values may be observed for the 1:100 WHO dilutions. In this case, use the following WHO dilutions to perform the regression curve.

Washing

- Wash buffer: dilute the concentrated washing solution (W) 1:10 in distilled or demineralized water.
- Carefully remove the adhesive film and wash 4 times.

Addition of conjugate

- Dilute the concentrate (CJ) 1:10 in the conjugate diluent (CD). 2 ml are needed for one strip, meaning 200 μ l of CJ in 1.8 ml of CD.
- Add 100 μ l of diluted conjugate to all the wells and cover with a new piece of adhesive film.
- Incubation of conjugate: 1 hour \pm 5 min. at $+37 \pm 3^\circ\text{C}$.
- Carefully remove the adhesive film and wash 4 times.

Addition of the substrate

- Add 100 μ l of buffered peroxidase substrate (PS) per well. Do not cover with adhesive film at this stage. Mix by gentle shaking the plate manually or use a plate agitator to ensure correct homogenisation.
- Incubate for 30 \pm 5 min. at laboratory temperature ($+23 \pm 5^\circ\text{C}$), shielded from light.

Addition of the Stop Solution

- Add 50 μ l of stop solution (S) per well.
- Mix by gentle shaking the plate manually or by using a plate agitator. Make sure that no bubbles occur in the wells.

Measure of the optical density

- Measure the optical density (OD) bichromatically at 450 and 630 nm or monochromatically at 450 nm (in the yellow band).
- Reading bichromatically is strongly recommended. Should a monochromatic reader be used, ensure the cleanliness of the bottom of the wells prior to reading.

7. RESULT INTERPRETATION AND REPORTING

Titer calculation using the regression curve

- 7.1 (We recommend the use of an Excel spreadsheet. Synbiotics will provide you with a ready-to-use file upon request)
- 7.2 Calculate the average OD value for each sample tested and each WHO serum dilution.
- 7.3 Calculate the Neperian logarithm (ln) value for each average OD and the ln value of the Rabies Ab concentration for each WHO dilution (from 6.7 to 0.0223 IU/ml, without taking into account the 1:100 testing dilution factors).
- 7.4 Plot the ln (OD) (Y-axis) as a function of the ln (Rabies Ab concentration) (X-



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- axis) in order to draw the reference curve for the *WHO* standard serum.
- 7.5 Using all individual results obtained for the *WHO* standard serum dilutions, perform a linear regression curve between \ln Rabies Ab concentrations (expressed in EU/ml) (equivalent units per ml) and \ln (OD), to establish the corresponding mathematics model: \ln [Rabies Ab concentration (EU/ml)] = a + b * \ln OD
- 7.6 For each tested sample, calculate the average OD value and then the Rabies antibody concentration of the sample expressed as «equivalent units per ml» (EU/ml), from the established model: Sample Rabies Ab concentration (EU/ml) = e (a + b* \ln OD)

Rabies antibodies quantities are reported as protected or non-protected.

EXAMPLE

- * Positive control: well B1 = 0.610; well B2 = 0.690 Average OD P = 0.650
- * Negative control: well A1 = 0.190; well A2 = 0.210 Average OD N = 0.200
- * Sample 1: OD 1 = 1.790, OD 2 = 1.750 Average OD = 1.770
- * Sample 2: OD 1 = 0.350 OD 2 = 0.390 Average OD = 0.370
- * Test validation: OD P = 0.650 > 0.300 Average OD N = 0.200 < 0.50 x 0.650 = 0.325, therefore valid test.

Samples (final dilution)	Ab	OD 1	OD 2	averag	\ln	\ln
WHO 1:100	6.7	over	over	over	1.9021	-
WHO 1:250	2.233	1.280	1.237	1.259	0.8033	0.2299
WHO 1:600	0.67	0.809	0.751	0.780	-0.4005	-0.2485
WHO 1:800	0.447	0.600	0.620	0.610	-0.8052	-0.4943
WHO 1:1700	0.2233	0.406	0.425	0.416	-1.4992	-0.8783
WHO 1:4000	0.067	0.214	0.217	0.216	-2.7031	-1.5348
WHO 1:8000	0.0223	0.148	0.154	0.151	-3.8032	-1.8905
Sample 1	unknow	1.790	1.750	1.770	unknow	0,5710
Sample 2	unknow	0.350	0.390	0.370	unknow	-0,9943

Mathematics Model:

$$\ln [\text{Rabies Ab}] = 0.255 + 2.063 * \ln \text{OD}$$

* Test validation: correlation coefficient $r = 0.996 > 0.95$, therefore valid test

* Rabies Ab concentration in Sample 1:

$$e (0.255 + 2.063 * \ln \text{OD}) = e (0.255 + 2.063 * 0.5710) = 4.19 \text{ EU /ml} \quad \text{protected}$$

* Ab Concentration in sample 2:



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$e^{(0.255 + 2.063 * \ln OD)} = e^{(0.255 + 2.063 * -0.9943)} = 0.17 \text{ EU /ml}$ not protected
(FAVN confirmation).

8. WASTE DISPOSAL

Since the kit and the samples are highly infectious materials, dispose off properly following a SOP.

9. RISK ASSESSMENT

- 9.1 Place all reagents at laboratory temperature for at least 1 hour prior to use.
- 9.2 Handle all reagents and samples as biohazardous material.
- 9.3 Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- 9.4 Never pipette by mouth.
- 9.5 Avoid inter sample contamination during sample collection, storage or transport. Use separate disposable pipette tips for each sample.
- 9.6 Avoid contamination of the substrate solution with metallic ions, oxidizing agents or detergents. Make sure that all containers are clean. Do not use the same container or the same pipette tip for the conjugate and the substrate.
- 9.7 It is recommended to dispose reagents and contaminated material according to the applicable regulations. The safety data sheets for the product are available upon request.

10. TROUBLESHOOTING

- 10.1 The results of each test run (or for each plate) are valid
- 10.2 When the optical density (OD) obtained with the positive control is 0.300, and
- 10.3 When the optical density (OD) obtained with the negative control is 0.50 x OD P
- 10.4 When the correlation coefficient between the Neperian logarithm (ln) ODs and ln Rabies Ab concentrations for the *WHO* standard serum is 0.95.

11. REFERENCES

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SOP No: NCAH/LSU/SERO 11

Title: SOP on Rose Bengal Test (RBT) for *Brucella*

Version No: 3, *Total Pages:*3

Issue Month/Effective Date: March 2026

Revision: Summary:

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Prepared by: Serology

Laboratory service unit

National center for Animal Health

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Approved by:

Application/Distribution: NCAH/NVH/RVLs/SVL/DVLs



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1. SCOPE

The Rose Bengal Test (RBT) is a rapid slide agglutination test used for the qualitative screening of antibodies against *Brucella* species in serum samples of livestock. It is primarily applied at field and laboratory levels for early detection and surveillance of brucellosis. The test is suitable for screening large numbers of samples due to its simplicity, cost-effectiveness, and quick results, but positive findings require confirmation by more specific diagnostic methods.

2. PRINCIPLES

The Rose Bengal test is a simple agglutination test in which a standardized antigen with added rose Bengal dye is mixed with serum on a standardized, clear glass or plastic substrate. After standardized mixing for a standard time at a standard temperature, the degree of agglutination is estimated over a light box in comparison with positive controls.

The Rose Bengal test is relatively simple to perform and interpret and can be done by laboratories with basic resources.

3. APPLICATION

The Rose Bengal test is a spot agglutination test used to screen herds for *Brucella abortus*. For the diagnosis of individual cattle the test is oversensitive. This test is also used to test for *B. suis*.

4. OBJECTIVE

To describe the procedures for screening the herd against Brucellosis.

5. APPARATUS/TEST KIT/REAGENTS

- 5.1 *B. abortus* Rose Bengal antigen.
- 5.2 *B. abortus* positive reference control serum
- 5.3 *B. abortus* negative reference control serum
- 5.4 Agglutination plates, flat, Perspex
- 5.5 Pipettors and tips
- 5.6 Spatula for mixing serum and antigen
- 5.7 Timer

6. TEST PROCEDURE

- 6.1 Bring antigen and test sera to room temperature ($20^{\circ}\text{C} \pm 5^{\circ}\text{C}$).
- 6.2 Pipette 25-30 μl of test serum, and positive and negative reference control serum onto a labelled agglutination plate.
- 6.3 Add 25-30 μl of antigen to each well. One plate at a time is set up to minimise delay between the addition of antigen to the first and last serum.
- 6.4 Mix antigen and sera with a mixer / spatula, wiping the implement between each mix.
- 6.5 Place the agglutination plate on a tray rocker (approximately 30 oscillations / minute) and mix for 4 min.
- 6.6 Read the results using a light box.
- 6.7 Read results of reference sera first, then the remaining wells.



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7. RESULT INTERPRETATION AND REPORTING

A scoring system is used to allow correlation with the CFT results. The following allows distinction of degrees of reaction:

Negative	No agglutination, no "ringing", a uniform pink color.
1+	Barely perceptible agglutination and/or some "ringing". Also any doubtful reaction.
2+	Fine agglutination, definite ringing, and some clearing.
3+	Coarse clumping, definite clearing.

Results are recorded as Negative or Positive in standard laboratory report format.

8. WASTE DISPOSAL

The samples and the kits items contain infectious materials hence, should be disposed properly.

9. RISK ASSESSMENT

All Positive and Suspect samples are retested to confirm results using *B. abortus* ELISA test. It can infect the handlers if adequate precautions are not taken.

10. TROUBLESHOOTING NA

11. REFERENCES

- Alton, G.G., Jones, L.M., Angus, R.D. and Verger, J.M. *Techniques in the Brucellosis Laboratory*. Institute National de la Recherche Agronomique; Parris. 1988.
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SOP No: NCAH/LSU/SERO 12

Title: SOP on SAT for Salmonella

Version No: 3, *Total Pages:*5

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

Prepared by: Serology

Laboratory service unit

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Approved by:

Application/Distribution: NCAH



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1. SCOPE

This SOP outlines the procedure for the Slide Agglutination Test (SAT) used for the rapid serological identification of *Salmonella* organisms based on antigen–antibody reactions. The test is applied to confirm the presence of *Salmonella* spp. from bacterial isolates obtained from clinical, food, or environmental samples at the laboratory level.

2. PRINCIPLES

The Standard Tube Agglutination Test (STAT) is a simple, tube agglutination test in which a standardized antigen solution is incubated with test serum. Specific *Salmonella* antibody if present will agglutinate this antigen causing precipitation and clearing compared with a standard tube.

The results of the agglutination tests are expressed in international units (IU), which are defined by reference to OIE Standard.

3. APPLICATION

The test is used for screening the poultry flock against salmonella. Agglutination reveals the presence of circulating antibodies.

4. OBJECTIVE

To describe the procedure for conducting Standard Tube Agglutination Test (TSAT).

5. APPARATUS/TEST KIT

- 5.1 Plastic tubes (5 ml volume).
- 5.2 Pipettors and tips
- 5.3 1 ml and 10 ml Pipettes
- 5.4 37°C Incubator

6. REAGENTS, SOLUTION AND BUFFER

- 6.1 *Salmonella* sp. Antigen
- 6.2 0.5 % (w/w) Phenol Saline (See Media Manual)
- 6.3 *Salmonella* sp. Positive control serum
- 6.4 *Salmonella* sp. Negative control serum

7. PROCEDURE

- 7.1 Prepare a General Serology Work Sheet for the test serum samples (plus positive and negative reference control serum).
- 7.2 Each test serum and the negative reference control serum is tested at serum dilutions of 1/10, 1/20 and 1/40 (See Fig 1). These dilutions are the final serum dilution after addition of antigen. The positive reference control serum is tested at 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320.
- 7.3 Prepare three (3) tubes for each test serum and the negative reference control serum and six (6) tubes for the positive reference control serum.
- 7.4 Add 0.8 ml Phenol Saline to Tube 1 and 0.5 ml Phenol Saline to all other tubes for each sample.
- 7.5 Add 0.2 ml of test serum and reference control serum to the respective tubes (Tube 1)



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- and mix thoroughly
- 7.6 Transfer 0.5 ml of diluted serum from Tube 1 to Tube 2 and mix thoroughly.
- 7.7 Transfer 0.5 ml of diluted serum from Tube 2 to Tube 3, mix and discard 0.5 ml. For the positive reference controls continue on to Tube 6 and then discard 0.5 ml.
- 7.8 Add 0.5 ml of antigen to all tubes and mix thoroughly.
- 7.9 NB. The antigen is used at ten times the dilution as used in the CFT and is diluted with phenol saline (i.e if the antigen is used at 1:100 in the CFT it is used at 1:10 in the SAT).
- 7.10 Incubate the tubes at 37°C for 20 □ 1 hour

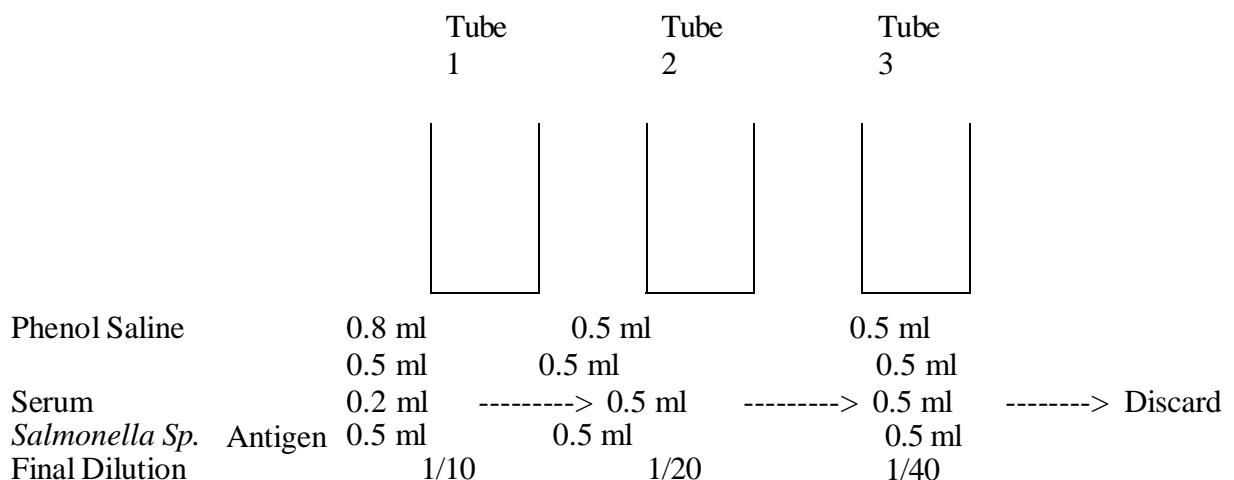
8. RESULT INTERPRETATION

- 8.1 Read results of the reference sera first. If the positive and negative reference results are satisfactory proceed to next step. If reference results are unsatisfactory, tests are repeated. Responses to further test failures are determined by detailed examination of the various control elements in the procedure.
- 8.2 The tubes are examined without being shaken against a black background. By comparison with the positive control, read and record the degree of clearing in the test sera tubes.
- 8.3 Read tubes as follows:

Result	Agglutination	% Clearing
Negative	No agglutination	no clearing
1+	Some agglutination	25% clearing
2+	Marked agglutination	50% clearing
3+	Nearly complete agglutination	75% clearing
4+	Complete agglutination	100% clearing

- 8.4 Convert the agglutination reading to International Units [IU] (Appendix B).
- 8.5 Retest all positive test samples using a six (6) tube dilution series (1/10 – 1/320).

Figure 1.





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9. WASTE DISPOSAL

Consider the material as infectious waste and dispose properly as per the SOP.

10. RISK ASSESSMENT

The antigen may be infectious hence, proper disinfection should be carried out after the tests and the materials should be handled with precaution.

11. TROUBLESHOOTING

NA

12. REFERENCES

- Alton, G.G., Jones, L.M., Angus, R.D. and Verger, J.M. *Techniques in the Brucellosis Laboratory*. Institute National de la Recherche Agronomique; Parris. 1988.
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13. Appendix: Conversion of SAT Titres to International Units (IU)/ml.

Final dilution of serum	End point reading	IU/ml
1/10	1+	17
	2+	20
	3+	23
	4+	27
1/20	1+	34
	2+	40
	3+	47
	4+	53
1/40	1+	67
	2+	80
	3+	93
	4+	106
1/80	1+	134
	2+	160
	3+	186
	4+	212
1/160	1+	268
	2+	320
	3+	372
	4+	424
1/320	1+	536
	2+	640
	3+	744
	4+	848
1/640	1+	1072
	2+	1280
	3+	1488
	4+	1696



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SOP No: NCAH/LSU/SERO 13

Title: SOP on SAT for Mycoplasma

Version No: 3, *Total Pages:*5

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

Prepared by: Serology

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Reviewed by: Dr. Dezang Wangchuk, Sr. Veterinary Officer

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Mr. Roshan Rai, Sr. Lab Tech

Approved by:

Application/Distribution: NCAH



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1. SCOPE

This SOP describes the procedure for the Slide Agglutination Test (SAT) used for the rapid detection of antibodies against *Mycoplasma* species in animal sera. The test is applied as a screening tool at the laboratory level to identify exposure to *Mycoplasma* infections, particularly in poultry and livestock.

2. PRINCIPLES

The SAT is a simple, tube agglutination test in which a standardized antigen solution is incubated with test serum. Specific *Mycoplasma* antibody if present will agglutinate this antigen causing precipitation and clearing compared with a standard tube.

The results of the agglutination tests are expressed in international units (IU), which are defined by reference to OIE Standard.

3. APPLICATION

It is used to screen poultry against *Mycoplasma* infection.

4. OBJECTIVE

To describe the procedure for carrying out simple tube agglutination test for *Mycoplasma*.

5. APPARATUS/TEST KIT

- 5.1 Plastic tubes (5 ml volume).
- 5.2 Pipettors and tips
- 5.3 1 ml and 10 ml Pipettes
- 5.4 37⁰C Incubator

6. REAGENTS, SOLUTION AND BUFFER

- 6.1 *Mycoplasma sp.* antigen
- 6.2 0.5 % (w/w) Phenol Saline (See Media Manual)
- 6.3 *Mycoplasma sp.* positive control serum
- 6.4 *Mycoplasma sp.* negative control serum

7. PROCEDURE

- 7.1 Prepare a General Serology Work Sheet for the test serum samples (plus positive and negative reference control serum).
- 7.2 Each test serum and the negative reference control serum is tested at serum dilutions of 1/10, 1/20 and 1/40 (See Fig 1). These dilutions are the final serum dilution after addition of antigen. The positive reference control serum is tested at 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320.
- 7.3 Prepare three (3) tubes for each test serum and the negative reference control serum and six (6) tubes for the positive reference control serum.
- 7.4 Add 0.8 ml Phenol Saline to Tube 1 and 0.5 ml Phenol Saline to all other tubes for each sample.
- 7.5 Add 0.2 ml of test serum and reference control serum to the respective tubes (Tube 1) and mix thoroughly
- 7.6 Transfer 0.5 ml of diluted serum from Tube 1 to Tube 2 and mix thoroughly.
- 7.7 Transfer 0.5 ml of diluted serum from Tube 2 to Tube 3, mix and discard 0.5 ml. For



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Calculation and Expression of Results

- Interpretation of results with Bovine serum:

Negative	<50 IU/ml
Suspect	>50 and <100 IU/ml
Positive	>100 IU/ml

- Results are entered as Negative, Suspect or Positive with the International Units [IU] attached.
- Data recording and document tracking is described in the Specimen Receipt/Processing document.
- Quality assurance procedures and repeat testing policies are outlined in the Quality Assurance of Test Methods document.

9. WASTE DISPOSAL

Since the kit contains antigen, it should be properly disinfected after the tests and dispose properly.

10. RISK ASSESSMENT

NA

11. TROUBLESHOOTING

NA

12. REFERENCES

- Alton, G.G., Jones, L.M., Angus, R.D. and Verger, J.M. *Techniques in the Brucellosis Laboratory*. Institut National de la Recherche Agronomique; Parris. 1988.
- Corner, L.A. Bovine Brucellosis: Serology. In “Standing Committee on Agriculture and Resource Management, *Australian Standard Diagnostic Techniques for Animal Diseases*”, Eds Corner, L.A. and Bagust, T.J., 1993.
- Veterinary Laboratory User’s Guide. Edited by R A McKenzie, ARI



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13. Appendix: Conversion of SAT Titres to International Units (IU) /ml.

Final dilution of serum	End point reading	IU/ml
1/10	1+	17
	2+	20
	3+	23
	4+	27
1/20	1+	34
	2+	40
	3+	47
	4+	53
1/40	1+	67
	2+	80
	3+	93
	4+	106
1/80	1+	134
	2+	160
	3+	186
	4+	212
1/160	1+	268
	2+	320
	3+	372
	4+	424
1/320	1+	536
	2+	640
	3+	744
	4+	848
1/640	1+	1072
	2+	1280
	3+	1488
	4+	1696



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SOP No: NCAH/LSU/SERO 14

Title: SOP on ELISA for Infectious Bursal Disease

Version No: 3, *Total Pages:*3

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

Prepared by: Serology

Laboratory service unit

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Reviewed by: Dr. Dezang Wangchuk, Sr. Veterinary Officer

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Approved by:

Application/Distribution: NCAH



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1. SCOPE

This ELISA procedure is intended for the detection and quantification of antibodies against Infectious Bursal Disease Virus (IBDV) in poultry serum or plasma samples. It is applicable for surveillance, flock immunity assessment, vaccine efficacy monitoring, and epidemiological studies.

2. TEST PRINCIPLES

This assay is designed to measure the relative level of antibody to IBD in chicken serum. Viral antigen is coated on 96-well plates. Upon incubation of the test sample in the coated well, antibody specific to IBD forms a complex with the coated viral antigens. After washing away unbound material from the wells, a conjugate is added which binds to any attached chicken antibody in the wells. Unbound conjugate is washed away and enzyme substrate is added. Subsequent color development is directly related to the amount of antibody to IBD present in the test sample.

3. APPLICATION

The Infectious Bursal Disease virus antibody test kit idexx is used for detection of antibody against IBD virus in chicken serum.

4. OBJECTIVE

To outline the procedure for conduction the immunoassay for detection of antibody against IBDV in chicken serum.

5. APPARATUS/TEST KIT/REAGENTS

- 5.1 IBD Antigen Coated Plates
- 5.2 Positive Control
- 5.3 Negative Control
- 5.4 Conjugate
- 5.5 Sample Diluent
- 5.6 TMB Substrate
- 5.7 Stop Solution

6. PROCEDURE

All the reagents must be allowed to come to 18-26°C before use. Mix reagents by gently inverting or swirling.

- 6.1 Obtain coated plate and record the sample position.
- 6.2 Dispense 10 µL of the un diluted Positive control into duplicate wells
- 6.3 Dispense 10 µL of the un diluted Negative control into duplicate wells
- 6.4 Dispense 10 µL of samples in to appropriate wells. Samples may be tested in duplicate but a single well is acceptable. None of the samples are diluted for testing.
- 6.5 Cover the plate and incubate for 30 minutes (\pm 2minutes) at 18 - 26°C.
- 6.6 Wash each well with 350 µL of distilled water 3-5 times. Aspirate completely.
- 6.7 Dispense 100 µL conjugate in to each well.



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- 6.8 Incubate for 30 minutes at at 18 - 26°C.
- 6.9 Repeat step 6
- 6.10 Dispense 100 µL of TMB substrate into each well.
- 6.11 Incubate at 18 - 26°C for 15 minutes ((± 2minutes).
- 6.12 Dispense 100 µL of stop solution into each well.
- 6.13 Read the results at a wavelength of 650 nm.

7. RESULT INTERPRETATION and REPORTING

$$\text{SP ratio} = \frac{\text{Sample mean} - \text{NC}}{\text{PC} - \text{NC}}$$

Negative - SP ratio ≤ 0.20

Positive - SP ratio ≥ 0.20

8. WASTE DISPOSAL

Consider it as infectious agent and dispose properly.

9. RISK ASSESSMENT

NA

10. TROUBLESHOOTING

NA

11. REFERENCES

- Infectious Bursal Disease Virus Ab test kit int 06-02470-08 manual



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SOP No: NCAH/LSU/SERO 15

Title: SOP on ELISA for Infectious Bovine Rhinotracheitis

Version No: 3, *Total Pages:*3

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

Prepared by: Serology

Laboratory service unit

National center for Animal Health

Reviewed by: Dr. Dezang Wangchuk, Sr. Veterinary Officer

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Mr. Roshan Rai, Sr. Lab Tech

Approved by:

Application/Distribution: NCAH



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1. SCOPE

This ELISA procedure is designed for the detection and quantification of antibodies against Bovine Herpesvirus-1 (BHV-1), the causative agent of Infectious Bovine Rhinotracheitis (IBR), in bovine serum or plasma samples.

2. TEST PRINCIPLES

Microplates are coated with an ultra-purified BHV-1 lysate. Samples to be tested are diluted and incubated in the wells. Upon incubation of the test sample in the coated wells, BHV-1 specific Antibodies form immunocomplexes with BHV-1 Antigen.

3. APPLICATION

The Infectious Bovine Rhinotracheitis Antibody test kit (idexx Trachitest serum screening) is used to detect the antibody to bovine herpes virus 1 (BHV-1), the virus of the Infectious Bovine Rhinotracheitis, in individual serum or plasma samples and pools up to 10 serum samples from bovines.

4. OBJECTIVE

To describe the procedure for carrying out the enzyme immunoassay for detection of antibody against BHV-1.

5. APPARATUS/TEST KIT/REAGENTS

5.1	BHV-1 Antigen coated plates	10
5.2	Positive control	2ml
5.3	Negative control	2ml
5.4	(Anti-Ruminant IgG-HRPO) conjugate	110 ml
5.5	Sample diluent	100ml
5.6	TMB substrate N 12	100ml
5.7	Stop solution N3	100ml
5.8	Wash concentrate (10X)	480ml

Materials Required but Not provided

- 5.9 Pipettes (10-1000 μ l)
- 5.10 Disposable pipette tip
- 5.11 500ml; graduated cylinder for wash solution
- 5.12 96-well plate reader equipped with 450nm filter
- 5.13 Distilled or de-ionized water
- 5.14 Device for delivery and aspiration of wash solution
- 5.15 A trap for retaining aspirate and disinfectant
- 5.16 Humid chamber/incubator capable of maintaining a temperature + 37⁰C (+3⁰C)
- 5.17 Plate sealer lids
- 5.18 Vortex mixer

6. PROCEDURE



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All the reagents must be allowed to come to 18-26°C before use. Mix reagents by gently inverting or swirling.

- 6.1 Dispense 90µL of samples diluents into each well of the microtiter plate.
- 6.2 Add 10 µL of the undiluted samples and control into the appropriate wells of the micro titter plate. (Final dilution = 1:10).
- 6.3 Mix the contents within each well by gently shaking the microtiter plate briefly.
- 6.4 **Short Incubation-** Cover the plate with lid and incubate for 60 minutes (\pm 5 mins) at 37°C in a humid chamber.
- 6.5 **Overnight Incubation-** Cover the plate with lid and incubate for 4 – 18 hours at 2 - 8°C in a humid chamber.
- 6.6 Wash each well with 300 µL of wash solution 3 times. Avoid the plate drying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
- 6.7 Dispense 100 µL conjugate in to each well.
- 6.8 Incubate for 60 minutes at 37°C in a humid chamber using plate covers.
- 6.9 Repeat step 6
- 6.10 Dispense 100 µL of TMB substrate N.12 into each well.
- 6.11 Incubate at 18-26°C for 15 minutes.
- 6.12 Dispense 100 µL of stop solution N.3 into each well.
- 6.13 Read the results at a wavelength of 450 nm.

7. RESULT INTERPRETATION and REPORTING

- 7.1 The OD of the Positive control should not exceed 2.00.
- 7.2 The OD of the Negative control should not exceed 0.500.
- 7.3 The difference between Positive OD and Negative OD must be \geq 0.300.

8. WASTE DISPOSAL

The samples and the reagents after use should be treated as infectious agent and dispose it properly.

9. RISK ASSESSMENT

NA

10. Troubleshooting

NA

11. REFERENCES

- a. IBR-06-40659-01 manual



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<i>SOP No:</i> NCAH/LSU/SERO 16
<i>Title:</i> SOP on ELISA test for Equine Infectious Anemia (EIA)
<i>Version No:</i> 3, <i>Total Pages:</i> 3
<i>Issue Month/Effective Date:</i> March 2026
<i>Revision: Summary:</i>
<i>Supersedes Version No:</i> 2018.1
<i>Prepared by:</i> Serology Laboratory service unit National center for Animal Health
<i>Reviewed by:</i> Dr. Dezang Wangchuk, Sr. Veterinary Officer Mr. Karma Tsheten, Sr. Lab Tech Mr. Roshan Rai, Sr. Lab Tech
<i>Approved by:</i>
<i>Application/Distribution:</i> NCAH



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1. SCOPE

This ELISA procedure is intended for the detection of antibodies against Equine Infectious Anemia Virus (EIAV) in equine serum or plasma samples. It is applicable for routine surveillance, screening of equine populations, and certification of horses for trade or movement, and epidemiological studies.

2. TEST PRINCIPLES

The IDEXX cELISA kit contains microtiter pre-coated with monoclonal antibody specific for p26, the major group specific antigen of equine infectious anemia virus.

3. APPLICATION

The kit is used for EIA antibody in equine serum.

4. OBJECTIVE

To describe the ELISA procedure for EIA.

5. APPARATUS/TEST KIT/REAGENTS

- 5.1 Anti-EIA Antibody Coated Plate
- 5.2 Positive control
- 5.3 Negative control
- 5.4 EIAV antigen Conjugate
- 5.5 TBM substrate
- 5.6 Stop Solution

6. PROCEDURE

All the reagents must be allowed to come to 18-26oC before use. Mix reagents by gently inverting or swirling.

- 6.1 Dispense 100µL of each serum samples into appropriate wells.
- 6.2 Dispense 100µL of Negative Control and 100µL of Positive into their respective wells.
- 6.3 Add 50 µL of EIAV antigen Conjugate into all wells.
- 6.4 Mix the contents by gently shaking the microtiter plate briefly.
- 6.5 Incubate uncovered for 30 minutes (± 2 mins) at 37oC.
- 6.6 Wash each well with plate washer. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
- 6.7 Dispense 100 µL of TMB substrate into each well. Mix thoroughly 10 times.
- 6.8 Incubate at 18-26oC for 15 minutes. (± 1 mins)
- 6.9 Dispense 100 µL of stop solution into each well.
- 6.10 Read the results at a wavelength of 650 nm.

7. RESULT INTERPRETATION

- Positive control must be ≥ 0.150 OD
- Positive control must be $\leq 70\%$ of Negative control OD.

8. WASTE DISPOSAL



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Dispose properly as per the SOP/guidelines

9. RISK ASSESSMENT

- Test samples may contain infectious agents and pose risk through skin contact, cuts, ingestion, or aerosols
- Exposure to chemical reagents (acids, substrates, buffers) may cause skin and eye irritation or burns
- Risk of injury from sharp pipette tips, broken materials, and spills leading to contamination
- Electrical hazards may arise from improper use of ELISA equipment
- Possibility of cross-contamination between samples during handling
- Errors in pipetting, washing, or incubation may affect test accuracy
- Generation of infectious biological waste and chemical waste
- Risk of environmental contamination if waste is not handled properly

10. TROUBLESHOOTING

NA

11. REFERENCES

- a. World Organization for Animal Health (WOAH). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – Equine Infectious Anemia.*
- b. Food and Agriculture Organization. *Guidelines for Diagnosis of Equine Diseases and Laboratory Techniques.*
- c. Manufacturer's ELISA Kit Manual (e.g., IDEXX EIA ELISA, IDvet, VMRD).
- d. Sellon, D.C., & Long, M.T. *Equine Infectious Diseases.*
- e. Radostits, O.M., Gay, C.C., Hinchcliff, K.W., & Constable, P.D. *Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs, and goats.*



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<i>SOP No: NCAH/LSU/SERO 17</i>
<i>Title: SOP on Antibody ELISA for Classical Swine Fever Virus</i>
<i>Version No: 3, Total Pages:4</i>
<i>Issue Month/Effective Date: March 2026</i>
<i>Revision: Summary:</i>
<i>Supersedes Version No: 2018.1</i>
<i>Prepared by: Serology</i> Laboratory service unit National center for Animal Health
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<i>Approved by:</i>
<i>Application/Distribution: NCAH</i>



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1. SCOPE

This ELISA procedure is designed for the detection and quantification of antibodies against Classical Swine Fever Virus (CSFV) in porcine serum samples. It is applicable for routine surveillance, monitoring of herd immunity, evaluation of vaccination efficacy, and epidemiological investigations.

2. TEST PRINCIPLES

IDEXX CSFV Ab is IDEXX's immunoassay for the detection of CSFV specific antibodies in swine serum or plasma. The assay is a blocking ELISA which utilizes Microplates coated with CSF antigen.

3. APPLICATION

To test for antibody against Classical Swine Fever Virus (CSFV) in pig serum.

4. OBJECTIVE

To describe the procedure for conducting antibody ELISA for CSFV.

5. APPARATUS/TEST KIT/REAGENTS

- 5.1 CSFV Antigen coated plate
- 5.2 Positive control
- 5.3 Negative control
- 5.4 Conjugate
- 5.5 Sample diluents
- 5.6 TMB Substrate N.12
- 5.7 Stop Solution N.3
- 5.8 Wash Concentration (10X)

6. PROCEDURE

All the reagents must be allowed to come to 18-26°C before use. Mix reagents by gently inverting or swirling.

- 6.1 Dispense 50 µL of the sample diluents to all well to be tested and to the control wells.
- 6.2 Dispense 50 µL of Negative Control and 50µL of Positive into duplicate wells
- 6.3 Dispense 50 µL of the serum or plasma to all the remaining wells.
- 6.4 Mix the content gently by taping the plate
- 6.5 Incubate 2 hours (\pm 5 mins) or overnight (12-18 hours) at 18-26⁰C with either option. The plates should be tightly sealed or incubated in a humid chamber using plate covers to avoid any evaporation.



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- 6.6 Wash each well with 300 μ L of wash solution 3 times. Avoid the plate drying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
- 6.7 Add 100 μ L of Conjugate into all wells.
- 6.8 Mix the contents by gently shaking the microtiter plate briefly. The plates should be tightly sealed or incubated in a humid chamber using plate covers to avoid any evaporation
- 6.9 Incubate for 30 minutes (\pm 2 mins) at 18-26⁰C.
- 6.10 Repeat step 6
- 6.11 Dispense 100 μ L of TMB substrateN.12 into each well.
- 6.12 Incubate 10 minutes. (\pm 1 mins) at 18-26⁰C away from direct sunlight.
- 6.13 Dispense 100 μ L of stop solution into each well.
- 6.14 Read the results at a wavelength of 450 nm.

7. RESULT INTERPRETATION and REPORTING

- ✓ Negative OD- >0.500
- ✓ Positive OD- < 50

$$\text{Blocking \%} = \frac{\text{Sample mean - NC}}{\text{PC-NC}}$$

- ✓ **Negative Blocking % ≤ 30**
- ✓ **Suspect Blocking % $= < 40$**
- ✓ **Positive Blocking % ≥ 40**

8 WASTE DISPOSAL

- a. Treat all samples as infectious (may contain Classical Swine Fever virus).
- b. Put used tips, tubes, and ELISA plates in biohazard bags.
- c. Autoclave before disposal (121⁰C, 15–20 min).
- d. Liquid waste \rightarrow add bleach (0.5–1%) for 30 minutes before discarding.
- e. Acid (stop solution) \rightarrow neutralize before disposal.
- f. Sharps \rightarrow dispose in sharps container (do not recap needles).

2. RISK ASSESSMENT

- a. biological risk: Infection from samples.
- b. Chemical risk: Acid (burns), TMB (irritant).



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c. Work risk: Pipetting mistakes, contamination, wrong incubation.

Safety Measures

- a. Wear gloves, lab coat, eye protection.
- b. Do not touch samples directly.
- c. Use clean tips for each sample.
- d. Follow correct timing and temperature.

3. TROUBLESHOOTING

- a. No color → reagents expired or wrong procedure
- b. Too much color (high background) → washing not proper
- c. Positive in negative control → contamination
- d. Weak positive → wrong incubation time/temp
- e. Different results in duplicates → pipetting error
- f. Whole plate not working → substrate or conjugate problem

8. REFERENCES

- a. IDEXX CSFV Ab 06-43230-09
- b. World Organisation for Animal Health – CSF Manual
- c. Food and Agriculture Organization – CSF Guidelines
- d. ELISA Kit Instruction Manual (IDEXX / IDvet / Bio-X)



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SOP No: NCAH/LSU/SERO 18

Title: SOP on ELISA for Classical Swine Fever Virus Antigen test

Version No: 3, *Total Pages:*4

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

Prepared by: Serology

Laboratory service unit

National center for Animal Health

Reviewed by: Dr. Dezang Wangchuk, Sr. Veterinary Officer

Mr. Karma Tsheten, Sr. Lab Tech

Mr. Roshan Rai, Sr. Lab Tech

Approved by:

Application/Distribution: NCAH



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1. SCOPE

This ELISA procedure is intended for the detection of Classical Swine Fever Virus (CSFV) antigen in clinical samples such as blood, tissue homogenates, or swabs from infected pigs. It is applicable for early diagnosis, outbreak investigation, and confirmation of active infection in pigs, supporting rapid disease control measures.

2. TEST PRINCIPLES

A microtitration format has been configured by immobilizing specific monoclonal antibodies on the plates. CSFV AG of the sample is captured on the plates.

3. APPLICATION

IDEXX CSFV AG serum Plus is IDEXX's enzyme immunoassay for the detection of CSFV antigens in swine serum, plasma and tissue (preferably tonsil, spleen, mesenteric lymph node or kidneys) samples

4. OBJECTIVE

To outline the procedure of IDEXX CSFV AG serum Plus is IDEXX's enzyme immunoassay for the detection of CSFV antigens in swine serum, plasma and tissue (preferably tonsil, spleen, mesenteric lymph node or kidneys) samples.

5. APPARATUS/TEST KIT/REAGENTS

- 5.1 Anti-E^{ms}mAB coated plate
- 5.2 Positive control
- 5.3 Negative control
- 5.4 Conjugate
- 5.5 Tissue Soaking Buffer Concentrate (2X)
- 5.6 TMB Substrate N.12
- 5.7 Stop Solution N.3
- 5.8 Wash Concentration (10X)

6. PROCEDURE

Sample preparation

Use preferably fresh tissue but, if necessary, tissue can be stored frozen. Process one or two tissues from each animal submitted, preferably tonsil, spleen, mesenteric lymph node or kidneys.

- a) With scissors, cut tissue into small pieces (250mg)
- b) Place the tissue into appropriate centrifuge or an Eppendorf tube and add 1mL Tissue soaking Buffer. Vortex at 18-26°C for 1-2 hours.
- c) Centrifuge at 1500xg for 10 minutes and use 50 µL of the clean supernatant for the testing as described in the test procedure.

Please use the overnight incubation protocol for tissue samples



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All the reagents must be allowed to come to 18-26°C before use. Mix reagents by gently inverting or swirling.

- 6.1 Dispense 50 µL of detection solution in to all wells to be tested and to the control wells.
- 6.2 Dispense 50 µL of Negative Control and 50µL of Positive into duplicate wells
- 6.3 Dispense 50 µL of the samples to all the remaining wells.
- 6.4 Mix the content gently by taping the plate
- 6.5 Incubate Sample
 - A. **Serum and plasma:** Incubate for 2 hours (± 5 mins) at 37° C. The plates should be tightly sealed or incubated in a humid chamber using plate covers to avoid any evaporation.
 - B. **Tissue samples:** Incubate overnight (12-18 hours) at 2-8oC. The plates should be tightly sealed or incubated in a humid chamber using plate covers to avoid any evaporation.
- 6.6 Wash each well with 300 µL of wash solution 5 times. Avoid the plate drying between plate wash. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
- 6.7 Add 100 µL of Conjugate into all wells.
- 6.8 Incubate for 10 minutes (± 3 mins) at 18-26oC away from sun light.
- 6.9 Repeat step 6
- 6.10 Dispense 100 µL of TMB substrateN.12 into each well.
- 6.11 Incubate 10 minutes. (± 1 mins) at 18-26oC away from direct sunlight.
- 6.12 Dispense 100 µL of stop solution into each well.
- 6.13 Blank the spectrophotometer on air
- 6.14 Read the results at a wavelength of 650 nm.

7. RESULT INTERPRETATION and REPORTING

For the assay to be valid, the difference (P-N) between the positive and negative control mean must be greater than or equal to 0.150 OD.

- Negative mean= ≤ 0.25

- ✓ **Sample with S-N values equal or less than 0.300 are classified as Negative.**
- ✓ **Sample with S-N values greater than 0.300 are classified as Positive**



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8. WASTE DISPOSAL

- Treat all samples as infectious (Classical Swine Fever virus may be present).
- Used tips, tubes, ELISA plates → biohazard bag → autoclave (121°C, 15–20 min) before disposal.
- Liquid waste → add bleach (0.5–1%) for 30 min before discarding.
- Stop solution (acid) → neutralize before disposal.
- Sharps (needles, broken glass) → sharps container (do not recap needles).

- **RISK ASSESSMENT**
- Biological risk: CSFV exposure through sample contact.
- Chemical risk: Stop solution (acid) can burn; substrate (TMB) irritant.
- Operational risk: Pipetting errors, cross-contamination, incorrect incubation.
- Safety Measures
- Wear lab coat, gloves, eye protection.
- Use fresh tips for each sample.
- Avoid direct contact with samples and reagents.
- Follow proper incubation times and temperatures.
- Clean spills immediately with 1% bleach solution.

- **TROUBLESHOOTING**
- No color → reagents expired or wrong procedure
- Too much color (high background) → washing not proper
- Positive in negative control → contamination
- Weak positive → wrong incubation time/temp
- Different results in duplicates → pipetting error
- Whole plate not working → substrate or conjugate

9. REFERENCES

- a. IDEXX CSFV AG serum Plus , CSFV-06-43230-09 manual
- b. World Organization for Animal Health – CSF Manual
- c. Food and Agriculture Organization – CSF Guidelines
- d. ELISA Kit Instruction Manual (IDEXX / IDvet / Bio-X)



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SOP No: NCAH/LSU/SERO 19

Title: SOP on Hemagglutination test for Newcastle Disease

Version No: 3, Total Pages:5

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

*Prepared by: Serology,
Laboratory Service Unit
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*Reviewed by: Dr. Dezang Wangchuk, Sr. Veterinary Officer
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Approved by:

Application/Distribution: NCAH



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1. SCOPE

This procedure is intended for the detection and quantification of Newcastle Disease Virus (NDV) antigen by Hemagglutination (HA) and the corresponding antibodies by Hemagglutination Inhibition (HI) in poultry serum or clinical samples. It is applicable for routine surveillance, monitoring of flock immunity, assessment of vaccination efficacy, and outbreak investigations.

2. PRINCIPLES

Hemagglutination (HA) is the agglutination of red blood cells (RBC), which can be caused by some viruses. This method is done to find presence or absence of haemagglutinin. The property of some viruses to agglutinate the red blood cells of poultry and certain other animals is used in a range of systems to detect viruses and to titrate antibodies. Hemagglutinating properties of viruses are most often used in serology to detect and titrate antibodies by using the hemagglutination inhibition test (HI). However, before the HI test can be carried out, the hemagglutinating strength of the virus must be determined. Doubling dilutions of antigen are mixed with washed RBC and allowed time to agglutinate; the dilution at which agglutination stops is considered 1 hemagglutinating unit (1 HAU). By counting back two doubling dilutions the dilution is reached which contains 4 HAU and this is the strength of antigen usually used in the HI test. Imagine that neat virus solution has 100 agglutinating units, then a 1:2 dilution will have 50, and a 1:4 will have 25 etc. A point will be reached when there is less than 1 unit in the diluted virus and at this point the RBC will no longer agglutinate. In other words, it takes at least one unit to agglutinate RBC. For e.g. if a 1:64 dilution was the last dilution to have at least one unit then 1:32 must be 2 unit and 1:16 must be 4 HA units. Therefore 1:16 is the dilution of antigen (virus), which must be used in subsequent HI test.

Hemagglutination inhibition (HI) is the inhibition of HA, for example by using hyperimmune serum specific to a hemagglutination virus. In this test, wells contain equal volumes and strengths of antigen, an equal volume of 1% RBC and the same volume of doubling dilutions of test serum (antibody). hemagglutination properties of the viruses are blocked by antibody binding to the virus. Blocking takes place up to the dilution of serum (antibody) where there is no longer sufficient antibody to combine with, and eliminate the virus. At this dilution virus will be free to agglutinate RBCs.

3. APPLICATION

The test is used to assess the efficacy of Newcastle disease vaccine in laboratory and field trials and also to assess the level of Newcastle disease virus antibodies in the field.

4. OBJECTIVE

To outline the method of the HA and HI.

5. APPARATUS/TEST KIT

HA

- 5.1 Antigen
- 5.2 V bottom microtiter plate and lid
- 5.3 Micropipette and tips



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- 5.4 1% RBC
- 5.5 Discard tray Microtitre plate recording sheet
- 5.6 Phosphate Buffer solution
- 5.7 Glucose 2.05 g
- 5.8 NaCl 0.42 g
- 5.9 Trisodium citrate 0.80 g
- 5.10 Distilled water 100 ml

HI

- 5.11 Thawed serum samples in racks
- 5.12 V bottom microtitre plates
- 5.13 Lids for microtitre plates
- 5.14 1% washed red blood cells
- 5.15 V-bottom reagent trough
- 5.16 25 μ L single and multichannel pipettes and tips
- 5.17 Microtitre plate recording sheet
- 5.18 Newcastle disease virus antigen diluted to contain 4 HA units
- 5.19 Standard positive and negative serum

6. PROCEDURE

- 6.1 Dispense 25 μ L PBS into each well of a plastic V-bottomed microtitre plate.
- 6.2 Add 25 μ L antigen to first well and mix well
- 6.3 Make two-fold serial dilutions along the row until the last well. Discard the 25 μ L this well.
- 6.4 Each well now contains a dilution of 1 in 21 22 23... and so on
- 6.5 Add 25 μ L of 1% red blood cells to each well
- 6.6 Mix by tapping the plate gently and allow to stand at room temperature for 45 minutes.
- 6.7 Record on microtitre recording sheets the position of samples in corresponding wells of the microtitre plates.
- 6.8 Read settling patterns
- 6.9 HA is determined by tilting the plate and observing the presence or absence of tear shaped streaming of the RBC.
- 6.10 Calculate the number of plates required to titrate and test antigen and test all the serum samples including the positive and negative serum. Check the plates are clean and number each plate with a marker pen.
- 6.11 Fill each well of each plastic V-bottomed microtitre plate with 25 μ L of PBS.
- 6.12 Dispense the serum samples. Shake before dispensing. Place 25 μ L of a sample into the first well and the last (control) well of each row of a microtitre plate.
- 6.13 Use a multichannel pipette to make two-fold serial dilutions along the row until the second last well from the end. Discard the 25 μ L taken from this well. Do not dilute the last well, which is the serum control.
- 6.14 Add 25 μ L of the 4 HA dilution of antigen to each well excluding the control wells in the last column.
- 6.15 Mix gently and allow standing for 30 minutes at room temperature.
- 6.16 Add 25 μ L of 1% chicken red blood cells (RBC) to each well including the control wells in the last column.



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- 6.17 Gently tap the sides of the microtitre plates to mix the reagents. Stack the plates and cover with a lid and let the microtitre plates stand at room temperature for 45 minutes.
- 6.18 Read the settling patterns for each serum sample. Read the control serum well first then read the patterns in the other wells.
- 6.19 Record the patterns observed in each well on a microtitre plate-recording sheet.
- 6.20 Determine the end point. This is the point where there is complete haemagglutination inhibition. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBC 'stream' at the same rate as the control wells (containing 25 μ L RBC and 0.05 ml PBS only) should be considered as showing inhibition.
- 6.21 Record the antibody level for each sample. This is expressed as a log base 2. For convenience, the titre is often recorded as just the log index. For example a titre of 26 would be recorded as 6.

7. RESULT INTERPRETATION

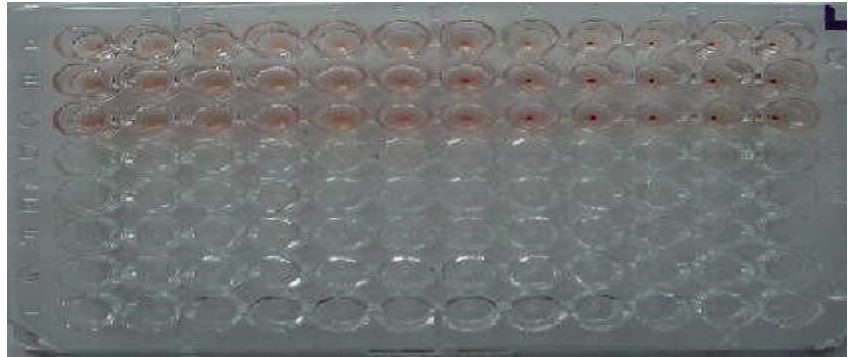
- 7.1 The setting patterns of single and agglutinated red blood cells are different. Single cells roll down the sides of the V-bottom well and settle as a sharp button. Agglutinated cells do not roll down the sides of the well to form a button. Instead they settle as a diffuse film.
- 7.2 Identify the end point. This will be last well to show complete hemagglutination of the red blood cells. In the hemagglutination titration the last well that shows complete agglutination is the well that contains one HA unit. One HA unit is the minimum amount of virus in the assay that will cause complete agglutination of the red blood cells.
- 7.3 *Interpretation*
 - The HI titre is the highest dilution of serum causing complete inhibition of 4HAU of antigen.
 - In the wells where antibodies are present there will be hemagglutination inhibition. The red blood cells will settle at a bottom.
 - In the wells where antibodies are absent, the red blood cells will agglutinate.
 - The end point is not always easy to determine. Look at the size of the button as an indication of the degree of hemagglutination inhibition. Use the control well as a point of comparison. Be consistent in deciding which well shows complete hemagglutination inhibition. The validity of results should be assessed against a negative control serum, should not give a titre >22, and a positive control serum for which the titre should be within one dilution of the known titre.
 - Negative HA result (-) = a sharp button
 - Positive HA result (+) = a diffuse film



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Figure: Photograph of a plate showing HA test (note the diffuse film till well no 6)



8. WASTE DISPOSAL

Consider as infectious material and dispose off properly.

9. RISK ASSESSMENT

Newcastle disease viruses, whether virulent field viruses or live vaccine, can produce a transitory conjunctivitis in humans, but the condition has been limited primarily to laboratory workers and vaccination teams exposed to large quantities of virus.

10. TROUBLESHOOTING

- 10.1 Antibodies not detectable for several days of infection
- 10.2 Limited value because of routine use of vaccine

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SOP No: NCAH/LSU/SERO 20

Title: SOP on AGID for New Castle Disease

Version No: 3, *Total Pages:*4

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

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Application/Distribution: NCAH



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1. SCOPE

This procedure is intended for the detection of antibodies against Newcastle Disease Virus (NDV) in poultry serum using the Agar Gel Immunodiffusion (AGID) technique. It is applicable for Sero-surveillance, evaluation of flock immunity, monitoring vaccination programs, and epidemiological investigations.

2. PRINCIPLES

Agarose plates are poured with 18mL of agarose. Wells of 6.5 mm diameter with a 3mm distance between wells are cut in agar plates in patterns of 7 wells consisting of a circular centre-well and six surrounding circular wells forming the points and centre of a regular hexagon. Six patterns may be cut around the periphery of the agar plate using a template for spacing. Antigen is placed in the centre well. Sera to be tested and reference antiserum are placed in the 6 surrounding wells. Antigen and serum antibody diffuse toward each other through the agar. Where specific antibody and antigen meet at approximately equal concentration, a visible precipitin line forms.

3. APPLICATION

The Agar Gel Immunodiffusion test (AGID) is a simple and reliable technique for the diagnosis of New Castle Disease (NCD).

4. OBJECTIVE

To outline the method of AGID for NCD.

5. APPARATUS/TEST KIT

- 5.1 Agar plates for NCD AGID test (Labelled NCD -See Media Manual).
- 5.2 NCD Reference Sera (standardized).
- 5.3 NCD Positive Control Sera. The NCD Reference Sera is used as the Positive Control Serum if a separate Positive Control is not provided.
- 5.4 NCD Negative Control Serum (standardized). Kit Negative antisera is used if provided. If not, Fetal Bovine Serum known to be negative to NCD is suitable.
- 5.5 NCD Antigen (standardized).
- 5.6 Well cutter
- 5.7 Template – locates 6 x 7 well patterns on the agarose plate.
- 5.8 Vacuum pump.
- 5.9 50 µL pipettor.
- 5.10 Humidified ambient temperature (15-25°C) incubating chamber.
- 5.11 Fibre optic light source.

6. PROCEDURE

- 6.1 Samples are placed on the plate according to the plate layout described in 6.11 below. Details of each sample or control are recorded against the appropriate Specimen Number on the work sheet to indicate the position of the sample on each plate. Samples 1-18 are placed on Plate 1, samples 19-36 on Plate 2 etc. Reagent sera wells are not included in the numbering system as recorded on the work sheet.
- 6.2 Remove the required number of NCD plates from sealed plastic bags stored at 4°C.
- 6.3 Use the template, if necessary, to locate a maximum of 6 x 7 well patterns around the



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- periphery of the NCD plate. The area in the centre of the plate should not be used.
- 6.4 With the location marker on the well cutter directed to the outer edge of the agarose plate, firmly push the well cutter through the agarose.
 - 6.5 Repeat this procedure for all 6 positions on the plate.
 - 6.6 Suck the agarose plugs from all wells on the plate using a vacuum pump with a pipette tip attached to the end of a hose. Be careful to avoid lifting the agarose from the plastic plate as this may result in leakage of reagents under the agarose.
 - 6.7 Number the centre of the base of each plate sequentially. Label Plate 1 on its lid with the date and the type of AGID (i.e. NCD). Mark the location of one well with an easily recognisable spot on the rim of the plastic plate adjacent to it. This will be the first sample position for the plate.
 - 6.8 Samples are placed sequentially from the marked well in a clockwise pattern using every second well only. The outermost well is the first sample well for each pattern (Well 1 in 6.11 below).
 - 6.9 Wells are loaded with 50 μ L of reagents or sample according to the following:
 - i. Central Well: NCD Antigen
 - ii. Wells 1, 3 and 5: Test Samples or Controls
 - iii. Wells 2, 4 and 6: Reference Serum
 - 6.10 Three test sera are tested per 7-well pattern. Thus, with a maximum of 6 patterns per plate, a maximum of 18 sera may be tested per plate.
 - 6.11 Plates are placed in foam boxes with moist paper towel in its base to maintain a humid atmosphere and are incubated at ambient temperature (15-25 $^{\circ}$ C) for 24-72 hours.
 - 6.12 AGID tests performed on different days are placed in different boxes to ensure plates do not get mixed up and that samples can be traced back to the appropriate work sheet.
 - 6.13 The NCD AGID test is read after 24-72 hours incubation. If distinct lines of identity can be seen at the time of viewing the result can be reported.
 - 6.14 Plates are read using a fibre optic light source in a darkened room against a dark background. The light is adjusted to an angle of approximately 45 $^{\circ}$ to the bottom of the plate. Precipitin lines should be clearly visible where specific antibody and antigen meet at approximately equal concentration.

7. RESULT INTERPRETATION

For antibody detection, antibody precipitation turns the reference line inwards towards the antigen well.

The strength of the test reaction is determined by comparison with the reference line.

Interpretation

- Negative:** The control lines continue into the test sample well without bending or with a slight bend away from the antigen well and toward the positive control serum.
- **Weak positive (1+):** The control line bends slightly towards the antigen and may or may not form a complete line between the antigen and the test serum. This reaction occurs very close to the test well. These reactions require careful observation.
 - **Positive (2+-3+):** The control line joins with and forms a continuous line with the line formed between the test serum and antigen.
 - **Very strong positive (4+):** The control lines will turn toward the antigen well



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before they reach the well containing the test serum and continue on as a broad or hazy between test serum and antigen. This line is situated very near the antigen well especially if the plate is observed at 24 hours. This reaction should not be confused with those obtained by use of inactive or weak control agents which produce short, faint, control lines extending equal distances on each side of the antigen, but not continuing into the test wells. A more distinct line will usually form if the very strong positive samples are diluted and retested.

8. WASTE DISPOSAL

Consider as infectious material and dispose off properly.

9. RISK ASSESSMENT

Newcastle disease viruses, whether virulent field viruses or live vaccine, can produce a transitory conjunctivitis in humans, but the condition has been limited primarily to laboratory workers and vaccination teams exposed to large quantities of virus.

10. TROUBLESHOOTING

Non-specific lines: These lines are observed between the antigen and test serum well. However, the reference positive control lines will pass through the non-specific line and continue on into the test serum well of negative sera. The non-specific line does not form a continuous line with the reference positive control lines. The reference positive control lines will form more acute angles with a non-specific line than with an NCD-specific line of identity. The non-specific lines are formed by sample-antibody reactions with antigens other than with NCD p26. A sample serum may produce a specific NCD line as well as a non-specific line. Care must be taken to be certain a specific reaction is not obscured by a non-NCD line. Retesting such samples and observing the reactions at frequent intervals may facilitate making determinations if the samples are positive or negative.

Haze Around Well: Occasionally a haze, due to lipids or other material in the serum, will form around the test serum well that may obscure the reference positive control lines near the sample well. If the test is read at 24 and 48 hours, sometimes the results can be determined before the haze obscures the reaction.

- Semi quantitative
- Moderate sensitivity
- Subjective interpretation
- Requires 24 hours
- Further testing of positives
- Antibodies not detectable for several days

11. REFERENCES

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SOP No: NCAH/LSU/SERO 21

Title: SOP on ELISA for detection of antibody to *Mycoplasma mycoides* (CBPP)

Version No: 3, *Total Pages:*6

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

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Application/Distribution: NCAH



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1. SCOPE

This ELISA procedure is designed for the detection and quantification of antibodies against *Mycoplasma mycoides* subsp. *mycoides*, the causative agent of Contagious Bovine Pleuropneumonia (CBPP), in bovine serum or plasma samples. It is applicable for routine surveillance, monitoring herd immunity, evaluation of vaccination efficacy, and epidemiological studies.

2. PRINCIPLES

The principle of the test is:

- 2.1 The microplates wells are coated with an MmmSC lysate.
- 2.2 Serum samples to be tested are diluted and mixed with the specific monoclonal antibody (Mab 117/5) in a dilution plate or “pre-plate”. This mixture is then transferred into the MmmSCcoated microplate. Any specific antibodies present in the test sera will bind to the MmmSC antigen, competing with the Mab for the specific epitope.
- 2.3 After washing, an anti-mouse IgG serum conjugated to horseradish peroxidase (HRP), which will bind to any Mab fixed to the wells, is added. If specific MmmSC antibodies are present in the bovine sera, they will displace the Mab and the conjugate will not be able to bind.
- 2.4 Following another series of washes, the HRP substrate (TMB) is added, forming a blue compound that will turn to yellow when the reaction is stopped. The intensity of the color is an inverse measure of the proportion of MmmSC antibodies present in the test sera.

The cut-off point is calculated using the results obtained from a monoclonal control (Cm, 0% inhibition) and a conjugate control (Cc, 100% inhibition). Positive and negative control sera are delivered within the kit. They must be included in each microplate in order to validate the results.

3. APPLICATION

The kit is used to detect antibody against *Mycoplasma mycoides* in serum of cattle.

4. OBJECTIVE

To describe the procedure for serological detection of *Mycoplasma mycoides sub species mycoides in cattle*.

5. APPARATUS/TEST KIT/REAGENTS

- | | | |
|-----|---|------------------|
| 5.1 | 96-well coated microplate | |
| 5.2 | Wash concentrate (20x) | 2 x 100ml bottle |
| 5.3 | Dilution buffer 24 | 3 x 120ml bottle |
| 5.4 | Strong positive control (CP ++) | 1 x 0.5ml vial |
| 5.5 | Weak positive (CP +) | 1 x 0.5ml vial |
| 5.6 | Negative control (CN) | 1 x 0.5ml vial |
| 5.7 | Monoclonal anti-MmmSC antibody (Mab 117/5) freeze dried | 1 x 1 ml |
| 5.8 | Anti-mouse IgG HRP-conjugated | 1 x 1.2ml |
| 5.9 | TMB substrate solution 3 | 1 x 120ml bottle |



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5.10 Stop solution (0.5M H₂SO₄)

1 x 120ml bottle

Materials required but not provided:

- 5.11 Refrigerator +5°C (+3°C) and freezer -16°C
- 5.12 Incubator at +37°C (+3°C)
- 5.13 Plate agitator
- 5.14 Microplate reader
- 5.15 Microplate washing system that distributes 300µl per well (optional)
- 5.16 Vortex or similar (optional)
- 5.17 Precision micropipettes and multichannel micropipettes (the precision required must be lower than or equivalent to 10% for volumes lower or equal to 10µl and to 5% for all the other volumes indicated)
- 5.18 Disposable micropipette tips
- 5.19 Reagent reservoirs for multichannel pipettes
- 5.20 96-well microplates for dilutions
- 5.21 Microplate covers (lid, aluminium foil or adhesive)
- 5.22 Distilled water. The water used for the reconstitution of controls and of wash solution can be produced by a conventional distillation system or any other high-performance water purification system (reverse osmosis, resin or activated charcoal purification...)
- 5.23 Disinfectant for safe disposal of test sera (when required)

6. TEST PROCEDURE

6.1 DISTRIBUTION OF TEST AND CONTROL SERA Prior to transferring the sera into the coated microplate, they are diluted and mixed with the monoclonal anti-MmmSC antibody (Mab 117/5) in a 96-well microplate, called the “pre-plate”, which should be made out of normal plastic without any absorption activity.

a) Distribution of controls and test sera

These dilutions must be carried out just before the test: - Distribute 100µl "Dilution buffer 24" in all the wells of the pre-plate. - Distribute 110µl "Dilution buffer 24" in wells A1 and A2 (Conjugate control, Cc) - Distribute 11µl of the three control samples: CP++ in B1, B2, C1, C2 and CP+ in D1, D2, E1, E2 and CN in H1, H2. - Distribute 11µl of each of the test sera in the remaining wells (A3 to H12, as required).

b) Reconstitution and distribution of the monoclonal antibody (Mab 117/5)

Reconstitute the Mab 117/5 with 1 ml of distilled water. Dilute the necessary quantity of the Mab to 1/120 in "Dilution buffer 24" (i. e.: 100µl Mab in 11.9 ml "Dilution buffer 24" for one plate). - Distribute 110µl diluted Mab 117/5 in all the wells except for A1 and A2. Note: If the whole amount is not immediately used, the monoclonal antibody must be stored in aliquots at a temperature $\geq 16^{\circ}\text{C}$ (before dilution in “Dilution buffer 24”).

c) Incubation of the serum/Mab mixture

- Mix by pipetting and transfer 100µl of the serum/Mab mixture from the pre-plate into the coated plate by using a multi-channel pipette (see fig. 1). - Cover the plate with a lid, aluminium foil or adhesive and incubate for 1 hour (+/- 5 minutes) at 37°C ($\pm 3^{\circ}\text{C}$) under gentle agitation.



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6.2 WASHING

- Dilute a bottle of "Wash concentrate (20x)" in 1900 ml of distilled water. This solution is hereafter called the "wash solution". The dilution can be carried out before the disappearance of the crystals which may appear at +5C (+ 3C) as long as the whole 100 ml bottle is used. However, if the whole amount is not to be used immediately, prepare only 400ml of wash solution per plate tested (i. e.: dilute 20 ml "Wash concentrate (20x)" in 380 ml water).
- Empty the contents of the plate by inversion or by another manual or automatic method.
- Fill all the wells of the plate with wash solution; then empty them again.
- Repeat step c) (a total of 2 washes).

Note: When performing the washes manually, empty the plates completely by tapping them upside-down on an absorbent towel.

6.3 DISTRIBUTION OF THE CONJUGATE

- Dilute the conjugate to 1/100 in "Dilution buffer 24" (i.e.: 120 l conjugate in 11.88 ml "Dilution buffer 24" for one plate).
- Distribute 100µl of diluted conjugate in each well.
- Cover the plate with a lid, aluminum foil or adhesive and leave to incubate for 30 minutes (± 3 minutes) at 37C (± 3 C) under gentle agitation.

6.4 WASHING

- Empty the content of the plate by returning or by another manual or automatic method.
- Fill all the wells of the plate with the wash solution; then empty them again.
- Repeat step b) twice (a total of 3 washes). Note: The care brought to the last washing is essential for a good implementation of the test. If the washing is manual, the plate can be inverted and tapped on an absorbent support, in order to completely empty the wells after the last washing.

6.5 REVELATION

- Distribute 100µl of ready to use "TMB substrate 3" per well.
- Cover the plate and incubate for 30 minutes (± 10 minutes) at +37C (± 3 C) under gentle agitation.
- Add 100µl of "Stop solution" per well.
- Gently shake the plate until the colored solution is homogenized. Wipe carefully the bottom of the plate.

Notes:

1. A 30-minute incubation with the substrate provides the O.D. values given in the paragraph "INTERPRETATION" when implemented in our laboratories. However, the rate of color development can be slightly modified by different factors (quality of the washes and of the water used, pipetting precision, temperature of the reaction...). Depending on working conditions, assay development may result in OD values higher or lower than those expected. Therefore, the user may stop the reaction after 30 minutes 10 minutes.

2. Reading may be performed up to 1 hour after the reaction has been stopped as long as the plates are kept in the dark.



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7. RESULT INTERPRETATION

The Optical density (OD) to be read in an ELISA reader at 450 nm and the cut off points to be calculated to validate the results. All sera with percentage Inhibition (PI) > 50% should be considered as positive. Sera with PI between 40-50% to be considered doubtful and those sera with PI less than 40% should be considered as negative (cELISA-Version P05410/02 from

OD Sample – OD negative

$$\frac{\text{OD Sample} - \text{OD negative}}{\text{OD Positive serum} - \text{OD negative serum}} \times 100 = \text{OD}\%$$

OD Positive serum – OD negative serum

OD Cm – OD Test

$$\frac{\text{OD Cm} - \text{OD Test}}{\text{OD Cm} - \text{OD Cc}} \times 100 = \text{PI (Percentage inhibition)}$$

OD Cm – OD Cc

CIRAD / Institut POURQUIER).

VALIDATION CRITERIA

- The reaction is considered valid when the following criteria are obtained:
- The OD of **Cm** must be between 0.5 and 2.0 (preferably near 1.0)
- The OD of **Cc** must be below 0.3
- The PI of CN must be equal to or lower than 35%
- The PI of CP+ must be between 50 and 80%
- The PI of CP++ must be between 60 and 90%

8. INTERPRETATION

8.1 Sera with a percentage of inhibition equal to or lower than **40%** are considered negative.

8.2 Sera with a percentage of inhibition between **40 and 50%** are considered doubtful.

8.3 Sera with a percentage of inhibition equal to or greater than **50%** are considered positive.

9. WASTE DISPOSAL

Consider as infectious and toxic materials hence, dispose the samples and kits properly after the test.

10. RISK ASSESSMENT

- 10.1 Never pipette by mouth.
- 10.2 Stop dilution contains 0.5M H₂SO₄ that causes serious burns.
- 10.3 Avoid contact of the substrate (TMB) with skin mucous membrane and eyes.
- 10.4 Control sera contain sodium azide and may be toxic if ingested.

11. TROUBLESHOOTING

NA



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12. REFERENCES

Antibodies to Mmm CBPP serum is based on competitive ELISA - Version P05410/02 from CIRAD / Institut POURQUIER.



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SOP No: NCAH/LSU/SERO 22

Title: SOP on ELISA for detection of Bovine Viral Diarrhoea Virus (BVDV) Antigen

Version No: 3, *Total Pages:*7

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

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1. SCOPE

This ELISA procedure is intended for the detection and quantification of antibodies against Bovine Viral Diarrhea Virus (BVDV) in bovine serum or plasma samples. It is applicable for routine surveillance, monitoring herd immunity, assessment of vaccination efficacy, and epidemiological investigations

2. PRINCIPLES

IDEXX BVDV Ag/Serum Plus is an enzyme immunoassay designed to detect BVDV antigens in serum bovine serum, plasma, whole blood and ear notch tissue samples. A microtitration format has been configured by immobilising specific monoclonal antibodies for BVDV (Ems) on the plates. BVDV Ag of the sample is captured on the plates. After incubation of the test sample in the well, captured BVDV Ag is detected by specific antibodies and a horseradish-peroxidase conjugate. Next unbound conjugate is washed away and a substrate/chromogen solution is added. In the presence of enzyme, substrate is converted into a product which reacts with chromogen to generate blue colour. Upon addition of the stop solution, a yellow colour is generated. The absorbance at a single wavelength of 450nm or a dual wavelength of 450nm and 650nm is measured using spectrophotometer. The corrected OD value of the sample is calculated by using the absorbance obtained with the test sample and corrected for the absorbance of the negative control.

3. APPLICATION

IDEXX BVDV Ag/Serum Plus is IDEXX's enzyme immunoassay for the detection of BVDV antigens in bovine serum, plasma, whole blood and ear notch tissue samples.

4. OBJECTIVE

To describe the procedure for IDEXX BVDV Ag/Serum Plus is IDEXX's enzyme immunoassay for the detection of BVDV antigens in bovine serum, plasma, whole blood and ear notch tissue samples.

5. APPARATUS/TEST KIT/REAGENTS

1	Anti-E ^{ms} mAb Coated Plates	2	5	30
2	Positive Control	1.6 mL	2 mL	6.5 mL
3	Negative Control	1.6 mL	2 mL	6.5 mL
4	Conjugate	25 mL	60 mL	350 mL
5	Ear Notch Tissue Soaking Buffer	80 mL	2×80 mL	2× 480 mL
6	TMB Substrate N. 12	20 mL	60 mL	400 mL
7	Stop Solution N. 3	20 mL	60 mL	400 mL
8	Wash Concentrate (10 ×)	125 mL	480 mL	3 × 480 mL
9	Detection Solution	15 mL	30 mL	180 mL

Materials required but not provided:



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1. Centrifuge (capacity 2000xg)
2. Precision micropipettes and multichannel micropipettes (the precision required must be lower than or equivalent to 5% for all the other volumes indicated)
3. Disposable micropipette tips
4. Microplate shaker
5. Distilled water or deionized water
6. Microplate washer
7. Microplate covers (lid, aluminium foil or adhesive)
8. 96-well microplates reader equipped with a single wavelength filter of 450nm or dual wavelength of 450nm and 650nm.
9. Humid chamber/incubator capable of maintaining temperature of about $\pm 37^{\circ}\text{C}$ ($+3^{\circ}\text{C}$)
10. Tubes for soaking ear notch tissue samples.

6. TEST PROCEDURE

Preparation of Reagents

Wash Solution

The Wash Concentrate (10 \times) should be brought to 18-26 $^{\circ}\text{C}$ and mixed to ensure dissolution of any precipitated salts. The wash solution (10 \times) must be diluted 1 to 10 with distilled/deionized water before use (e.g., 30 mL of wash Concentrate (10 \times) plus 270 mL of water per plate to be assayed). When prepared under sterile conditions, the Wash Solution can be stored for one week at 2-8 $^{\circ}\text{C}$.

Preparation of Samples

Fresh or frozen serum, plasma, whole blood or ear notch tissue samples can be tested.

Ear Notch Tissue Samples

- . Use ear notch tissue plugs (samples) of 2-3 mm diameter in size (e.g., sampled by applying ear tags with attached ear notch tissue sampling device).
- . If applicable, ear notch samples can be remained in the sampling device for incubation.
- . **Note:** Fresh, humid, desiccated or frozen ear notch tissue samples can be tested.
- . Add 150-250ul of IDEXX Ear Notch Soaking Buffer to the ear notch tissue. Make sure the tissue sample is completely covered with the solution (gently tap or mix).
- . Seal the tube and allow the samples to soak in soaking buffer between 12 and 24 hours at 18-26 $^{\circ}\text{C}$ or over weekend up to 72 hours at 18-26 $^{\circ}\text{C}$ (or at 2-8 $^{\circ}\text{C}$) in a humid chamber.
- . Aspirate 50ul of the soaking buffer for testing.

Note: Remaining soaking buffer can be separated from the ear notch tissue sample and stored frozen (-20 $^{\circ}\text{C}$) for later testing or for retesting.

Test Procedure

All reagents must be allowed to come to 18-26 $^{\circ}\text{C}$ before use.

Reagents should be mixed by gently swirling or vortexing. Use a separate pipette tip for each sample.

1. Obtain coated plates and record the sample position on a worksheet.
2. Add 50uL of the Detection antibodies to each well. A multichannel pipette (8-12



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- Channel) can be used for this step.
3. Add 50 μ L Negative Control into the appropriate wells.
 4. Add 50 μ L Positive Control into the appropriate wells.
 5. Add 50 μ L samples into the remaining wells. Use a separate pipette tip for each sample.
 6. Mix the content of the microwells by gently tapping the plate or use a shaker for microtiter plates.
 7. Incubate for 2 hours (\pm 5 mins.) at 37⁰C (\pm 3⁰C) or overnight (12-18 hours) at 2-8⁰C (in a refrigerator). With either option, the plates should be tightly sealed or incubate in a humid chamber using plate covers to avoid any evaporation.
 8. Aspirate the liquid contents of all the wells into an appropriate waste reservoir.
 9. Wash each well with approximately 300 μ L of Wash Solution five times. Aspirate the liquid contents of all the wells after each wash. Following the final aspiration, firmly tap residual wash fluid from each plate onto absorbent material. Avoid plate drying between washes and prior to the addition of the next reagent.

Important: Control carefully that no traces of blood are left on the walls or edges of the wells. Additional 2-3 washes can be necessary to remove the blood before proceeding to the next step.

10. Dispense 100 μ L of Conjugate into each well.
11. Incubate for 30 minutes (\pm 2 min.) at 18-26⁰C.
12. Repeat steps 8 and 9.
13. Dispense 100 μ L of TMB Substrate N. 12 solution into each well.
14. Incubate for 10 minutes (\pm 1 min.) at 18-26⁰C in darkness. Begin timing after the first well is filled.
15. Dispense 100 μ L of stop solution N. 3 into each to stop the reaction. Add the stop solution in the same order as the substrate solution was added in step 13.
16. Blank the spectrophotometer on air.
17. Measure and record the absorbance of the samples and controls at 450 nm or using a dual wavelength of 450 nm and 650 nm.
18. Calculate the results.

For the assay to be valid, the difference (P-N) between the Positive Control mean (PCX) and the Negative Control mean (NCX) must be greater than or equal to 0.150 optical density (OD). In addition, the Negative Control mean (NCX) must be less than or equal to 0.250 OD.

For invalid assays, techniques may be suspect and the assay should be repeated following a thorough review of the package insert. The presence or absence of BVDV antigen in the sample is determined by the corrected OD value (S-N) for each sample.

See the Calculations section for examples.

Note: IDEXX has instrument and software systems available that calculate means and S-N and provide data summaries.

Calculation



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a. Calculation of Negative Control mean (NCX)

$$\text{NCX} = \text{NC1 A450} + \text{NC A450}/2$$

Example:

$$0.056 + 0.060/2 = 0.058$$

b. Calculation of Positive Control mean (PCX)

$$\text{PCX} = \text{PC1 A450} + \text{PC2 A450}/2$$

Example:

$$1.100 + 1.090/2 = 1.095$$

c. Calculation of test samples

$$\text{S-N} = \text{Sample A450} - \text{NCX}$$

Example sample

$$\text{A450} = 1.558 \quad \text{S-N} = 1.558 -$$

$$0.058 = 1.500$$

9 RESULT INTERPRETATION

Serum, Plasma and Whole Blood Samples

Samples with S-N values equal or less than 0.300 are classified as **negative** for BVDV Ag.

Samples with S-N values greater than 0.300 are classified as **positive** for BVDV Ag. **Positive** results from this assay are valid for calves for any age. Circulating high titers of maternal BVDV antibodies might interfere with the detection of BVDV antigen in serum, plasma and whole blood. Detection of BVDV antigen in serum, plasma and whole blood samples can be less sensitive after antibody intake via colostrum. “False-negative” results can occur after colostrum intake (“diagnostic gap”). In order to exclude influence of colostrum antibodies, it is recommended to test calves before colostrum intake. Negative results of calves after colostrum intake should be confirmed by re-testing at age of more than 30 days. Refer to regulation in your country if different from this description.

Ear Notch Tissue Samples

Samples with S-N values equal or less than 0.200 are classified as **negative** for BVDV Ag.

Samples with S-N values greater than 0.200 but equal or less than 0.300 are considered **suspect** and should be retested.

Samples with S-N values greater than 0.300 are classified as **positive** for BVDV Ag. **Suspect** samples should be retested by using another 50 μL of the same soaking buffer. If there is less than 50 μL remaining, retesting can be done by soaking a second time the ear notch tissue sample with 250 μL of new soaking buffer between 12 and 24 hours at 18-26°C in a humid chamber. If the sample tests are suspect again, a blood sample should be taken and tested by IDEXX BVDV Ag/Serum ELISA Plus, virus isolation or PCR for BVDV. If there is any doubt about the status of a valuable, positive live animal, retest another sample collected 7-14 days after the initial sample was collected to confirm persistent infection. We recommend that positive results obtained with whole blood should be confirmed using serum or plasma from the same animal. When testing ear notch samples, there is no diagnostic gap.



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10 WASTE DISPOSAL

- Liquid waste: All used reagents, wash solutions, and diluted samples should be collected in a labeled biohazard container. Autoclave or disinfect with 10% bleach before disposal.
- Solid waste: Used ELISA plates, pipette tips, gloves, and paper towels should be placed in biohazard bags and autoclaved or incinerated according to lab policy.
- Sharps: Any broken glass or sharp materials must be disposed of in designated sharps containers.

11 RISK ASSESSMENT

Hazard	Risk	Control Measures
Handling BVDV positive samples	Risk of exposure to infectious agents	Wear PPE (gloves, lab coat, and eye protection). Handle in BSL-2 conditions. Avoid aerosols.
Chemical reagents (substrates, stop solution)	Corrosive/irritant	Use gloves and eye protection; handle carefully. Follow MSDS instructions.
Spillages	Contamination risk	Clean immediately with 10% bleach. Decontaminate work surfaces.
Broken plates/tips	Injury and contamination	Dispose in sharps or biohazard containers.

Precautionary Notes:

- Only trained personnel should perform ELISA.
- Maintain proper sample labeling to prevent mix-ups.
- Do not eat, drink, or apply cosmetics in the lab.



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12 TROUBLESHOOTING

NA

13 REFERENCES

- a. IDEXX BVDV Ag/Serum Plus manual version 06-43860-08
- b. Smith, D.R., & Givens, M.D. (2012). *Detection of Bovine Viral Diarrhea Virus by ELISA*. *Journal of Veterinary Diagnostic Investigation*, 24(2), 205–212.
- c. OIE (World Organisation for Animal Health). (2023). *Bovine Viral Diarrhoea Virus (BVDV) Diagnostic Manual*.
- d. Manufacturer's ELISA Kit Protocol (e.g., IDEXX BVDV Antigen ELISA).
- e. Quinn, P.J., et al. (2011). *Veterinary Microbiology and Microbial Disease*. Wiley-Blackwell.



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SOP No: NCAH/LSU/SERO 23

Title: SOP on ELISA test for *Leptospira interrogans serovar hardjo*

Version No: 3, *Total Pages:*7

Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No: 2018.1

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Approved by:

Application/Distribution: NCAH



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1. SCOPE

This ELISA procedure is designed for the detection and quantification of antibodies against *Leptospira interrogans* serovar Hardjo in bovine serum or plasma samples. It is applicable for routine herd surveillance, assessment of exposure, evaluation of vaccination efficacy, and epidemiological studies.

2. PRINCIPLE

A microtitre plate is coated with inactivated antigen. Serum/milk are samples are dispensed in coated wells of the microtitre plate. Antibodies directed against *L. hardjo* that are present in the test samples will bind to the antigen during incubation. The bound antibodies are detected using an anti-bovine monoclonal antibody conjugated to the enzyme horseradish- peroxidase. Subsequently, the bound conjugate is visualized by incubation with the chromogen (TMB) substrate. After the chromogen (TMB) substrate incubation addition of the stop solution will terminate the colour. The OD can be measured in an ELISA reader. Test results are expressed in percentage positivity (PP).

3. APPLICATION

The ELISA kit (Cypress) is used for detection of antibodies in serum and milk against *Leptospira interrogans* serovar hardjo (*L. Hardjo*).

4. OBJECTIVE

To describe the procedure for detection of antibodies in serum and milk against *Leptospira interrogans* serovar hardjo (*L. Hardjo*).

5. APPARATUS/TEST KIT/REAGENTS

Materials provided in the kit

- Five coated test plates
- Conjugate, 30x concentrated
- Lyophilized reference serum 1 (=positive serum)
- Lyophilized reference serum 2 (=negative serum)
- Lyophilized reference serum 3 (=weak positive serum)
- Lyophilized horse serum
- Dilution buffer, 5x concentrated
- Chromogen (TMB) substrate solution (ready to use)
- Demineralized water
- Washing solution, 200x concentrated
- Stop solution
- Additional materials required
 - Dummy plates to make pre-dilutions of milk and serum samples. U-bottom shaped plates are recommended; however, also other non-binding plates or tubes can be used
 - General laboratory equipment
 - Plate reader 450nm filter
 - Plate washer (optional)
 - Plate sealer



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6. TEST PROCEDURE

A. Solutions to be made in advance

a. Dilution buffer working solution

- The concentration dilution buffer must be diluted 5 times (1 part concentrated dilution buffer + 4 parts demineralized water); e.g. for 1 plate prepare 55 mL (add 11 mL concentrated dilution buffer to 44 mL demineralized water). The total volume of dilution buffer that can be prepared is 300 ml. The dilution buffer working solution is stable for 4 hours at room temperature (20-25⁰C).

b. Horse serum

- Reconstitute horse serum 100 times with 3, 5 demineralized water (supplied in the kit). The reconstitute horse serum can be stored at -20⁰c until expiry date.

c. ELISA buffer

- Dilute reconstituted horse serum 100 times in dilution buffer working solution; E.g. for 1 plate prepare 40 ml (add 400µl horse serum to 39.6 ml dilution buffer working solution). The ELISA buffer can be stored up to 4 hours at room temperature (20-25⁰C).

d. Conjugate dilution

- Dilute the concentrated conjugate solution 30 times in dilution buffer working solution; e.g. for 1 plate prepare 12 ml (add 400µl horse serum to 11.6 ml dilution buffer working solution).
- NOTE: The conjugate dilution is not stable, prepare just before use.

e. Reference sera

- Reconstitute each reference serum with 0.5 ml demineralized water (supplied in the kit).
- Reconstituted reference sera are preferably aliquot and can be stored at -20⁰C until expiry date. Mix sera gently after thawing and do not refreeze.

f. Reconstitution of lyophilized reagents should be performed as follows:

1. Equilibrate the vials at room temperature
2. With the vials in an upright position, tap the vial gently against the worktop to ensure that the content is on the bottom of the vial.
3. Open the vial
4. Add the specific amount of demineralized water (see label on vial).
5. Allow the lyophilized material to dissolve.
6. Replace the stopper on the vial and gently agitate so that any remaining dry material will be dissolved.
7. Allow the lyophilized material to stand at least 15 minutes at room temperature. Occasionally gently invert vial.

g. Washing solution

- Dilute the concentrated washing solution 200 times in demineralized water; e.g. for 1 plate prepare 400ml diluted washing solution (add 2ml concentrated washing solution fluid to 388ml demineralized water). The



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total volume of diluted washing solution that can be prepared is 12 liters. The diluted washing solution is stable for 1 week when stored at room temperature (20-25⁰C).

Test procedure

B. Pre-treatment of test plates (for milk samples only)

- Dispense 100 µl ELISA buffer to all wells of the test plates(s).
- Seal or cover the test plates and incubate for 1 hours (\pm 2 minutes) at 37⁰C.
- Discard the ELISA buffer and wash the test plate(s) 6X with washing solution.

C. Pre-diluted (1:20 of reference sera

- Dispense 190µl ELISA buffer to the wells of column 1 of the dummy plate.
- Dispense 10 µl of the reference 1 to the wells C1 and D1 of the dummy plate.
- Dispense 10 µl of the reference 2 to the wells E1 and F1 of the dummy plate.
- Dispense 10 µl of the reference 3 to the wells G1 and H1 of the dummy plate.

D. Pre-dilution of test sera

- Dispense 190 µl ELISA buffer to the wells of columns 2 to 12 of the dummy plate.
- Dispense 10 µl of test serum to the wells of columns 2 to 12 of the dummy plate according to your test schedule.
- Shake the dummy plate.

E. Dispensing of the reference samples to the test plate

- Dispense 100 µl buffer to wells A1 and B1 (blanks) of the test plate(s).
- Dispense 90 µl ELISA buffer to the remaining wells of column 1 (C1 to H1) of the test plates.
- Transfer 10 µl of 1:20 pre-diluted reference 1 from the dummy plate to wells C1 and D1 of the test plates (final dilution 1:200)
- Transfer 10 µl of 1:20 pre-diluted reference 2 from the dummy plate to wells E1 and F1 of the test plates (final dilution 1:200)
- Transfer 10 µl 1:20 pre-diluted reference 3 from the dummy plate to wells G1 and H1 of the test plates (final dilution 1:200).

F. Dispensing of test samples

- *Only for serum samples*
 - Dispense 90 µl ELISA buffer to the remaining empty wells of the test plate. Transfer 10 µl of the pre-diluted sera from the dummy plate to the appropriate wells of the test plate (final dilution 1:200) according to your test schedule (e.g. sample 1 in A2, S2 in B2, etc).
 - Serum samples can be titrated by making two-fold dilutions in dilution buffer. Cover, then shake the test plates gently and incubate for 1 hour (\pm 5 minutes) at 37⁰C.
 - Continue from 6.
- Only for individual Milk samples



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- Dispense 75 µl ELISA buffer to the remaining empty wells of the test plate. Dispense 25 µl milk of a test plate (to achieve a final dilution 1:4) according to your test schedule. Take defatted milk from underneath the creamy layer.
- Cover, then shake the test plate gently and incubate for 1 hour (±5 minutes) at 37 °C.
- Continue from 6.
- Only for Bulk milk samples
- Dispense 100 µl of (undiluted) milk samples to the remaining empty wells of the plate according to your test schedule. . Take defatted milk from underneath the creamy layer.
- Cover, then shake the test plate gently and incubate for 1 hour (±5 minutes) at 37 °C.
- Continue from 6.

G. Incubation with conjugate

- Wash the plate 6 times with diluted washing solution. Dispense 100 µl of diluted conjugate to all wells. Cover, then shake the test plate gently and incubate for 1 hour (±5 minutes) at 37 °C.
- Cover the test plate and incubate for 1 hour (±5 minutes) at 37 °C.

H. Incubation with chromogen (TMB substrate solution)

- Wash the test plate 6 times with diluted wash buffer solution Dispense 100 µl of the chromogen solution to all wells.
- Incubate the plates for 15 minutes at room temperature (20-25 °C). Add 100 µl stop solution
- Agitate the plate to mix the content of the well of the test plate prior to measuring.

7. Reading the test and calculating the result

- Measure the optical density (OD) of the wells at 450 nm within 15 minutes of stopping the colour development.
- Calculate the mean OD₄₅₀ value of the blank (well A1 and B1)
- Calculate the corrected OD₄₅₀ value of all samples by subtracting the mean OD₄₅₀ of the blanks.
- Calculate the percentage positivity (PP) of the reference samples 2, 3 and the test samples according to the formula given below.
- The corrected OD₄₅₀ values of all samples are expressed as percentage positivity (PP) or relative to the corrected mean OD₄₅₀ value of reference 1 in wells C1 and D1.

Corrected OD₄₅₀ test sample

Percentage positivity (PP) = $\frac{\text{Corrected OD}_{450} \text{ test sample}}{\text{Corrected OD}_{450} \text{ reference serum 1}} \times 100 \%$



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Calculate the PP of the test samples

Validation Criteria

- The mean of the blanks must be < 0.150 .
- The corrected OD₄₅₀ of reference 1 must be ≥ 1.000 .
- The mean percentage positivity (PP) of reference 2 must be $< 20\%$.
- The mean percentage positivity (PP) of reference 3 must be between 20% and 60 %.
- The above-mentioned criteria have to be met in order to validate the results of test samples.

8. RESULT INTERPRETATION

- Interpretation of the percentage positivity of the test samples Serum samples
 - PP $< 20\%$: Test sample is Negative for L. Hardjo specific antibodies.
 - PP 20 % - 45 %: Inconclusive (antibodies may be present)
 - PP $> 45\%$: Test sample is positive to L. Hardjo specific antibodies
 - Milk (individual and bulk) samples
 - PP $< 40\%$: Test sample is Negative for L. Hardjo specific antibodies. PP 40 % - 60 %: Inconclusive (antibodies may be present)
 - PP $> 60\%$: Test sample is positive to L. Hardjo specific antibodies
- Bulk milk samples with a doubtful test result must be retested.
- When an inconclusive result is confirmed, it may be followed by collection blood samples of the infected herd.

9. WASTE DISPOSAL

Treat it as the infectious material and dispose accordingly.

10. RISK ASSESSMENT

- Handle all reagents and samples as bio-hazardous material.
- Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontaminated with bleach or other strong oxidizing agent before disposal
- Take special care not to contaminate any of the test re-agents with serum or bacterial agents.
- The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
- Never pipette by mouth. Allow all reagents to come to room temperature before starting.
- Control, TMB substrates and wash concentrate solutions can cause eye irritation
- Stop solution can cause severe skin burns and eye damage



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11. TROUBLESHOOTING

Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be compromised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.

12. REFERENCES

- *Leptospira interrogans* serovar Harjo, Insert Ref. VB066 2009



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<i>SOP No: NCAH/LSU/SERO 24</i>
<i>Title: SOP on Antibody ELISA for Peste Petits Ruminants</i>
<i>Version No: 1, Total Pages:5</i>
<i>Issue Month/Effective Date: March 2026</i>
<i>Revision: Summary:</i>
<i>Supersedes Version No: 2018.1</i>
<i>Prepared by: Serology, Laboratory Service Unit National Center for Animal Health</i>
<i>Reviewed by: Dr. Dezang Wangchuk, Sr. Veterinary Officer Mr. Karma Tsheten, Sr. Lab Tech Mr. Roshan Rai, Sr. Lab Tech</i>
<i>Approved by:</i>
<i>Application/Distribution: NCAH</i>



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1. SCOPE

This SOP outlines the procedure for the Enzyme-Linked Immunosorbent Assay (ELISA) used for the detection of antibodies against Peste des Petits Ruminants (PPR) virus in serum samples of small ruminants. The test is applied at the laboratory level for sero-surveillance, assessment of immune status following vaccination, and confirmation of prior exposure to the virus in sheep and goats.

2. TEST PRINCIPLES

The wells are coated with purified recombinant PPR nucleoprotein (NP). The samples to be tested and the controls are added to the microwells. Anti-NP antibodies, if present, form an antibody-antigen complex which masks the NP epitopes. An anti-NP-peroxidase (HRP) conjugate is added to the microwells. It fixes to the remaining free NP epitopes, forming an antigen-conjugate -HRP complex. After washing in order to eliminate the excess conjugate, the substrate solution (TMB) is added. The resulting coloration depends on the quantity of specific antibodies present in the sample to be tested.

In the absence of antibodies, a blue solution appears which becomes yellow after addition of the stop solution. In the presence of antibodies, no coloration appears.

The microplate is read at 450nm.

3. APPLICATION

To test for antibody against Peste des Petits Ruminants (PPR) virus in sheep/goat serum.

4. OBJECTIVE

To describe the procedure for conducting antibody ELISA for PPR.

5. APPARATUS/TEST KIT/REAGENTS

- 5.1 PPRV Antigen coated plate
- 5.2 Positive control
- 5.3 Negative control
- 5.4 Conjugate 10X
- 5.5 Dilution Buffer
- 5.6 Substrate solution
- 5.7 Stop Solution
- 5.8 Wash Solution

6. PROCEDURE

Allow all the reagents to come to room temperature (210C ± 50C) before use.

Homogenize all reagents by inversion or Vortex.

- a. Dispense 25 µl of Dilution Buffer 13 to each well.
- b. Dispense 25µl of the Positive Control to wells A1 and B1.
- c. Dispense 25µl of the Negative Control to wells C 1 and D1



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- d. Dispense 25µl of each sample to be tested to the remaining wells.
- e. Incubate for 1 hour at room temperature 18-25⁰C.
- f. Wash each well 3 times with approximately 300µl of the Wash Solution. Avoid drying of the wells between washings
- g. Prepare the Conjugate 1X by diluting the Conjugate 10X to 1/10 in Dilution Buffer 4.
- h. Add 100µl of the Conjugate 1X to each well. Incubate 45 min at room temperature (18 -25⁰C)
- i. Wash each well 3 times with approximately 300µl of the Wash Solution. Avoid drying of the wells between washings.
- j. Add 100µl of the Substrate solution to each well. Incubate 15 min at 37⁰C) in the dark room.
- k. Add 100µl of the Stop solution to each well in order to stop the reaction.
- l. Read and record the OD at 450nm.

7. RESULT INTERPRETATION and REPORTING

Interpretation

1.1 Validation and Optical density (OD) CB and NC.

- Average OD of wells CB (OD_{cb}) ≤ 0.1
- Median OD of wells NC (OD_{nc}) ≥ 0.7

1.2 Calculation of the percentage of Inhibition (PI)

$$PI (\%) = \left(\frac{OD_{nc} - OD_x}{OD_{nc} - OD_{cb}} \right) \times 100$$

OD_x = OD Control OR Sample

1.3 Validation PI of control PC and NC

- Positive control (PC): All wells with PI > 70%
- Negative Control (NC): At least 3 wells with PI < 15%

1.4 Interpretation of result

- Negative Serum: PI ≤ 30%



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- Doubtful Serum: $30\% < PI < 35$
- Positive serum: $PI \geq 35\%$

8. WASTE DISPOSAL

- Liquid waste: Collect used samples, wash solutions, and reagents in a labeled biohazard container. Disinfect with 10% bleach before disposal.
- Solid waste: Dispose of used ELISA plates, tips, gloves, and paper towels in biohazard bags. Autoclave or incinerate as per lab SOPs.
- Sharps: Broken glass, needles, or other sharp items must go in designated sharps containers and autoclaved/incinerated

9. RISK ASSESSMENT

Hazard	Risk	Control Measures
Handling PPR-positive sera or samples	Exposure to infectious agents	Use PPE (gloves, lab coat, eye protection). Work in BSL-2 conditions. Avoid aerosol generation.
Chemical reagents (substrates, stop solutions)	Irritation/corrosive	Wear gloves and eye protection. Follow MSDS guidelines.
Spills	Contamination	Clean immediately with 10% bleach; decontaminate surfaces.
Broken plates or tips	Injury or contamination	Dispose in sharps or biohazard containers.

Precautions:

- Only trained personnel should perform the test.
- Keep samples properly labeled to avoid mix-ups.
- Do not eat, drink, or apply cosmetics in the lab.

9. TROUBLESHOOTING

NA

10. REFERENCES

- a. Development of competitive ELISA for detection antibodies to the Peste des petits ruminant's virus using a recombinant nucleoprotein. Libeau G, Prehaud C, Lancelot R, Colas F, Guerre L, Bishop DH, Diallo A, Res Vet Sci. 1995 Jan; 58(1): 50-5.
- b. Banyard, A.C., et al. (2010). *Peste des Petits Ruminants Virus: Diagnostics and Serology*. Veterinary Microbiology, 164(1-2), 14-22.
- c. OIE (World Organisation for Animal Health). (2023). *Peste des Petits Ruminants (PPR) Diagnostic Manual*.
- d. Manufacturer's Antibody ELISA Kit Protocol (e.g., IDEXX PPR Antibody ELISA).



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SOP No: NCAH/LSU/SERO 25

Title: SOP on ELISA for Detection of Porcine Circovirus 2 (PCV 2)

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Issue Month/Effective Date: March 2026

Revision: Summary:

Supersedes Version No:

Prepared by: Dr. Dezang Wangchuk, Sr. Veterinary Officer

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Application/Distribution: NCAH



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1. SCOPE

This SOP applies to all laboratory personnel working in serology laboratories at the National Veterinary Laboratory responsible for testing porcine serum samples for PCV2 antibodies. The test is used for disease surveillance, monitoring vaccination programs, and supporting diagnosis of porcine circovirus associated diseases.

2. PRINCIPLE

The ELISA test is based on the indirect immunoassay technique. Microtiter plate wells are coated with PCV2 antigen. When serum samples containing antibodies against PCV2 are added, the antibodies bind to the antigen. After washing, an enzyme-labelled secondary antibody specific to pig immunoglobulin is added. A chromogenic substrate is then added which reacts with the enzyme to produce a color change. The intensity of the color is proportional to the amount of antibody present and is measured using an ELISA plate reader.

3. OBJECTIVES

To describe the procedure for conducting antibody ELISA for Porcine Circovirus 2 (PCV2).

4. Kit Components and Materials required

- 5.1. ELISA Microtiter Plate – 96 wells
- 5.2. Dilution Plate – 96 wells
- 5.3. HRP Conjugate – 11 ml
- 5.4. Sample Diluent – 50 ml
- 5.5. 20x Concentrated Wash Buffer – 40 ml
- 5.6. Substrate Reagent A – 6 ml
- 5.7. Substrate Reagent B – 6 ml
- 5.8. Stop Solution – 6 ml



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5.9. Positive Control – 1 ml

5.10. Negative Control – 1 ml

5.11. Plate Sealer – 3 pieces

5.12. Sealed Bag – 1 piece

5.13. Manual – 1 copy

5. Test Procedure

6.1. Sample Preparation

6.1.1. Use the conventional method to prepare animal serum, the serum must be clear, no hemolysis and no pollution. Samples can be conserved at 2 - 80 C in 1 week, and should be stored at -200 C for a long term storage.

6.1.2. Diluted Serum: Dilute the sample serum with the sample diluent at 1:39 (5 μ L sample serum and 195 μ L of sample diluent, mix fully). The negative and positive control do not need to be diluted.

6.1.3. Wash Buffer: The 20xConcentrated Wash Buffer Should be adjusted to room temperature to make the sediment dissolve fully before use, then dilute it with deionized water at 1:19.

6.2. Assay Procedure

Restore all reagents and samples to room temperature (250C) before use. All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. The unused ELISA Microtiter plate should be sealed as soon as possible and stored at 2 – 80 C.

6.2.1. Number the sample and control in order (Multiple well), and keep a record of control wells and sample wells. Set 2 wells for negative/positive control respectively. Samples need test in duplicate.

6.2.2. Add 100 μ L of Positive/Negative control to Positive/Negative control well. Add 100 μ L of diluted serum to the sample wells.

6.2.3. Cover the plate sealer and mix thoroughly, incubate at 370 C for 30 minutes in shading light.



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- 6.2.4. Remove the liquid in each well. Immediately add 300 μ L of wash buffer to each well and wash. Repeat wash procedure for 5 minutes, 30 s intervals/time. Invert the plate and pat it against the thick clean absorbent paper (If bubbles exist in the wells, clean tips can be used to prick them).
- 6.2.5. Add 100 μ L of HRP conjugate into each well. Cover the plate sealer and incubate at 37 $^{\circ}$ C for 30 minutes in shading light.
- 6.2.6. Repeat step 4 for washing.
- 6.2.7. Add 50 μ L of Substrate reagent A and 50 μ L of Substrate reagent B into each well. Cover the plate sealer and incubate at 37 $^{\circ}$ C for 10 minutes in shading light.
- 6.2.8. Add 50 μ L of stop solution into each well, mix thoroughly.
- 6.2.9. Measure the absorbance value (A-value) of each well by using a Microplate Reader with 450 nm (it is recommended to set the dual wavelength at 450 nm/630nm) wavelength.

6. Result Interpretation and reporting

Reference value

Normally, the A-value of negative control ≤ 0.3 and the A-value of positive control ≥ 0.6 .

Interpretation of the results

Average absorbance of sample – average absorbance of negative control

S / P =

Average absorbance of positive control – average absorbance of negative control

1. Positive result: S / P ≥ 0.2
2. Negative result: S / P < 0.2
3. Unimmunized animal: Positive result indicates that it may be infected with PCV2
4. Immunized animal: The antibody level at the time of the sample were monitored and recorded and distribution of antibody levels and the trend of immune status of the flock were analyzed based on the result.



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7. Risk Assessment

Serum samples may contain infectious pathogens. Laboratory personnel must follow biosafety guidelines including:

- 8.1. Wearing laboratory coat, gloves, and protective equipment.
 - 8.2. Avoiding direct contact with samples and reagents.
 - 8.3. Following biosafety procedures recommended by the World Organization for Animal Health.
8. Waste Disposal
- a. Dispose of used pipette tips, plates, and contaminated materials in biohazard waste containers.
 - b. Liquid waste should be treated with disinfectant before disposal.
 - c. Solid waste should be autoclaved or incinerated according to laboratory waste management procedures.

1. Trouble Shooting

NA

2. References

- a. World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.
- b. Food and Agriculture Organization Laboratory Diagnostic Manuals for Animal Diseases.
- c. Manufacturer's instructions for PCV2 Antibody ELISA kit



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SOP No: NCAH/LSU/SERO 26

Title: SOP on ELISA for Detection of antibodies against the West Nile Virus (Japanese Encephalitis)

Version No: 1, Total Pages:4

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Revision: Summary:

Supersedes Version No:

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Application/Distribution: NCAH



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1. SCOPE

This SOP describes the procedure for detection of antibody against Japanese Encephalitis viruses and the Tick-bone encephalitis viruses from equine and avian serum or plasma.

2. OBJECTIVES

To detect antibodies against the WNV prE protein in equine or avian serum or plasma via Competitive ELISA

3. PRINCIPLE

Microtiter plates are coated with a purified extract of West Nile virus. Dilutions of the samples to be tested are incubated in the wells of these plates. Any antibody specific for Japanese Encephalitis binds to the antigen in the wells and forms an antigen/antibody complex on the plate well surface. Unbound material is removed from the wells by washing. An anti-pr-E antibody peroxidase (HRP) conjugate is added. Unbound conjugate is removed by washing and TMB substrate added to the wells. The degree of color that develops (Optical density) measured at 450nm) is directly proportional to the amount of antibody specific for West Nile virus present in the sample. The result is obtained by comparing the optical density (OD) that develops in wells containing the samples with the OD from wells containing the Positive Control.

4. Equipment and Consumables

- 4.1 ELISA test kit
- 4.2 ELISA reader
- 4.3 Refrigerator
- 4.4 Freezer
- 4.5 Incubator,
- 4.6 Shaking machine
- 4.7 Micropipette multi-channel 20-300ul
- 4.8 Micropipette single channel 10-100ul Micropipette tips-10-100ul,20-300ul
- 4.9 Wash tub
- 4.10 Distill water
- 4.11 Measuring cylinder
- 4.12 Conical flask
- 4.13 Beaker
- 4.14 Timer
- 4.15 Marker pen,
- 4.16 Tissue paper
- 4.17 Towel
- 4.18 Adhesive taps
- 4.19 Nitrile gloves



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5. Testing Procedure

Follow the protocol as described in WNC ver 1014 EN ID Screen, West Nile Competition Multispecies

6. Result Interpretation

Follow the protocol as described in WNC ver 1014 EN ID Screen, West Nile Competition Multi-species

- Waste Disposal
- Liquid waste:
 - Collect used reagents and samples in biohazard container
 - Disinfect with 10% bleach before disposal
- Solid waste:
 - ELISA plates, tips, gloves → biohazard bags
 - Autoclave or incinerate
- Sharps: Dispose

7. Risk Assessment

Hazard	Risk	Control Measures
Serum/plasma samples	Exposure to zoonotic pathogens	Wear PPE; handle at BSL-2
ELISA reagents (TMB, stop solution)	Irritant/corrosive	Use gloves and eye protection
Spills	Contamination	Clean with 10% bleach immediately
Poor handling	Cross-contamination	Use proper pipetting and labeling

General Precautions:

- Only trained personnel should perform the test
- Avoid aerosol generation



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- No eating/drinking in

7. Troubleshooting

NA

8. References

- Competitive ELISA for the detection of antibodies against the MNV prE protein in equine or avian serum or plasma. WNC ver 1014 EN
- IDvet (Innovative Diagnostics). *ID Screen® West Nile Competition Multi-species ELISA Kit Manual (WNC ver 1014 EN)*.
- WOAH (OIE). (2023). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – West Nile Virus*.
- Beck, C., et al. (2013). *Serological Diagnosis of West Nile Virus*. Veterinary Microbiology.
- Quinn, P.J., et al. (2011). *Veterinary Microbiology and Microbial Disease*. Wiley-Blackwell.



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<i>SOP No:</i> NCAH/LSU/SERO 27
<i>Title:</i> SOP on ELISA for Detection of antibodies against the PRRS (Porcine Reproductive and Respiratory Syndrome)
<i>Version No:</i> 1, <i>Total Pages:</i> 5
<i>Issue Month/Effective Date:</i> March 2026
<i>Revision:</i> Summary:
<i>Supersedes Version No:</i>
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<i>Application/Distribution:</i> NCAH



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1. Scope

This SOP describes the procedure for the qualitative detection of antibodies against Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in swine serum or plasma using an indirect ELISA method.

2. Objective

- To detect anti-PRRSV antibodies in swine serum/plasma and to assist in diagnosis of PRRS infection, monitoring vaccination efficacy and sero-surveillance programs rapid, reliable, and sensitive qualitative results using ELISA.

3. Principle

This kit is based on the Indirect Enzyme-Linked Immunosorbent Assay (ELISA) principle.

Working Principle:

1. The ELISA plate is pre-coated with PRRS viral antigen.
2. Serum/plasma samples are added:
 - If PRRS antibodies are present, they bind to the antigen.
3. An HRP-conjugated secondary antibody binds to the antigen-antibody complex.
4. After washing, TMB substrate (chromogen) is added:
 - HRP catalyzes color development (blue → yellow after stop solution).
5. The intensity of color is measured at 450 nm (reference 630 nm).

Interpretation:

- Color intensity \propto amount of antibody present
- Result is qualitative (positive/negative)

4. Equipment and Consumables

4.1 Equipment

- ELISA microplate reader (450/630 nm)
- Micropipettes (10 μ L, 100 μ L, 1000 μ L)
- Multichannel pipette (optional)
- Incubator (37°C)
- Plate washer (manual/automatic)
- Vortex mixer
- Timer

4.2 Kit Components

- Pre-coated ELISA microplate (96 wells)
- Dilution plate
- HRP conjugate
- Sample diluent
- 25 \times wash buffer
- Substrate Reagent A (TMB)



- Substrate Reagent B
- Stop solution
- Positive control
- Negative control
- Plate sealers
- Instruction manual

4.3 Additional Consumables

- Disposable pipette tips
- Microcentrifuge tubes
 - Distilled/deionized water
 - Absorbent paper towels
 - PPE (gloves, lab coat, goggles)

5. Testing Procedure

5.1 Preparation

1. Bring all reagents and samples to room temperature (20–25°C).
2. Dilute wash buffer (25× → 1×) using distilled water.
3. Label wells for:
 - Blank
 - Negative control
 - Positive control
 - Test samples

5.2 Sample Preparation

- Serum/plasma samples should be:
 - Clear (non-hemolyzed, non-lipemic)
 - Stored at 2–8°C (short term) or -20°C (long term)
- Dilute samples as per kit instructions (usually 1:10 or as specified).

5.3 Assay Procedure

Step 1: Sample Addition

- Add:
 - 50 µL negative control
 - 50 µL positive control
 - 50 µL diluted sample
- Incubate at 37°C for 30 minutes

Step 2: Washing

- Wash plate 3–5 times with 1× wash buffer
- Ensure no residual liquid remains



Step 3: Add HRP Conjugate

- Add 100 μ L HRP conjugate to each well
- Incubate at 37°C for 30 minutes

Step 4: Washing

- Repeat washing step (3–5 times)

Step 5: Substrate Reaction

- Add:
 - 50 μ L Substrate A
 - 50 μ L Substrate B
- Incubate at 37°C for 10–15 minutes (dark)

Step 6: Stop Reaction

- Add 50 μ L stop solution
- Color changes from blue \rightarrow yellow

Step 7: Reading

- Measure absorbance at 450 nm (reference 630 nm) within 10 minutes

5.4 Result Interpretation

Calculate S/P ratio or Cut-off value (as per kit manual):

- Positive: Sample OD \geq Cut-off
- Negative: Sample OD $<$ Cut-off

6. Risk Assessment

Hazard	Risk	Mitigation
Biological samples	Infection risk	Use PPE, biosafety cabinet
Chemical reagents (TMB, stop solution)	Irritation/corrosive	Avoid contact, use gloves
Cross-contamination	False results	Use sterile tips, avoid splashing
Improper waste disposal	Environmental hazard	Follow biohazard disposal protocols

7. Precautions

General

- Read manual thoroughly before use
- Do not mix reagents from different batches
- Use calibrated pipettes

Technical

- Avoid air bubbles in wells
- Ensure complete washing (critical step)



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- Incubation time and temperature must be strictly followed
 - Do not expose substrate to light

Sample Handling

- Avoid repeated freeze-thaw cycles
- Use clean, non-contaminated samples

Quality Control

- Always include positive and negative controls
- Results are valid only if controls meet criteria

8. Reference

1. Elabscience Biotechnology Inc.
 - *Porcine Reproductive and Respiratory Syndrome Virus Antibodies ELISA Kit Manual (Cat. No. E-AD-E006)*
2. Product technical specifications:
 - Indirect ELISA, qualitative detection, serum/plasma samples, detection at 450 nm
3. General ELISA principles and laboratory practices (standard immunodiagnostic guidelines)