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HARAMBEE

A Laboratory Manual on Standard Operating Procedures (SOPs) for Laboratory Tests and Testing procedures for Trade Related Transboundary Animal Diseases

Compiled as a component of USAID/KEA/AU-IBAR, Standard Methods and Procedures in Animal Health (SMP-AH) project

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A Laboratory manual on Standard Operating Procedures (SOPs) for Laboratory Tests and Testing procedures for Trade Related Transboundary Animal Diseases

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This manual was collaboratively developed by laboratory experts from the participating countries in the Greater Horn of Africa (GHoA). We would like to thank the following for their contribution.

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FOREWORD

The arid and semi-arid lands of the Horn of Africa (HOA) are home to poor and vulnerable populations, the majority of whom rely on livestock to sustain livelihoods. However, the performance of livestock in the region remains low, given the widespread occurrence of transboundary animal diseases (TADs) that are responsible for production losses, and reduced performance of intra- and inter-regional trade in livestock and livestock products. Because of disease outbreaks, live animal exports have been severely constrained during the past two decades, by bans imposed by importing countries to reduce risks associated with these diseases.

To address the negative impact of TADs on livestock trade, AU-IBAR and ICPALD together with the participating countries in the region, with financial support from the United States Agency for International Development (USAID), developed a framework to support harmonization and coordination of the control of the diseases, referred to as the Standard Methods and Procedures (SMP) Approach. This involves the implementation of disease control programs, the Standard Methods and Procedures, which define the minimum standards, procedures, methods and goals for a particular disease in areas of surveillance, laboratory procedures and disease control.

In order to operationalize the laboratory component of each SMP, Standard Operating Procedures (SOPs) for laboratory tests and testing procedures have been developed. The SOPs provide a step-by-step guide on how to carry out tests for screening or confirmation for each trade related disease. SOPs usually lead to achievement of comparable laboratory disease diagnosis and test interpretation.

This manual presents the laboratory SOPS for Brucellosis, Contagious Bovine Pleuropneumonia (CBPP), Contagious Caprine Pleuropneumonia, Foot and Mouth Disease (FMD), Peste des Petits Ruminants (PPR), Rift Valley Fever (RVF), African Swine Fever and Newcastle Disease.

The compilation of the materials in this Manual was a collaborative effort and contributors were drawn from countries in the Greater Horn of Africa, AU-IBAR, AU-PANVAC, ICPALD, FAO among others. AU-IBAR is indebted to the many contributors (see the next page) and especially to Dr. James Wabacha the coordinator of the SMP-AH project for coordinating the preparation of the SOPs.

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1. SAMPLE COLLECTION, PACKAGING, TRANSPORTATION AND STORAGE

Standard Operating Procedure **APPROVED**			**SOP No:** **Version: Original** **Supersedes: None** **Effective Date:** **Review Date:**
Title: SAMPLE COLLECTION, PACKAGING, TRANSPORTATION AND STORAGE			
	Name	**Signature**	**Date**
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE/INTRODUCTION:

1.1. PURPOSE

The purpose of this SOP is to describe the procedures for collecting, handling, transportation and storage of samples so as to ensure accurate test results from the sample in the laboratory.

1.2. INTRODUCTION:

1.2.1. Proper sample collection and sample handling until delivery to the laboratory is critical to ensure accurate test assay results. Also quality specimens relate directly to reliable and quality diagnostic results.

2.0 SCOPE / RESPONSIBILITY:

2.1. SCOPE :

The scope of this SOP is to outline sampling procedures including collection, handling ,transportation, and storage. This SOP is intended to be used by all staff that carries out sampling of animals for diagnostic tests.

2.2. RESPONSIBILITY:

- It is the responsibility of the sample collectors to follow this procedure while collecting and handling samples.
- Lab personnel should ensure that samples submitted meet the sample acceptance criteria for the laboratory testing (optimal test samples).

3. 0 DEFINITIONS AND ABBREVIATIONS:

3.1. ABBREVIATIONS

- EDTA: Ethylene diamine tetra acetic acid
- FTA: Fast technology for analysis of nucleic acids.
- GLP: Good laboratory practices
- GPS: Global positioning system
- ID: Animal identification
- IATA: International air transport association
- PPE: Personal Protective equipment
- VTM: viral transport media

3.2. DEFINITIONS NOT APPLICABLE

4.0 SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
• Whole blood, • Blood serum, • Swab sample • Tissue sample	See section 7.2 for proceduresv	see section 4.1

5.0 EQUIPMENT / SUPPLIES/ REAGENTS:

5.1 EQUIPMENT

- Centrifuge
- Cold chain
- Means of transport
- GPS

5.2 SUPPLIES:

- Gloves appropriate sizes,
- Appropriate Personal Protective Equipment (PPE),
- Vacutainer tubes plain for serum, heparinzed / EDTA
- Vacutainer needles and holders,
- Screw caped 2ml vials,
- Self sealing plastic bags,
- Sealing tape,
- Filter paper/FTA cards,
- Pasteur pipettes 3ml or 5ml,
- Syringes 5ml and 10ml with needles 19 gauge 11/2 inch,
- Absorbent cotton,
- Sample collection/submission forms,
- Biohazard label,
- Specimen category symbol
- Swab,

- Racks for serum tubes,
- Racks for vacutainer tubes,
- Permanent laboratory marker,
- Appropriate transport containers.
- Cool box,
- Ice packs,
- Scissors
- Surgical blade
- Forceps

5.3 PRESERVATIVES AND DISINFACTANTS

- Viral Transport Media (VTM)
- Ethyl Alcohol
- Methyl alcohol
- Phosphate buffered saline
- 50% glycerol saline Virkon
- 10% bleach

SAFETY PRECAUTIONS

5.4. Follow safety practices, Good Laboratory Practice (GLP) and wear appropriate PPE when handling diagnostic specimens.

5.5. Wastes should be disposed in environmental friendly manner. Items to be discarded should be properly decontaminated and packed using biohazard bags before disposal.

6.0 METHODOLOGY:

6.1 TEST PRINCIPLE

Not applicable

6.2 PROCEDURES:

6.3 COLLECTION OF BLOOD/SERUM SAMPLE

Note: For serum samples use plain vacutainer tubes, for plasma or whole blood use heparinized or EDTA tubes.

Blood samples may be taken for culture, in which case it is usual to use anticoagulants, such as ethylene diamine tetra-acetic acid (EDTA) or heparin. They may also be taken for serology, which requires a clotted sample.

6.3.1 SAMPLES FOR SERUM

Note: Sera samples can be used for serological tests such as Enzyme Linked Immunosorbent Assay (ELISA), Agar Gel Immuno diffusion (AGID), Complement fixation fest (CFT), haemagglutination, and haemagglutination Inhibition (HA/HI).

These tests can be done on samples from animals suspected to be infected with PPR, Brucellosis, FMD, RVF and CBPP.

- Collect 5 to 7 ml whole blood using appropriate needle in plain vacutainer tube.
- Properly label a blood collection tube with animal ID, collection date and place of collection.
- Avoid hemolysis when serum sample is collected as hemolysed serum may have effect on test results.
- For serum allow blood to clot
- Centrifuge sample at 1000g for 10-15 minutes to separate serum from clot.

This can also be accomplished by storing the whole blood sample, in an upright position, overnight at room temperature (22 – 250°C).

- Properly label a 2 ml cryovial with complete animal ID; serum collection date, and place of collection.
- Transfer 1.0 – 2.0 ml of serum to the cryovials.
- Serum samples can be stored in the refrigerator (2 – 6°C) for up to one week.
- Serum samples can be stored frozen (-20^{0}C)

6.3.2 SAMPLES FOR WHOLE BLOOD

Note: whole blood can be collected for plasma and preparing blood meal (blood on filter paper / FTA cards). These samples can be used for molecular diagnostic tests. The test can be done on animals suspected to be infected viral diseases and bacterial diseases

Using jugular vein puncture technique, collect 5ml to 7ml whole blood using EDTA vacutainer tubes and needles with needle holder.

- Properly label the blood collection tube with animal ID, collection date and place of collection.
- Keep the samples chilled if the samples have to be transported to the laboratory.
- Once in the laboratory the whole blood can be stored in at 2-8 OC or use Pasteur pipette to transfer the whole blood for preparing blood smear on filter paper/FTA cards.

6.3.3 COLLECTION OF SWAB SAMPLE

Note 1: Swab samples may be taken with sterile dacron, cotton or gauze swabs, preferably on wire handles as wood is inflexible and may break. It may be helpful if the swab is first moistened with transport medium.

Note 2: Ensure that swab samples submitted to bacteriology laboratory should not have viral transport media. The antibiotic added in the VTM can hamper the diagnosis.

- Properly label the viral/bacterial transport sample tubes with complete animal identification, collection date, place of collection and the swab site. (e.g. Nasal, , Oropharyngal, lesions and eye).
- Use separate sterile swab for each collection site.

- Place each swab into separate sample tubes containing 1 – 3.0 ml of viral/bacterial transport media. If the shaft is longer than the tube, break or cut it.
- Store Swab samples in the refrigerator (2 – 8°C) for up to one week.
- For longer storage keep swab samples frozen (- 70°C to - 80°C).

6.3.4 COLLECTION OF TISSUE SAMPLE

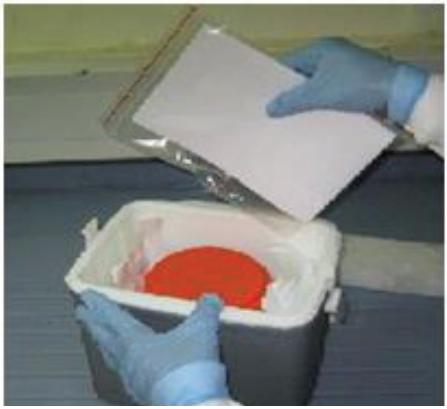
- Sample should be collected from clinically sick and freshly dead animals or sacrificed animals.
- Sample should be obtained from the edge of lesions and include some macroscopically normal tissue. Microbial replication will be most active at the periphery of the lesion.
- Always use sterile equipment/ scissors, scalpel blade and forceps whenever bacteriological sample is collected
- It is important to collect samples as aseptically as possible. Submission of adequate volume and number of samples have high probability for detection of the causative agent/s.
- Sample should be submitted individually in sterile separate leak proof container.
- Screw caped containers should be clearly marked indicating the tissue enclosed, animal identification, place of collection and the date of collection.
- Tissue sample should be transported under cold chain (+40C).
- Some viral species are fragile. Hence, special transport media should be used to keep the viability.

7.4 PROCEDURE NOTES

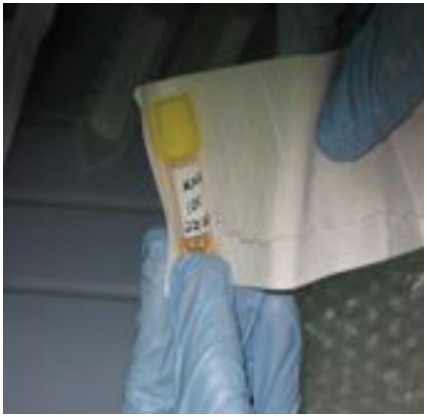
7.4.1 IN-COUNTRY SAMPLE PACKAGING

- Wash hands with soap and water.
- Wear appropriate personal protective equipment
- Have the needed sample packaging tools on hand (see section 5).
- Before packaging ensure that sample(s) you should has/have the required accompanying information in place.
- Use three (triple) packaging layers – First packaging layer (primary container) should be leak-proof and all layers (containers) should contain absorbent materials in case there are any leaks.
  - » Wrap every container (primary container) with an absorbent material like paper towels
  - » Place the primary container(s) into a secondary container
  - » Use additional absorbent material to cushion multiple containers.
  - » Place the secondary container(s) into a leak-proof larger tertiary container.
  - » Place four to eight frozen ice packs (from a -20°C compartment of the refrigerator) depending on the size of the ice packs, at the bottom, at the top, and on each side to maintain 2-8°C temperature
- Were necessary place the tertiary container into a box.
- Place shipping documents in zip-lock bag to keep from becoming contaminated or becoming wet.

- Place the zip-lock bag in the cooler box.
- Close and seal the cooler box by packing tape.
- Use waterproof ink to label the cooler box clearly indicating destination facility, Contact
- information (both for shipper and receiver) and affix "Infectious Substances" label.
- Disinfect the outside part of the cooler box with 10% bleach.
- Notify the receiving Laboratory on the mode of transport and itinerary
- Label the outer container with contact details (addresses) of the shipper and the consignee and transport the cooler box containing samples to the designated Laboratory



First packaging layer (primary container) should be leak-proof and all layers (containers) should contain absorbent materials in case there are any leaks



Wrap each primary container

Put in the secondary container

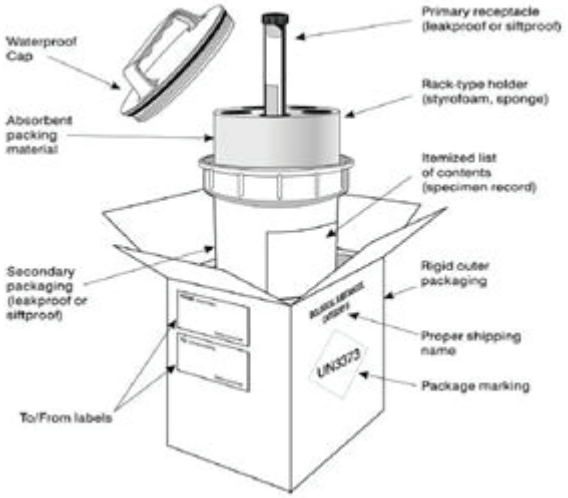
Put in the tertiary container with frozen ice packs with absorbent materials

NOTE: Alternatively swabs and tissues for viral samples can be transported using Liquid Nitrogen containers/dry shippers if available.

7.4.2 INTERNATIONAL SHIPPING

- WHO/OIE guidelines for the safe transport of infectious substances and diagnostic specimens
- IATA packaging specifications

Example of Packing and Marking of a "650 package" for Category B (UN 3373) Infectious Substances



NOTE: Alternatively samples can be transported using dry shippers without liquid phase

7.4.3 HANDLING OF SAMPLES

- All samples submitted to the laboratory should be accompanied by a completed sample submission form.
- Register samples received on the log book immediately on arrival.
- Keep samples which could not be registered at the time of receipt in the refrigerator until registered the next day.
- Samples collected for entomological teaching or reference purposes.

8.0 CRITERIA FOR ACCEPTANCE/ REJECTION OF SAMPLES

- Only samples which meet acceptance criteria shall be accepted for testing.
- If the sample does not meet sample acceptance criteria, it will not be considered fit for testing and shall therefore be rejected based on the fact that poor sample will not allow for accurate test results.

8.1 SAMPLE ACCEPTANCE CRITERIA

- Specimen with completed submission form.
- Proper packed and preserved specimen.
- Proper storage specimen eg on ice.

- Proper labeled specimen containers.
- Fresh specimen.
- Adequate amount of specimen
- Proper collected specimen
- Specimen collected in correct/appropriate container
- Specimen in good quality
- Specimen in good integrity
- Correct/appropriate specimen for the test required/requested

8.2 SAMPLE REJECTION CRITERIA

- Unlabelled samples
- Mislabeled samples
- Insufficient/inadequate volume/quantity for the test requested
- hemolysed/decomposing sample depending on the test requested
- Samples collected in unsuitable containers, leaking containers or use of wrong preservative or non sterile container
- Submission of wrong samples
- Poor handling of the samples with respect to temperature, timing and storage and requirements.
- Contaminated sample depending on the test requested
- Lack of sample information/biodata

9.0 SAMPLE DISPOSAL

Refer to sample retention and disposal policy

10.0 QUALITY CONTROL

Not applicable

11.0 QUALITY CONTROL MATERIAL

Not applicable

12.0 CALIBRATOR

Not applicable.

13.0 CALIBRATION

Not applicable

14.0 RESULTS

Not applicable.

14.1 QUALITY CONTROL RESULTS.

Not applicable

15.0 REFERENCES:

1. ***OIE manual 2008.*** Chapter 1.1.1, chapter 2.4.18
2. ***OIE Terrestrial Manual 2010*** Chapter 2.1.17
3. ***P.J. Quinn et al., 1999*** Clinical Veterinary Microbiology,

16. APPENDICES:

DOCUMENT CHANGE HISTORY:

16.1 Appendix .1:

Original Title: SAMPLE COLLECTION, TRANSPORTATION AND STORAGE	**Dated:**	**SOP No.: LQM**	**No. Pages:**
Version 1: Title	Dated	SOP No.:	No. Pages:
Version 2: Title	Dated	SOP No.:	No. Pages:
Version 3: Title	Dated	SOP No.:	No. Pages:
Version 4: Title	Dated	SOP No.:	No. Pages:
Version 5: Title	Dated	SOP No.:	No. Pages:

16.2 APPENDIX 2:

SOP DISTRIBUTION LISTING:

This section is to be completed by the Document Coordinator in consultation with the Document Initiator and Laboratory Management indicating the Section / stations where controlled copies of this document shall be circulated.

Section / Area	**SOP Manual Number**	**Date Issued**

16.3 APPENDIX 3:

SOP TRAINING LOG

This section is to be used to document training of the SOP.

AFRICAN UNION **INTERAFRICAN BUREAU FOR ANIMAL RESOURCES**				**SOP No:** **Version:** Original **Supersedes:** None Effective **Date:** Review Date:
Title: SAMPLE COLLECTION, TRANSPORTATION AND STORAGE				
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breech of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.				
DATE	**TRAINEE NAME**	**SIGNATURE**	**TRAINER NAME**	**SIGNATURE**

Name of training approval authority:……..Signature…….Date……

2. EXTRACTION OF AFRICAN SWINE FEVER VIRUS (ASFV) NUCLEIC ACID FOR POLYERASE CHAIN REACTION (PCR)

Standard Operating Procedure **APPROVED**			**SOP No:** **Version: Original** **Supersedes: None** **Effective Date:** **Review Date:**
Title: EXTRACTION OF AFRICAN SWINE FEVER VIRUS (ASFV) NUCLEIC ACID FOR POLYERASE CHAIN REACTION (PCR)			
	Name	**Signature**	**Date**
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1. PURPOSE

The purpose of this Standard Operating Procedure (SOP) is to describe the nucleic acid extraction method of the African swine fever virus (ASFV) DNA in clinical materials using the commercial nucleic acid extraction kit for further amplification by PCR.

2. INTRODUCTION

The African swine fever virus is highly contagious and can spread very rapidly in pig populations by direct or indirect contact. This virus can persist for long periods in pig products and the environment. It can also become endemic in undomesticated or wild Suidae and in Ornithodoros ticks.

3. SCOPE

This Standard Operating Procedure is used for the extraction of ASFV DNA. The nucleic acid obtained is used as a template for further PCR.

4. RESPONSIBILITY:

4.1 The head of the laboratory is responsible for ensuring the correct application of this procedure by suitably trained staff.

4.2 The head of the laboratory is also responsible for ensuring that the laboratory staff are appropriately qualified and trained to safely and properly handle specimens for molecular analysis

4.3 The trained laboratory staff should perform the extraction procedure in accordance with the SOP.

5. DEFINITIONS AND ABBREVIATIONS:

5.1 ABBREVIATIONS

5.1.1	ASFV	African swine fever virus
5.1.2	CT	Cycle Threshold
5.1.3	DNA	Deoxyribonucleic Acid
5.1.4	E+	ASFV Positive extraction control:
5.1.5	E-	ASFV Negative extraction control:
5.1.6	PCR	Polymerase chain reaction
5.1.7	rpm	revolution per minute
5.1.8	SOP	Standard Operating Procedure

5.2. DEFINITIONS

Not applicable

6. SAFETY PRECAUTIONS

Consider all clinical specimen as infectious and thereforehandle them appropriately.

7. SPECIMEN

Recommended specimen	Collection Notes	Pre-Analytical Processing
• Whole blood • Tissues • Tissue homogenates • Cell culture supernatant • Homogenated soft ticks (*ornithodoros genus*)	Fresh	Freeze on arrival

8. EQUIPMENT / SUPPLIES/ REAGENTS

Equipment	Supplies	Reagents
• Micro-centrifuge • Vortex mixer • Freezers -70°C and below • Freezer -20°C • Refrigerator +4°C • Water-bath • Biosafety cabinet class 2	• Single channel pipettes (1-10µl, 10-20µl, 10-100µl, 200-1000µl). • Assorted micropipette tips with aerosol resistant filter • Non-powdered latex or nitrile gloves. • Micro-centrifuge tubes • (0.2ml, 0.5ml, 1.5ml, and 2ml) • Tube racks.	• Binding buffer • Proteinase-k • Inhibitor removal buffer • Wash buffer • High pure filter tubes • Collection tubes • Absolute isopropanol • Absolute ethanol • Nuclease free or • PCR grade water • Positive and Negative controls

Equipment	Supplies	Reagents
	• Permanent marker pens • sample labels/ stickers • Disposable absorbent paper towel • Ice packs / cold blocks	• Disinfectant e.g. Sodium hypochlorite **NB:** All reagents to be stored as per manufacturer's instructions

9.0 METHODOLOGY:

9.1 PRINCIPLE

Cells are lysed during a short incubation with proteinase-K in the presence of a chaotropic salt (guanidine HCL), which immediately inactivates all nucleases. Cellular nucleic acids bind selectively to special glass fibers pre-packed in the high pure purification filter tube. Bound nucleic acids are purified in a series of rapid "wash-and-spin" steps to remove contaminating cellular components. Finally, the nucleic acids are released from the glass fiber using sterile nuclease free water or elution buffer.

9.2 PREPARATION OF REAGENTS

9.2.1 LYOPHILIZED PROTEINASE-K

Dissolve proteinase-K according to the manufacturer's instructions.

9.2.2 WASHING BUFFER

Add absolute ethanol to the original vial according to the manufacturer's instructions, label and store at room temperature.

9.3 DNA EXTRACTION PROCEDURE

The following procedure is based on Qiagen® extraction kit protocol;

1. Pipette 200µl of binding buffer and 40 µl of 20mg/ml proteinase-K into a 1.5 micro-centrifuge tube
2. Add 200µl of the sample. Include in each extraction procedure the E+ (200µl ASFV extraction positive control) and E- (200µl H2O). Mix immediately and incubate for 10 minutes at 72±2oC.
3. Briefly centrifuge the 1.5ml micro-centrifuge tube to collect all the tube contents at the bottom.
4. Add 100µl of iso-propanol to the sample tube.
5. Place the high pure filter tube in a collection tube and pipette the sample in the upper reservoir.
6. Centrifuge for one minute at 8000 rpm. Note; for blood samples, repeat the centrifugation step if sample remains in the filter tube.
7. Discard the collection tube and place the filter tube into a clean collection tube.
8. Add 500µl of inhibitor removal buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.
9. Discard the collection tube and place the filter tube into a clean collection tube.

10. Add 450µl of the wash buffer to the upper reservoir and centrifuge for 1 min. at 8000 rpm.
11. Discard the collection tube and repeat the washing step.
12. Discard the collection tube and place the filter tube into a clean collection tube. Centrifuge for 10 seconds at 13000 rpm to remove residual wash buffer.
13. Discard the collection tube and place the filter tube in a clean 1.5 ml micro-centrifuge tube.
14. Elute the nucleic acids by adding 50ul of pre-warmed (72±20C) sterile nuclease free water or elution buffer to the upper reservoir. Centrifuge for 1min. at 8000 rpm.

10.0 QUALITY CONTROL FOR EXTRACTION

Include the positive and negative controls to monitor the success of the extraction process verifiable by PCR.

11.0 QUALITY CONTROL MATERIALS

- **Extraction positive control (E+):** ASFV positive sample (serum, EDTA-blood, 1/10 tissue homogenate or culture supernatants) diluted in nuclease free water. It is recommended that, the reference material (E+) is pre validated by real time PCR with CT value of 32±4,
- **(E-) Negative sample control for the extraction:** Nuclease free water.

12. REFERENCE

1. AFRICAN SWINE FEVER. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees) CHAPTER 2.8.1 OIE, 2012. http://www.oie.int/ileadmin/Home/eng/Health standards/tahm/2.08.01 ASF.pdf]
2. Aguero M, Fernandez J, Romero U, Zamora MJ, Sanchez C, Belak S, Arias M, Sanchez-Vizcaino JM. "A highly sensitive and specific gel-based multiplex RT-PCR assay for the simultaneous and differential diagnosis of African Swine Fever and Classical African Swine Fever in clinical samples". Vet Res. 2004 Sept-Oct; 35 (5):551-63.
3. Aguero M, Fernandez J, Romero U, Sanchez C, Arias M, Sanchez- Vizcaino JM. 2003. "A. highly sensitive PCR Assay for Routine Diagnosis of African Swine Fever Virus in Clinical Samples J. Clin. Microbiol", vol. 41, no.9, p4431-4434
4. Food and Agriculture Organization of the United Nations (FAO). RECOGNIZING AFRICAN SWINE FEVER. A FIELD MANUAL. 2000 Edition, Vol 9.
5. Qiagen extraction kit

2.1 DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY CONVENTIONAL POLYERASE CHAIN REACTION (PCR)

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY CONVENTIONAL POLYERASE CHAIN REACTION (PCR)			
	Name	Signature	Date
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Approval Authority			

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1. PURPOSE

The purpose of this SOP is to describe the test for detection of the specific presence of African Swine Fever Virus (ASFV) DNA material by conventional polymerase chain reaction (PCR) technique.

2. INTRODUCTION

African swine fever virus (ASFV) is highly contagious, and can spread very rapidly in pig populations by direct or indirect contact. This virus can persist for long periods in pig products and the environment. It can also become endemic in undomesticated or wild Suidae, and in Ornithodoros ticks. ASFV isolates vary in virulence from highly pathogenic strains that cause near 100% mortality to low–virulence isolates that can be difficult to diagnose. There is no vaccine or treatment.

3. SCOPE

- This SOP is applicable to the ASFV DNA extracted following the procedure described in the SOP/ASF/DNA EXTRACTION.
- The SOP is applicable for all trained laboratory staff in molecular biology laboratory involved in detection of ASFV DNA by conventional PCR

4 RESPONSIBILITY

4.1 The head of the laboratory is responsible for ensuring the correct application of this procedure by suitably trained staff.

4.2 The head of the laboratory is also responsible for ensuring that the laboratory staff are appropriately qualified and trained to safely and properly handle specimens for molecular analysis.

4.3 The laboratory staff are responsible for ensuring that the proper procedures are followed according to the SOP.

5 DEFINITIONS AND ABBREVIATIONS

5.1 ABBREVIATIONS

ASF	African swine fever
ASFV	African swine fever virus
bp	Base pairs
DNA	Deoxyribonucleic acid
E+	ASFV Positive extraction control:
E-	ASFV Negative extraction control
EDTA	Ethylene diamine tetra acetic acid
PCR	Polymerase Chain Reaction
R+	ASFV DNA reaction positive control
R-	ASFV DNA reaction negative control
rpm	Revolution per minute
TAE	Tris base, acetic acid and EDTA

6 EQUIPMENT / SUPPLIES/ REAGENTS:

Equipment	Supplies	Reagents
• Analytical balance • Convetional thermocycler with heated lid • Freezers -70oC and above • Freezers -20oC • Refrigerator +4oC • Microcentrifuge • Gel documentation system including a UV transilluminator) • Pipette aid or equivalent • Ice maker • Vortex mixture • Microwave oven • Magnetic stirrer • Power back up • Spirit level	• Adsorbent paper • Glass or plastic pipettes (1-10ml) • Powder free latex or nitrile gloves • PPE • Microcentrifuge tubes (0.2, 0.5, 1.5 and 2ml) • Micropipette - Single channel (1-10µl, 10- 100µl, 10-200µl and 200-1000µl) • Pipette tips (assorted) with aerosol resistant filter • Horizontal Gel casting trays for agarose gels • Gel tanks • Gel combs • Electrophoresis system • Face shield • Tube racks. • Permanent marker pen • sample labels/ stickers • Ice packs/cold blocks • Masking tape	• A) Reagents for DNA amplification step • AmpliTaq Gold® DNA polymerase with buffer II and MgCl2 [Ref: N8080243 (Roche) or similar characteristics)] • ASFV Primers at a concentration of 20 pmol/µl store in aliquots. • Primer PPA-1 sequence 5' AGTTATGGGAAACCCGACCC-3' (Forward primer) • Primer PPA-2 sequence 5' CCCTGAATCGGAGCATCCT-3' (Reverse primer) • Deoxyribonucleotide triphosphate (dNTP) mix containing 10Mm of each dNTP [Ref.:11581295001 (Roche) or similar characteristics)] • Nuclease free sterile H2O, PCR grade • Positive and negative control • Agarose (Molecular grade) • Bromophenol blue • Ethidium bromide or equivalent DNA dye • Glycerol 87% • Molecular weight marker

Equipment	Supplies	Reagents
		• TAE buffer 50x (Tris base, acetic acid and EDTA) • Xylene cyanol • Distilled water **NB:** All reagents to be stored as per manufacturer's instructions

7 SAFETY PRECAUTIONS

- Ethidium Bromide is carcinogenic and should be handled with care.
- Avoid exposure to UV light.
- Good Laboratory Practices should be followed.

8 METHODOLOGY:

8.1 TEST PRINCIPLE

Polymerase Chain Reaction (PCR) is a molecular technique that allows for the specific detection of DNA by enzyme–based amplification of a short viral genome fragment defined by a specific primer set. Under controlled conditions, multiple copies of DNA are generated by the action of the DNA polymerase enzyme that adds complementary deoxyribonucloetides (dNTPS) to a piece of DNA known as template. PCR is a three-step process that is carried out in repeated cycles. The initial step is the denaturation, or separation of the two strands of the DNA molecule, accomplished by heating the starting material to temperatures of about 95oC (203oF). Each strand is a template on which a new strand is built. In the second step the temperature is reduced to a predetermined annealing temperature so that the primers can anneal to the template. In the third step the temperature is raised to about 72oC (162oF) for the DNA polymerase to begin adding dNTPs to the 3' ends of each primer and generate a section of double-stranded DNA in the region of the gene of interest. At the end of the cycle the temperature is raised and the process begins again. The number of copies doubles after each cycle generating multiple copies of the target DNA. Finally, in the conventional PCR the amplified product will be detected by agarose gel electrophoresis, staining with ethidium bromide that intercalates the double-stranded DNA. This can be observed under UV light.

9 PROCEDURES:

9.1 REAGENTS PREPARATION

9.1.1 LOADING SAMPLE BUFFER 6X

Prepare or use ready to use loading buffer that is composed of bromophenol blue 0.25%, xylene cyanol 0.25%, glycerol 30%.

9.1.2 ELECTROPHORESIS BUFFER 1X

Dilute 40mls of TAE (50x) in 1960ml of distilled water. Store at room temperature.

9.1.3 MOLECULAR WEIGHT MARKER DNA

Add 200 µl of marker to 200 µl of loading buffer 6x and 400 µl of electrophoresis buffer 1x. Store at +4±3°C.

9.2 DNA AMPLIFICATION PROCEDURE

9.2.1 MASTER MIX PREPARATION

In a sterile 1.5ml micro-centrifuge tube prepare the PCR reaction mixtures described below for the number of samples to be assayed (including all the controls) allowing for at least two extra samples.

Pipetting step	**Master Mix Reagent**	**1X Volume (reaction 25µl)**	**Final concentration**
	Reagent	Volume for a single reaction	Final concentration
1	H2O	17.4 µl	
2	Buffer 10X	2.5 µl	1X
3	MgCl2 25 mM	2 µl	2mM
4	dNTPs 10 mM	0.5 µl	0.2mM
5	Primer PPA-1 20 µm	0.25 µl	0.2µM
6	Primer PPA-2 20 µm	0.25 µl	0.2µM
7	AmpliTaq Gold® 5 U/ µl	0.125 µl	0.025U/µl
	Master mix Volume	23 µl	
8	Add 2 µl of DNA template to each 0.2ml PCR tube. Include all the controls		

After addition of the template, close the reaction tube and spin down the PCR mix. Place all tubes in an automated thermocycler. Run the incubation program as detailed below.

PCR STEP	**TEMPERATURE**	**TIME**	**No of CYCLES**
Activation of AmpliTaq Gold®	95oC	10 minutes	1
DNA Denaturation	95 oC	15 sec	40
Primer annealing	62 oC	30 sec	
Elongation of DNA	72 oC	30 sec	
Extra elongation step	72 oC	7 minutes	1
Hold at +4°C until electrophoresis (maximum 18 hours)			

10 AGAROSE GEL ELECTROPHORESIS

1. Make a 2% agarose gel solution in 1x TAE buffer. Heat the solution in a microwave oven until the agarose is completely melted. Add the Ethidium bromide (BrEt) at a final concentration of 0.5 µg/ml. Shake carefully to homogenate.
2. Prepare the gel tray, seal the ends and place the comb for adequate number of wells. Pour the melted agarose into the gel tray. Wait until the gel becomes solid (approx. 20 minutes).

3. Carefully remove the sealing of the tray and place it in the tank. Add the electrophoresis buffer until the gel is covered. Carefully remove the comb.
4. Add 4µl of 6x loading buffer to each tube containing 25µl of the PCR amplified product.
5. Load 10µl of each sample to each well of the gel.
6. Add 6µl of DNA molecular weight marker to one well lane of the gel.
7. Connect to power supply and confirm direction of sample(s) movement (DNA samples will move towards the positive electrode) Run the gel at a constant voltage of 150-200volts for about 30-40 minutes.
8. Finally, place the gel on an ultraviolet trans-illuminator to visualize the bands.

Note: The voltage depends on the percentage and size of the agarose gel. As a general rule, it is considered that for 2% agarose gels set the voltage at 5-10v/cm2.

11 ANALYSIS AND INTERPRETATION OF RESULTS

When electrophoresis is completed, immediately examine the gel over a UV light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive controls (R+, E+). Compare the size of the PCR products in the test samples and the positive control by reference to the standard molecular weight marker. The PCR product of the positive control (R+, E+) has a size of 257bp. No band should be seen in the negative control (R-, E-)

12 PROCEDURE PRECAUTIONS

Since PCR is a highly sensitive technique, the most critical point along all analysis procedure is the considerable risk of carry-over contaminations, and the false positive results that could be obtained in this situation. The contamination could be due to the ASFV itself present in the positive analyzed samples or in the positive controls included in the DNA extraction procedure. It could also be due to ASFV DNA obtained after amplification and manipulated by agarose gel electrophoresis during the amplicon analysis of a previous PCR. It is mandatory that personnel working on PCR follow and carry out strict work-flow rules in order to minimize contamination risk associated to PCR technique.

- All steps of sample analysis by PCR must be performed in separate designated rooms or locations using equipment and material specific for each as follows: sample preparation, DNA extraction, PCR mix preparation, and analysis of PCR products by agarose gel electrophoresis.
- Personnel must always work with clean nitrile or latex gloves in the PCR laboratory. Whenever personnel go into a different PCR area, they should change PPE including gloves.
- All material/equipment must be used only at the designated area as per step for the PCR procedure to avoid cross contamination.
- Materials/equipment used in these procedure must be used in the designated area as per where is located/labelled.

- Use a new pipette tip each time that a tube containing any sample or DNA material is to be manipulated.
- Tubes containing amplified products should never be opened and manipulated in other laboratory areas except in the distinctly assigned areas for their electrophoresis and analysis, where they will be discarded.
- Ethidium bromide (BrEt) is a known mutagen in powdered form and should be handled as a hazardous chemical. It is highly recommended to order as dropper solution to minimize its manipulation. Ethidium bromide handling must be performed exclusively in the laboratory assigned to it while observing laboratory safety measures. In case of any unintended contact, wash immediately with abundant water and contact the biosafety officer.

13 QUALITY CONTROL.

The following quality assurance methods shall be employed on regular bases (at least once a year)

- Intra and inter analyst comparisons
- Inter laboratory tests
- Proficiency testing

13.1 QUALITY CONTROL MATERIALS.

13.1.1 Descriptions of reference materials.

- R+ ASFV positive control for the reaction is ASFV positive DNA. It's highly recommended that the positive control is about the detection limit of the technique to track the yield of the DNA extraction procedure. The R+ material is recommended to be regularly checked by real-time PCR and optimized to 26 ±2 CT values. This control should be sourced from the OIE.
- R- Negative DNA target control for the reaction is nuclease free water.

13.2 CALIBRATION.

All the equipment must be put under planned maintenance in accordance with the Quality Manual regulations.

13.3 QUALITY CONTROL RESULTS.

Results for quality control checks on implementation and compliance to this SOP will be filed in the Quality Manager's Random Check Result File.

14 REFERENCES:

1. AFRICAN SWINE FEVER. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees) CHAPTER 2.8.1 OIE, 2012. http://www.oie.int/ileadmin/Home/eng/Health standards/tahm/2.08.01 ASF.pdf]
2. Aguero M, Fernandez J, Romero U, Zamora MJ, Sanchez C, Belak S, Arias M, Sanchez-Vizcaino JM. "A highly sensitive and specific gel-based multiplex RT-PCR assay for the simultaneous and differential diagnosis of African Swine Fever and Classical African Swine Fever in clinical samples". Vet Res. 2004 Sept-Oct; 35 (5):551-63.

3. Aguero M, Fernandez J, Romero U, Sanchez C, Arias M, Sanchez-Vizcaino JM. 2003. "A. highly sensitive PCR Assay for Routine Diagnosis of African Swine Fever Virus in Clinical Samples J. Clin. Microbiol"., vol. 41,no.9,p4431-4434
4. Food and Agriculture Organization of the United Nations (FAO). RECOGNIZING AFRICAN SWINE FEVER. A FIELD MANUAL. 2000 Edition.

15 APPENDICES

15.1 APPENDIX 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **Detection of African Swine Fever Virus (ASFV) by Conventional Polyerase Chain Reaction (PCR)**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages: 8**
Version 1:Title	Dated:	SOP No.:	No. Pages:
Version 2:Title	Dated:	SOP No.:	No. Pages:
Version 3:Title	Dated:	SOP No.:	No. Pages:
Version 4:Title	Dated:	SOP No.:	No. Pages:
Version 5:Title	Dated:	SOP No.:	No. Pages:

15.2 APPENDIX .2: SOP DISTRIBUTION LISTING

This section is to be completed by the Document Coordinator in consultation with the Document Initiator and Laboratory Management indicating the Section / stations where controlled copies of this document shall be circulated.

Section / Area	**SOP Manual Number**	**Date Issued**

15.3 APPENDIX 3: SOP TRAINING LOG

This section is to be used to document training of the SOP.

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: **DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY CONVENTIONAL POLYERASE CHAIN REACTION (PCR)**		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand		

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

3. SEROLOGICAL DETECTION OF SPECIFIC ANTIBODIES TO BRUCELLA USING COMPETITIVE ELISA

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: SEROLOGICAL DETECTION OF SPECIFIC ANTIBODIES TO BRUCELLA USING COMPETITIVE ELISA			
	Name	**Signature**	**Date**
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1. PURPOSE

The purpose of this Standard Operating Procedure (SOP) is to describe the serological diagnostic test used to detect antibodies in animals infected with Brucella using c-ELISA.

2. INTRODUCTION:

Brucellosis is regarded as a highly contagious, zoonotic disease with worldwide distribution. The condition is caused by bacteria of the genus Brucella, which occur in different variants in different animal species. For example, Brucella abortus is mostly associated with cattle and B. melitensis with sheep, goats, camel and humans. In animals it is characterized by abortion, retained placenta, orchitis and epididymitis and in human it is associated with undulating fever, fatigue, malaise, headache, backache, and arthralgia.

3. SCOPE

This SOP is for use by the technical staff involved in the laboratory diagnosis of Brucellosis.

4. RESPONSIBILITY

4.1. It is the responsibility of the head of laboratory to ensure that all the staff performing the test receive copies of the SOP.

4.2 It is also the responsibility of the head of laboratory to ensure that all the staff using this SOP are trained and are competent.

5. DEFINITIONS AND ABBREVIATIONS

5.1 ABBREVIATIONS

BP	Brucella proteins
c-ELISA	Competitive Enzyme Linked Immunosorbent Assay
oC	Degrees Celsius
HRP	Horseradish peroxidase
LPS	Lipopolysaccharide
N/A	Not applicable
OD	Optical density
PBS	Phosphate buffered saline
RT	Room temperature
SOP	Standard Operating Procedure
TMB	3,3',5,5' tetramethylbenzidine

5.2 DEFINITION

- Not applicable

6. SAFETY PRECAUTIONS:

- The laboratory personnel should wear appropriate personal protective equipment while handling kit reagents or specimens; wash hands thoroughly
- Chromogens and some chemicals are mutagenic and carcinogenic; therefore gloves and facemasks must be used all the time when running the ELISA test.
- Reagents/chemicals should be stored safely and be inaccessible to unauthorized person.
- Brucellosis is a zoonotic disease and therefore samples must be handled using appropriate personal protective equipment.
- Follow the established good laboratory procedure.

7. SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
• Serum or plasma	• Collect whole blood from suspected livestock and wild animals either in plain or heparinized vacutainer tubes	• Blood is left to stand for 2 hours at 22-25oC and then centrifuged at 2000 rpm for 10 minutes. • Collect the serum in sterile vials, label and assign a laboratory number. • The serum can be stored at –200C before use. • Fresh serum can also be used directly after centrifugation. • Heparinized blood can be kept at +40C.

8. EQUIPMENT / MATERIALS/ REAGENTS:

Equipment	Supplies	Reagents
• ELISA reader • Orbital shaker • ELISA plate washer • Water purification system • Refrigerator • Incubator • pH meter • Computer • Printer	• Coated plates • Micropipettes • Micropipette tips • Laboratory glass ware • Cryovials • Absorbent towels • Laboratory marker pens • Laboratory timer • Vortex mixer • Reagent troughs	• A competitive ELISA kit • Plates (pre-coated with B. melitensis LPS antigen • Dilution buffer • Wash solution –Na2HPO4 • Conjugate (x10) • Chromogen • Substrate • Stopping solution • Controls

9.0 METHODOLOGY:

9.1 TEST PRINCIPLE

This procedure is based on a solid phase competitive ELISA. The sample together with monoclonal antibody (mAb) specific to an epitope on the o-polysaccharide portion of the S-LPS antigen, are exposed to Brucella abortus smooth o-polysaccharide (S-LPS) coated wells on micro titre plates. If Brucella antibodies are present in the test sample, they will bind to the antigen in the well and block these antigen sites. If Brucella antibodies are absent in the sample, these sites will remain free and the mAb which was added together with the sample will bind to these antigenic sites. After an incubation period the unbound material are removed by rinsing and conjugated IgG is added to the plate. The conjugate will bind to the specific mAb in the absence of Brucella antibodies in the sample. Unbound materials are removed by rinsing prior to the addition of the substrate. Subsequently a blue colour develops which is due to the conversion of the substrate by the conjugate. A negative result is indicated by the development of a blue colour. The reaction is stopped by addition of stop solution, the colour changes to yellow. In the presence of antibodies, no coloration appears. The test plate is read at 450nm.

9.2 PREPARATION OF REAGENTS

9.2.1 Washing solution

- Add one ampoule of Na2HPO4 and 1ml of Tween 20 to 10 litres of distilled water.
- Store at room temperature for not more than a month.

9.2.2 Diluent Buffer (PBST)

- Add 5 tablets of PBS, 0.5ml of phenol red indicator and 250µl of Tween 20 to 500ml of distilled water. The pH of the buffer must be between 7.2 and 7.6.
- Phenol red will turn yellow below pH 7.2 and violet above pH 7.6
- Store at 40C±30C for not more than 1 month

9.2.3 Conjugate

- Dilute conjugate to 1/10 for short incubation or to 1/20 for overnight incubation in dilution buffer 3.

Guideline of conjugate dilution 1:10 depending on the number of plates

No of plates	Conjugate (ml)	Diluent buffer 24 (ml)
1	1	9
2	2	18
3	3	27
4	4	36

9.2.4 Substrate buffer

Use the supplied ready to use 'TMB"

9.2.5 Stopping solution

Dilute the content of the ampoule of citric acid with 38ml of distilled water.
Store at 40C±30C for not more than 1 month

9.2.6 Controls

Reconstitute each of the strong and weak positive and negative control samples with 1ml of distilled water.

Store at 40C±30C in aliquots for not more than 1 week or -200C±50C for longer periods.

9.3 TEST PROCEDURE

Note: This test procedure is based on Idexx test kit.

1. All reagents should equilibrate to room temperature 18-25 0c before use.
2. Dispense 45 µl of sample dilution buffer into each well that will be used for serum sample, serum controls and conjugate controls.
3. Add 5 µl of positive, weak positive and negative serum controls, into each of the appropriate wells, respectively. For confirmation purpose it is recommended to run the control sera in duplicate.
4. Add 5 µl of sample dilution buffer into two appropriate wells (designated as Conjugate control, Cc)
5. Add 5 µl of sample to each of the appropriate wells. The sample can be tested in singlicates or in duplicates. However for confirmation purposes it is recommended to run the samples in duplicates.
6. Add 50 µl of mAb- solution into all wells used for control and samples.
   NB: The time difference between control/ sample and mAb- solution addition must not exceed 10 minutes.
7. Seal the plates and mix the reagents thoroughly for 5 minute, either by using a plate shaker or by tapping the sides of the plate.
8. Incubate the plates at room temperature 18- 250c for 30 minutes.
9. Rinse the plates/strips 4 times with PBS-Tween Buffer: fill up the wells at each rinse, empty the plates and tap hard to remove all the fluid that remains.

10. Add 100 µl of conjugate solution into each well. Seal the plates and incubate at room temperature 18- 250c for 30 minutes.
11. Repeat step no.9
12. Add 100 µl of Substrate solution into each well and incubate at room temperature 18- 250c for 10 minutes. Begin timing after the first plate is filled.
13. Stop the reaction by adding 50 µl of stop solution to each well and mix thoroughly. Remember to add the stop solution in the same order as the substrate solution was added in step no.12.
14. Measure the optical density (OD) of the controls and samples at 450nm in a micro plate photometer (use air as a blank.)
15. Measure the OD within 15 minutes after addition of stop solution to prevent fluctuation in OD values.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cc	Cc	**1**	**1**	**9**	**9**	**17**	**17**	**25**	**25**	**33**	**33**
B	CP++	CP++	**2**	**2**								
C	CP++	CP++	**3**	**3**								
D	CP+	CP+	**4**	**4**								
E	CP+	CP+	**5**	**5**								
F	Cm	Cm	**6**	**6**								
G	Cm	Cm	**7**	**7**								
H	CN	CN	**8**	**8**	**16**	**16**	**24**	**24**	**32**	**32**	**40**	**40**

Key
Cc-Conjugate control
Cm -Monoclonal antibody control
CP++- Strong positive control
CP+- Weak positive control
CN -Negative Control

9.4 PROCEDURE NOTES.

- Bring all the reagents to room temperature one hour before use.
- TMB substrate and wash solutions can cause eye irritation; so take appropriate precaution.

10. QUALITY CONTROL.

- Always use the positive and negative controls to compare with samples when doing the test
- The kit contains a strong and weak positive controls, and negative control.

10.1 CALIBRATOR.

- Not applicable

10.2 CALIBRATION.

- The ELISA reader should be calibrated 2 times a year or as recommended by the manufacturer.

11. READING AND INTERPRETATION OF TEST RESULTS

- Read test plate at 450 nm.

11.1 Analysis of Results

Calculations

Calculate the mean OD values for each of the controls and samples.

Calculate the percent inhibition (PI) values for controls as well as samples, using the following formula:

$$\textbf{PI} = 100 - \frac{(\textbf{OD}\ \text{sample or control} \times 100)}{\textbf{OD}\ \text{sample or control}}$$

11.2 Interpretation of the results.

- Criteria for test validity:

OD Cc	0.75 – 2.0
PI Positive control	80 – 100
PI Weak Positive control	30 – 70
PI Negative control	(-10) – 15

PI	**Status**
<30%	Negative
≥30%	Positive

NB: For invalid test results, the assay should be repeated.

12. TROUBLESHOOTING

- If there is no colour development at all, or even after 15 minutes incubation (Repeat test)
- If colour develops too slowly (Check dilutions)
- If colour develops all over plate (Check for contamination)

8. REFERENCES:

1. COMPELISA VLA UK Test Kit.
2. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals-OIE, 2013. Chapter 2.4.3. available at https://www.google.com/#q=oie+manual+of+diagnostic+tests+and+vaccines+for+terrestrial+animals+pdf
3. Mantur B.G, Amarnath S.K, Shinde R.S. Review of clinical and laboratory features of human brucellosis. Indian J Med Microbiol 2007; 25:188–202.

9. APPENDICES:

9.1 APPENDIX 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **SEROLOGICAL DETECTION OF SPECIFIC ANTIBODIES TO BRUCELLA USING COMPETITIVE- ELISA**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

9.2 APPENDIX 2: SOP DISTRIBUTION LISTING:

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Section / Area	SOP Manual Number	Date Issued

9.3 APPENDIX 3: SOP TRAINING LOG

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**

Title: **SEROLOGICAL DETECTION OF SPECIFIC ANTIBODIES TO BRUCELLA USING COMPETITIVE- ELISA**

Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

3.1 INDIRECT ELISA FOR THE DETECTION OF BRUCELLOSIS (MULTI SPECIES)

Standard Operating Procedure **APPROVED**			**SOP No:** **Version: Original** **Supersedes: None** **Effective Date:** **Review Date:**
Title: INDIRECT ELISA FOR THE DETECTION OF BRUCELLOSIS (MULTI SPECIES)			
	Name	**Signature**	**Date**
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE/INTRODUCTION:

1.1 PURPOSE:

The purpose of this SOP is to describe the diagnostic test for Brucellosis (multi species). The SOP describes the indirect enzyme immunosorbent assay (i-ELISA) used for the detection of serum antibodies against Brucellosis.

1.2 INTRODUCTION:

Brucellosis is a bacterial zoonotic disease of the reproductive system characterized by abortion, retained placenta, orchitis and epididymitis in livestock and by undulating fever in man.

2.0 SCOPE / RESPONSIBILITY:

2.1 SCOPE

This SOP is for use by the technical staff involved in the laboratory diagnosis of Brucellosis.

2.2 RESPONSIBILITY

- It is the responsibility of the head of the laboratory to ensure that all the staff carrying out this test implement this SOP.
- It is also the responsibility of the head of the laboratory to ensure that all the staff using this SOP are trained and competent.
- It is the responsibility of the head of the laboratory or the designated staff to approve the test result.

3.0 DEFINITIONS AND ABBREVIATIONS:

3.1 ABBREVIATIONS

C+	Positive Control
FIFO	First in First out
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
iELISA	Indirect Enzyme Linked Immunoassay
LPS	Lipopolyssacharide
N/A	Not applicable
nM	Nanometer
OD	Optical density
ODNC	Negative control
ODPC	Positive control OD
ODS	Test sample OD
oC	Degrees Celsius
PBS	Phosphate Buffered Saline
PPE	Personal Protective Equipment
QM	Quality Management
S/P	Sample percentage positivity
SOP	Standard Operating Procedure
TM	Test Methods
TMB	3,3',5,5' tetramethylbenzidine

3.2 DEFINITION

Not applicable

4.0 SAFETY PRECAUTIONS:

- The laboratory personnel should wear appropriate personal protective equipment (P P E) while handling kit reagents or specimens; wash hands thoroughly afterwards.
- Chromogens and some chemicals are mutagenic and carcinogenic; therefore gloves and facemasks must be used all the time when running the ELISA test.
- Reagents/chemicals should be stored safely and be inaccessible to unauthorized persons.
- Brucellosis is a zoonotic disease and therefore must be handled using appropriate personal protective equipment.
- When preparing laboratory solutions, always add acid to water, never water to acid.
- Every reagent/chemicals and equipment should be handled in the manner recommended by the manufacturer.
- Follow the established Good Laboratory Practice (GLP).
- Use the approved forms, manuals, SOPs, TMs accurately at all times.

5.0 SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
• Serum or plasma	• Collect whole blood from cattle, buffalos and other infected livestock either in plain or heparinized vacutainer tubes.	• Blood is left to stand for 2 hours at room temperature and then centrifuged at 2000rpm for 10 minutes. • Collect the serum in sterile vials, label and assign a laboratory number. • The serum can be stored at –200C before use. Fresh serum can also be used directly after centrifugation. • The heparinized blood can be kept at 4^0C.

6.0 EQUIPMENT / MATERIALS/ REAGENTS:

Equipment	Supplies	Reagents
• ELISA reader • Incubator shaker • ELISA plate washer • Pipettes • Water distillation or de-ionization system • Refrigerator • pH meter • Vortex mixer • Computer • Analytical balance • Printer	• Coated plates (96 wells) • Micropipettes (single and multi channel) • Beakers, cylinders • Cryovials • Absorbent paper towels • Laboratory marker pens • Laboratory timer • Reagent troughs • Pipette tips • PPE • ELISA calibration plate	• Substrate • Control sera C+ and C- • Conjugate • Stop solution • Wash solution • Distilled or de-ionized water. • PBS • Disinfectants

7.0 METHODOLOGY:

7.1. TEST PRINCIPLE

Indirect ELISA is a two-step test that involves two binding processes of primary antibody and labelled secondary antibody. The primary antibody (sample/test antibody) is incubated with the antigen followed by the incubation with the secondary antibody, thus the sample antibody is sandwiched between the antigen coated on the plate and an enzyme-labelled, anti-species globulin conjugate. In short, the microplate wells are coated with purified Brucella abortus lipopolysaccharide, LPS. Specific antibodies present in the test sera bind to the coated antigen on the microwells, the anti Brucella antibodies if present form an antibody – antigen complex. After washing, a multi species horseradish peroxidase conjugate (HRP) is added to the microwells. This fixes to the anti Brucella antibodies, forming an antigen – antibody conjugate peroxidase complex. The complex is revealed when HRP substrate (TMB) is added to form a blue compound that will turn yellow when the reaction is stopped. The intensity

of the colour depends on the quantity of antibodies present in the test sera. Thus the intensity of the colour formed is directly proportional to the amount of bound sample antibody.

7.2 PREPARATION OF REAGENTS

7.2.1 Washing solution

Dilute 100ml of wash concentrate (20x) in 1900 ml of distilled water. If it is not to be used immediately then prepare for 1 plate i.e. 9 ml "wash concentrate (20x) in 171ml of distilled water

Guideline for wash solution dilution 1:20 depending on the number of plates

No. of plates	Wash concentrate 20x (ml)	Distilled Water (ml)
1	9 mlml	171mlmlml
2	18	342
3	27	513
4	81	684

In the absence of Kit Wash concentrate

Prepare 0.05%Tween 20 in PBS by dissolving 5 PBS tablets in distilled water. Top up to 1 litre with distilled water to make the PBS solution.

Note: Volume of tween 20 in a 1 litre of PBS = (0.05/100) x 1000ml = 0.5ml of Tween. Therefore 0.5ml of Tween + 999.5ml of PBS make the 0.05% Tween 20 in PBS.

7.2.2 Diluent Buffer (PBS)

Supplied as ready to use.

7.2.3 Conjugate

Dilute conjugate to 1/10 for short incubation or to 1/20 for overnight incubation in dilution buffer 3.

Guideline of conjugate dilution 1:10 depending on the number of plates

No of plates	Conjugate (ml)	Diluent buffer 24 (ml)
1	1	9
2	2	18
3	3	27
4	4	36

7.2.4 Substrate buffer

Supplied as ready to use 'TMB".

7.2.5 Stopping Solution

Supplied as ready to use.

7.3 PROCEDURE

Note: This test procedure is based on IDVET test kit.

1. Dispense
   - 190µl of dilution buffer 2 in all wells
   - 10µl of negative control to wells A1 and B1
   - 10µl of positive control, P+ to C1 and D1
   - 10µl of test sera in the remaining wells E1 to H12;
2. Cover the plate and incubate for 45 (+/- 4) min at 22-25 oC;
3. Wash 3 times with 300µl wash solution, empty plate by tapping it upside down on an absorbent towel;
4. Dispense 100µl of diluted conjugate into all wells;
5. Cover plate and incubate for 30min (+/- 3) min at 21oC (+/- 5oC);
6. Wash 3 times with 300µl wash solution as in 3 above;
7. Dispense 100µl of ready to use TMB substrate in all wells;
8. Cover plate and incubate for 15min (+/- 2) min at 21oC (+/- 5oC) in the dark;
9. Add 100µl of stop solution to all wells to stop the reaction;
10. Gently shake the plate until the coloured solution is homogenised;
11. Wipe carefully the bottom of the plate;
12. Read and record the OD at 450nm;
13. Interpret and record the results.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	C-	S_5	S_{13}	S_{21}	S_{29}	S_{37}	S_{45}	S_{53}	S_{61}	S_{69}	S_{77}	S_{85}
B	C-	S_6	S_{14}	S_{22}	S_{30}	S_{38}	S_{46}	S_{54}	S_{62}	S_{70}	S_{78}	S_{86}
C	C+	S_7	S_{15}	S_{23}	S_{31}	S_{39}	S_{47}	S_{55}	S_{63}	S_{71}	S_{79}	S_{87}
D	C+	S_8	S_{16}	S_{24}	S_{32}	S_{40}	S_{48}	S_{56}	S_{64}	S_{72}	S_{80}	S_{88}
E	S_1	S_9	S_{17}	S_{25}	S_{33}	S_{41}	S_{49}	S_{57}	S_{65}	S_{73}	S_{81}	S_{89}
F	S_2	S_{10}	S_{18}	S_{26}	S_{34}	S_{42}	S_{50}	S_{58}	S_{66}	S_{74}	S_{82}	S_{90}
G	S_3	S_{11}	S_{19}	S_{27}	S_{35}	S_{43}	S_{51}	S_{59}	S_{67}	S_{75}	S_{83}	S_{91}
H	S_4	S_{12}	S_{20}	S_{28}	S_{36}	S_{44}	S_{52}	S_{60}	S_{68}	S_{76}	S_{84}	S_{92}

7.4 PROCEDURE NOTES.

- Bring all the reagents to room temperature one hour before use.
- TMB substrate and wash solutions can cause eye irritation; so take appropriate precaution.

7.5 QUALITY CONTROL.

- Homogenize all reagents by inversion or vortex
- Monitoring and tracking assay performance on quality control charts provides insight as to when it is necessary to troubleshoot problems.
- Equipment: - Keep preventive maintenance up-to-date; Calibrate and clean pipettes; Calibrate reader, sanitize and maintain wash system.
- Reagents: - Maintain inventory control - first in first out (FIFO); inspect components; avoid contamination; verify proper storage.
- Technique:- Monitor sample quality; verify reagent preparation; verify appropriate sample mixing; verify proper pipetting; check timing for multiple plate runs; check washing of assay plates; use in-house controls to verify results.
- Others: - Monitor laboratory temperature; use sterile disposable reservoirs; monitor assay performance; record in a log.

7.6 QUALITY CONTROL MATERIAL.

The kit contains a strong positive control, and negative control.

7.7 CALIBRATOR.

Not applicable

7.8 CALIBRATION.

The ELISA Reader should be calibrated 2 times a year as recommended by the manufacturer.

7.9 RESULTS

- Read the optical densities at 450 nm .
- Calculate the S/P positivity for each sample using the corrected sample and control values

$$S/P = \frac{(OD_S - OD_{NC})}{(OD_{PC} - OD_{NC})} \times 100$$

S/P = Sample percentage positivity
OD_S =Test sample OD,
OD_{PC} =Positive control OD,
OD_{NC} =Negative control OD
$OD_{PC} - OD_{NC}$

7.9.1 Results/Interpretation

For individual serum or plasma samples, after short or overnight incubations interpretation of results is as shown in the table below.

Result	Interpretation
S/P% ≤ 110%	Negative
110% < S/P% < 120%	Doubtful
S/P% ≥ 120%	Positive

For pools of ten sera or plasmas after short incubation interpretation of results is as shown in the table belowResult	Interpretation
S/P% ≤ 20%	Negative
S/P% ≥ 20%	Positive

7.10 QUALITY CONTROL RESULTS.

The test is valid only when;

- The mean corrected value of the Positive Control OD (ODPC) is greater than 0.350
  ODPC > 0.350
- The ratio of the mean corrected values of the Positive and Negative controls (ODPC and ODNC) is greater than 3,
  ODPC / ODNC > 3

8.0 REFERENCES:

1. Refer to ID VET kit insert; www.id-vet.com
2. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals-OIE, 2013. Chapter 2.4.3. available at https://www.google.com/#q=oie+manual+of+diagnostic+tests+and+vaccines+for+terrestrial+animals+pdf

9.0 APPENDIX 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **INDIRECT ELISA FOR THE DETECION OF BRUCELLOSIS (MULTI-SPECIES)**	**Dated:**	**SOP No.:**	**No. Pages:**
Version 1:Title	Dated:	SOP No.:	No. Pages:
Version 2:Title	Dated:	SOP No.:	No. Pages:
Version 3:Title	Dated:	SOP No.:	No. Pages:
Version 4:Title	Dated:	SOP No.:	No. Pages:
Version 5:Title	Dated:	SOP No.:	No. Pages:

9.2 APPENDIX 2: SOP DISTRIBUTION LISTING:

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Section / Area	SOP Manual Number	Date Issued

9.3 APPENDIX 3: SOP TRAINING LOG

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: **INDIRECT ELISA FOR THE DETECION OF BRUCELLOSIS (MULTI-SPECIES)**		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.		

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

3.2 ROSE BENGAL TEST FOR DIAGNOSIS OF BRUCELLOSIS

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: ROSE BENGAL TEST FOR DIAGNOSIS OF BRUCELLOSIS			
	Name	Signature	Date
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1. PURPOSE

This SOP describes the screening test used to detect antibodies in animals suspected to be infected with Brucella organisms.

2. INTRODUCTION

Brucellosis is a highly contagious zoonotic disease caused by various bacteria of the genus Brucella. The disease in animals is characterized by abortions or reproductive failure. Animals typically recover, and are able to have live offspring following the initial abortion but they may continue to shed the bacteria. Brucellosis in cattle is caused by B. abortus, in sheep, camel and goats B. melitensis and in swine B. suis. Brucellosis is a notifiable disease and is listed as one of the diseases that must be reported to the World Organisation for Animal Health (OIE). In man, Brucellosis manifests as an undulant fever commonly known as Malta fever.

3. SCOPE

- This SOP is applicable for the diagnosis of Brucellosis in serum specimens from Bovine, Caprine, Ovine, Camel and Porcine species suspected to be infected with Brucellosis.
- The SOP is applicable to all staff running the Rose Bengal test.
- The scope of this procedure is to provide instructions on how to carry out the Rose Bengal test (RBT) for Brucellosis diagnosis.

4. RESPONSIBILITY

4.1 It is the responsibility of the head of the laboratory to ensure that the SOP is distributed to all staff responsible for performing this test.

4.2 It is also the responsibility of the head of the laboratory to ensure that all staff using the SOP are trained and competent.

5. DEFINITIONS AND ABBREVIATIONS

5.1 ABBREVIATIONS

ID Identity
GLP Good Laboratory Practice
OIE World Organization for Animal Health
PPE Personal Protective Equipment
QC Quality control
RBT Rose Bengal test
rpm Revolutions per minute

5.2 DEFINITIONS

- Diagnostic specimen- Any sample submitted in the laboratory for analysis.
- Brucellosis- A disease caused by bacteria of the genus Brucella and is characterized by abortion.
- Antigen- Foreign substance that enters the body and starts a process that can provoke an immune response
- Rose Bengal Antigen- An antigen used for early detection of Brucella antibodies
- Antibody- Substance that the body produces in the body to fight the disease (A protein that a body produces to counter the foreign body)
- Serum- The liquid fraction of whole blood that forms after the blood is allowed to clot.

6. SAFETY PRECAUTIONS

- Follow Good Laboratory Practices (GLP) when performing this test.
- Consider all samples as potentially infectious.

7. SPECIMEN:

Recommended specimen	Collection Notes	Pre-Analytical Processing
• Serum	• Collected as whole blood in plain vacutainers or clot activator coated tubes.	• Blood is left to stand for 2 hours at room temperature and then centrifuged at 2000 rpm for 10 min. • Serum is collected and poured into sterile vials. The vials are then labelled and assigned a laboratory number. • The serum can be stored at –20°C. • Fresh serum can also be used directly after centrifugation.

8. EQUIPMENT / SUPPLIES/ REAGENTS:

Equipment	Supplies	Reagents
• Rocker machine • Centrifuge	• Gloves • Cryovials • Applicator sticks • Pasteur pipettes • White marked ceramic tiles • Permanent Lab marker pen • Sample labels • Micro titre pipettes • Pipette tips • Beakers • Paper towels • Timer	• Rose Bengal antigen • Positive & Negative controls (standardized) • Disinfectant (e.g. sodium hypochlorite)

9 METHODOLOGY:

9.1 TEST PRINCIPLE

This is a rapid agglutination technique, which uses a dense (8%) suspension of inactivated Brucella organisms stained with Rose Bengal antigen. Agglutination indicates presence of antibody in the serum sample.

9.2 PROCEDURES:

1. Bring the test serum, controls and reagents to room temperature (22- 250C) before use;
2. Using a marker pen, label each square on the white tile (s) with the sample ID;
3. Label two empty squares with control IDs (Positive and Negative);
4. Pipette and dispense 30µl (0.03ml) of test serum onto respective squares;
5. Pipette and dispense 30µl (0.03ml) of control sera onto respective squares;
6. Gently shake the Rose Bengal antigen bottle to ensure a uniform suspension;
7. Add an equal volume of 30µl (0.03ml) of the Rose Bengal antigen to each of the squares containing either test serum or control;
8. Mix the test serum/control and Rose Bengal antigen using applicator stick, changing the stick for every new square;
9. Start the timer immediately after mixing the first well contents and agitate the tile on the shaker for up to 4 minutes;
10. Observe the mixtures for any agglutination in the various squares;
11. Record the results obtained.

9.3 PROCEDURE NOTES

- Do not test more than 9 samples at the same time, to avoid drying up of the plates.
- Any agglutination after 4 minutes should be disregarded.
- A control serum that gives a minimum positive reaction should be tested before each day's tests are begun to verify the sensitivity of test conditions

10 QUALITY CONTROL.

- All the tests should be performed according to the SOPs provided;
- Positive and Negative Controls should be included in every test performed;
- The Quality Manager will do random checks to ensure that this SOP is implemented as written;
- Corrective and preventive measures will be initiated, implemented, and reviewed.
- Documentation will be done in the managers random check log.
- Non-conformance must be recorded.

10.1 QUALITY CONTROL MATERIAL.

- Standard control sera

10.2 CALIBRATOR.

- N/A.

10.3 CALIBRATION.

- N/A

11 RESULTS.

11.1 Validation of the test

- Examine the positive and negative control squares; If agglutination is present on positive control square and no agglutination on negative control square, then the test is valid;
- If agglutination is present on both positive and negative controls then the test is invalid.

11.2 Result interpretation

- Any visible agglutination indicates a positive test, and no agglutination indicates a negative test.

12 REFERENCES:

- Rose Bengal Test Kit protocol
- Mantur B.G, Amarnath S.K, Shinde R.S. Review of clinical and laboratory features of human brucellosis. Indian J Med Microbiol 2007; 25:188–202.
- Manual of Diagnostic Tests and Vaccines for Terrestrial Animals-OIE, 2013. Chapter 2.4.3. Available at https://www.google.com/#q=oie+manual+of+diagnostic+tests+and+vaccines+for+terrestrial+animals+pdf

13 APPENDICES:

13.1 Appendix 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **ROSE BENGAL PLATE TEST FOR BRUCELLOSIS DIAGNOSIS**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

13.2. Appendix 2: SOP DISTRIBUTION LISTING:

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Section / Area	**SOP Manual Number**	**Date Issued**

13.3 Appendix 3: SOP TRAINING LOG:

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: **ROSE BENGAL PLATE TEST FOR BRUCELLOSIS DIAGNOSIS**		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.		

DATE	**TRAINEE NAME**	**SIGNATURE**	**TRAINER NAME**	**SIGNATURE**

Name of training approval authority_____ Signature:_____Date: _____

4. CBPP COMPETITIVE ELISA ASSAY

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: CBPP COMPETITIVE ELISA ASSAY			
	Name	**Signature**	**Date**
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE / INTRODUCTION:

1.1 PURPOSE

The purpose of this standard operating procedure (SOP) is to describe one of the diagnostic tests for Contagious Bovine Pleuropneumonia (CBPP). The SOP describes the competitive enzyme immunosorbent assay (cELISA) used for the detection of serum antibodies against CBPP etiological agents.

1.2 INTRODUCTION

Contagious Bovine Pleuropneumonia is an acute, sub-acute or chronic respiratory disease of cattle caused by Mycoplasma mycoides sub species mycoides small colony. In cattle, it causes heavy losses to farmers such as weight loss, low milk production and death. The disease is characterized by anorexia, fever and respiratory signs, such as laboured breathing, grunting when breathing, dyspnoea, polypnoea, coughing and nasal discharges. The head and neck of the infected cattle are extended when standing with front legs apart. Lameness is observed in calves up to six months of age due to painful limb joints. Transmission occurs by direct contact between infected and healthy animals. CBPP also affects buffaloes.

2.0 SCOPE / RESPONSIBILITY

2.1 SCOPE

This SOP is for use by all the technical staff involved in the laboratory diagnosis of CBPP.

2.2 RESPONSIBILITY

2.2.1 It is the responsibility of the head of the laboratory to ensure that this SOP is distributed.

2.2.2 The head of laboratory should ensure that all the staff using this SOP are trained and competent.

3.0 DEFINITIONS AND ABBREVIATIONS

3.1 ABBREVIATIONS

CBPP	Contagious Bovine pleuropneumonia
cELISA	Competitive enzyme-linked immunoassay
oC	Degrees in Celsius
HRP	Horse Radish Peroxidase
mAb	Monoclonal Antibodies
MmmSC	Mycoplasma mycoides subspecies mycoides small colony
Nc	Negative Control
OD	Optical Density
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween 20
Pc	Positive Control
PI	Percentage Inhibition
PPE	Personnel Protective Equipment
RPM	Revolutions per minute
SOP	Standard Operating Procedure
TMB	3,3',5,5' tetramethyl benzidine

3.2 DEFINITIONS

Not applicable

4.0 SAFETY PRECAUTIONS

- All the safety measures must be observed while carrying out the test;
- Handle all biological material as potentially infectious;
- Wear personal protective equipment (PPE) when handling samples and reagents;
- The stop solution contains 0.5MH2SO4 that can cause serious burns on skin, mucous membrane and the eyes;
- The TMB substrate is harmful to the skin, mucous membrane and the eyes;
- Decontaminate the work area and pipettes in 5% freshly prepared sodium hypochlorite solution for at least for a duration of 1hour;
- Control sera contain sodium azide that may be toxic if ingested.
- In case of exposure of eyes with chemicals, use the eyewash if available or wash using running water for 5 minutes.

5.0 SPECIMEN

Recommended Specimens	Collection Notes	Pre-Analytical Processing
• Serum	• Whole blood is collected in plain vacutainer tubes or tubes with clot activator.	• Whole blood is left to stand for 2 hours at 22-25^{0}C, then centrifuged at 2000rpm for 10 minutes. • The serum is collected in sterile vials, labeled and assigned a laboratory number. • The serum is stored at -20^{0}C. The serum is brought to +4^{0}C and later to room temperature for one hour before testing. • Fresh serum can also be used directly after centrifugation and maintained at +4°C within 24 hours. • In cases where there is haemolysis or autolysis, the technician has the discretion to call for a fresh sample.

6.0 EQUIPMENT / MATERIALS/ REAGENTS

Equipment	Supplies	Reagents
• ELISA Reader. • Freezers -200C. • Incubators at +370C • Magnetic stirrer. • Micro titer plate-washers. • pH meter. • Plate agitator/ Shaker. • Refrigerated centrifuge. • Refrigerators. • Shaker Incubator 37°C. • Vortex mixer. • Water distiller or deionizer. • Micro plate washing system that distributes 300ul (Optional). • Printer	• Single channel pipettes10, 20, 100,1000µl. • Multi-channel pipettes (8 and 12), 50, 300 µl. • Pipette filler (electrical or manual). • Pipette tips assorted volumes. • 96-well micro plates for dilution. • Absorbent paper towels or lint free cloth. • Coated plates. • Micro plate covers (lid, aluminium foil or adhesive). • Cryovials. • Glassware (Pipettes, 5ml, 10ml, beakers, cylinders and flasks). • Lab-timer.	• Conjugate. • Control sera (C++, C+ and C-) • Disinfectant for decontamination (sodium hypochlorite, 70% alcohol). • Distilled water. • Monoclonal antibody (mAb). • Stop Solution (commercial). • Substrate, TMB. • Wash solution (commercial).

Equipment	Supplies	Reagents
	• Permanent laboratory marker pens. • Reagent reservoir for multi-channel pipettes – troughs.	

7.0 METHODOLOGY

7.1 TEST PRINCIPLE

The micro plate wells are coated with an antigen composed of Mycoplasma mycoides sub species mycoides small colony (MmmSC) biotype lysate. Antibodies against CBPP present in the test sera will bind to the MmmSC antigens coated on the micro-titre plate competing with the monoclonal antibodies for the specific epitopes. The conjugate composed of anti-mouse IgG serum conjugated to horseradish peroxidase (HRP) added binds to any monoclonal antibodies fixed on the wells. If antibodies specific to MmmSC antigen are present in the test sera, they will outcompete and displace the monoclonal antibodies. Hence, the conjugate will not be able to bind. The HRP substrate (TMB) when added forms a blue compound that will turn to yellow when the reaction is stopped. The intensity of the colour is an inverse measure of the proportion of MmmSC antibodies present in the test sera.

7.2 PREPARATION OF REAGENTS

7.2.1 Wash Buffer

Either dilute 100ml of wash concentrate (20x) in 1900ml of distilled water to make wash solution. If the buffer is not for immediate use, prepare the amount required for washing 1 plate i.e. 20ml "wash concentrate (20x) in 380mls of distilled water".

OR dissolve one PBS tablet in 1 litre of distilled water as recommended by the manufacturer, add 2.5 mls of Tween 20 and mix well. Transfer this to washing trough.

Dilute the buffer further by adding 4 litres of distilled water, mix well, label and store at room temperature (22-25oC) for not more than one week.

OR dissolve one PBS sachet in one litre of distilled water to give a buffer of 0.01M, pH 7.4 then add 2.5 mls of Tween 20 and mix well. Transfer this to wash fluid container with a tap to which tubing may be attached. Dilute the buffer further by adding 4 litres of distilled water, mix well, label and store at room temperature for not more than one week.

Guideline for dilution of Wash Concentrate depending on number of plates

No of plates	Wash concentrate (ml)	Distilled Water (ml)
1	9	171
2	18	342
3	27	513
4	36	684

Dissolve one tablet (IDEXX), per litre of distilled water. Add 500 µl of Tween 20 per litre and mix well. Label and store at + 40C for no longer than two weeks.

NOTE: Prepare wash buffer according to the workload.

7.2.2 Monoclonal antibody Dilution

This is supplied freeze-dried and requires reconstitution with 1ml distilled water. Working mAb is diluted in 1/120 with dilution buffer 24, i.e 100µl mAb in 11.9ml "Dilution buffer 24" (Institut Pourquier)

Guideline for dilution of monoclonal antibody depending on number of plates

No of plates	Conjugate (µl)	Diluent buffer 24 (ml)
1	100	9.9
2	200	19.8
3	300	29.7
4	400	39.6

7.2.4 Substrate buffer
Supplied as ready to use 'TMB"

7.2.5 Stop Solution:
Supplied as ready to use solution of 0.5MH2SO4

7.3 PROCEDURE
7.3.1 Pre-plate
1. Dispense 100µl of dilution buffer 24 in all wells of the pre-plate;
2. Dispense 110µl of dilution buffer 24 in wells A1 and A2 (conjugate control Cc);
3. Dispense 11µl of strong positive control, P++ in wells B1, B2, C1 and C2;
4. Dispense 11µl of weak positive control, P+ in wells D1, D2, E1 and E2;
5. Dispense 11µl of negative control, CN in wells H1, and H2;
6. Dispense 11µl of test sera in the remaining wells A3 to H12 as shown in the table below;
7. Dispense 110µl of diluted mAb in all wells except for A1, and A2.

7.3.2 Antigen-Coated Micro-titre plate

1. Mix by pipetting up and down and transferring 100µl of the serum/mAb mixture and the controls from the pre-plate into the coated micro-plate using a multi-channel pipette;
2. Cover the plate with a lid and incubate for 1hour (±5 minutes) at 37oC under gentle agitation;
3. Wash 3 times with wash solution; empty plate completely by turning it upside down and fill it with 300ul with wash solution. Repeat this 3 times. Empty the plate and tap it upside down on absorbent towel to remove the remaining wash buffer;
4. Dispense 100µl of diluted conjugate into all wells;
5. Cover with a lid and incubate for 30 minutes (±3 minutes) at 37oC under gentle agitation;
6. Wash 3 times as in 10 above;
7. Dispense 100µl of ready to use TMB substrate into all the wells;
8. Cover plate and incubate for 30minutes at 37oC under gentle agitation;
9. Add 100µl of stop solution into all wells;
10. Gently shake the plate until the coloured solution is homogenous;
11. Wipe carefully the bottom of the plate.
12. Pre-warmed ELISA reader for 10 minutes.
13. Read the ODs at 450nm.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cc	Cc	S_1	S_9	S_{17}	S_{25}	S_{33}	S_{41}	S_{49}	S_{57}	S_{65}	S_{73}
B	CP++	CP++	S_2	S_{10}	S_{18}	S_{26}	S_{34}	S_{42}	S_{50}	S_{58}	S_{66}	S_{74}
C	CP++	CP++	S_3	S_{11}	S_{19}	S_{27}	S_{35}	S_{43}	S_{51}	S_{59}	S_{67}	S_{75}
D	CP+	CP+	S_4	S_{12}	S_{20}	S_{28}	S_{36}	S_{44}	S_{52}	S_{60}	S_{68}	S_{76}
E	CP+	CP+	S_5	S_{13}	S_{21}	S_{29}	S_{37}	S_{45}	S_{53}	S_{61}	S_{69}	S_{77}
F	Cm	Cm	S_6	S_{14}	S_{22}	S_{30}	S_{38}	S_{46}	S_{54}	S_{62}	S_{70}	S_{78}
G	Cm	Cm	S_7	S_{15}	S_{23}	S_{31}	S_{39}	S_{47}	S_{55}	S_{63}	S_{71}	S_{79}
H	CN	CN	S_8	S_{16}	S_{24}	S_{32}	S_{40}	S_{48}	S_{56}	S_{64}	S_{72}	S_{80}

Key for plate layout

Cc: Conjugate control (without serum, without Mab = 100% inhibition)
Cm: Monoclonal control (without serum = 0% inhibition)
CP++: Strong positive serum
CP+: Weak positive serum
CN: Negative serum
S_1: Test serum No. 1
S_2: Test serum No. 2

7.4 PROCEDURE NOTES

- Bring all the reagents including the test sera to room temperature one hour before use.
- The tests should be done on dust free benches. They must be disinfected with 70% alcohol.
- The temperature of the room must not exceed 25oC.
- Reading may be performed up to 1 hour after the reaction has been stopped as long as the plates are kept in the dark.

7.5 QUALITY CONTROL

- The lyophilized controls must be reconstituted one day in advance with distilled water, aliquoted and kept <-16oC;
- Samples and controls are pre diluted on the U-shaped low protein binding pre plate (Uncoated);
- Selected sera will be retained in accordance to the retention policy for future use such as for teaching or research;

7.6 QUALITY CONTROL MATERIALS

- The kit contains a strong and a weak positive control, negative control which can be replaced with an in house, controls. The usefulness of these reagents as controls for the test is assured when the positive and negative controls produce results indicated in the cELISA kit.
- For internal control, if supplied controls show abnormalities, the results are not valid.
- External quality control should always be done by participating in proficiency tests coordinated by International Atomic Energy Agency (IAEA).

7.7 QUALITY CONTROL RESULTS.

a. OD of Cm must be between 0.5 and 2.0 (preferably near 1.0)
b. OD of Cc must be below 0.3
c. PI of CN must be equal to or lower than 35%
d. PI of CP+ must be between 50 and 80%
e. PI of CP++ must be between 60 and 90%

QC results will be filed in the Quality Manager's Random Check Result File

7.8 CALIBRATOR.

Not applicable

7.9 CALIBRATION.

7.9.1 The ELISA Reader should be calibrated at least once a year or in accordance with the manufacturer's instructions.

7.9.2 Validation Criteria

- Read the Optical densities at 450nm (blank with air)
- Calculate the mean value of the Cm (0% inhibition) and Cc (100% inhibition) controls
- Calculate the percentage of inhibition (PI) for each serum as follows: PI = 100 x ((OD Cm – OD Test) / (OD Cm – OD Cc))

7.9.3 Calibration of pipettes and other equipment should be done regularly by certified engineers.

7.10 RESULTS AND INTERPRETATION

- Sera with equal to or lower than 40 % Inhibition are considered negative for CBPP
- Sera with between 40 and 50 % Inhibition are considered doubtful
- Sera with above 50 % Inhibition are considered positive for CBPP

NB: repeat the tests for doubtful results.

8.0 REFERENCES

1. OIE TERRESTERIAL MANUAL – 2009 Edition
2. A competitive ELISA for the specific diagnosis of contagious bovine pleuropneumonia (CBPP) C. Le Goff, F. Thiaucourt Veterinary Microbiology 60 1998. 179–191
3. CBPP Kit Insert

9. APPENDICES

9.1 Appendix 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **CBPP cELISA Assay**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

9.2 Appendix 2: SOP DISTRIBUTION LISTING:

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Section / Area	SOP Manual Number	Date Issued

13.3 Appendix 3: SOP TRAINING LOG:

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: **CBPP COMPETITIVE ELISA ASSAY**		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.		

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

5. CCPP COMPETITIVE ELISA ASSAY

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: CCPP COMPETITIVE ELISA ASSAY			
	Name	Signature	Date
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE / INTRODUCTION:

1.1 PURPOSE

The purpose of the SOP is to describe the competitive ELISA for detection of antibodies against CCPP.

1.2 INTRODUCTION:

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats caused by Mycoplasma capricolum subspecies capripneumoniae (F38 strain). This organism is closely related to three other Mycoplasma: M. mycoides subsp. mycoides large colonies (LC), M. mycoides subsp. capri, and M. capricolum subsp. capricolum. Unlike the true CCPP, which is confined to the thoracic cavity, the disease caused by the latter three Mycoplasma species is accompanied by prominent lesions in other organs and/or parts of the body besides the thoracic cavity.

2.0 SCOPE / RESPONSIBILITY

2.1 SCOPE

This SOP shall be use in the laboratory for detection of CCPP antibodies from goats.

2.2 RESPONSIBILITY

2.2.1 It is the responsibility of the head of the laboratory to ensure that the SOP is distributed to all the staff.

2.2.2 It is also the responsibility of the head of the laboratory to ensure that all the staff using the SOP are trained and competent.

3.0 DEFINITIONS AND ABBREVIATIONS

3.1 ABBREVIATIONS

CCPP	Contagious caprine pleuropneumonia
cELISA	Competitive enzyme-linked immunosorbent assay
HRP	Horse radish peroxidase
mAb	Monoclonal antibodies
Nc	Negative control
0C	Degrees in celsius
OD	Optical Density
PBS	Phosphate buffered saline
PBST	Phosphate Buffered Saline with Tween 20
Pc	Positive control
PI	Percentage Inhibition
PPE	Personnel protective equipment
Rpm	Revolution per minute
SOP	Standard operating procedure

3.2 DEFINITIONS

N/A

4.0 SAFETY PRECAUTIONS

- Handle all biological material and chemicals as potentially infectious and hazardous respectively;
- Wear personal protective equipment (PPE) when handling samples and reagents-
- The stop solution contains H2SO4 (0.5M) that can cause serious burns if in contact with the skin, mucous membrane or the eyes;
- All waste should be disposed according to disposal procedure;
- Protect all reagents from direct sunlight and heat.

5.0 SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
Serum	Whole blood is collected from the jugular vein of the goat using plain vacutainer tubes or with clot activator	• Whole blood is left to stand for 2 hours at room temperature, then centrifuged at 2000rpm for 10 minutes • The serum is collected in sterile vials, labeled and assigned a laboratory number. • The serum can be stored at -20^{0}C. • The serum is brought to room temperature before use.

Recommended Specimens	Collection Notes	Pre-Analytical Processing
		• Fresh serum can also be used directly after centrifugation and maintained at +F4°C within 24 hours. • In cases where there is haemolysis or autolysis the technician has the discretion to call for a fresh sample

6.0 EQUIPMENT / MATERIALS/ REAGENTS

Equipment	Supplies	Reagents
• Analytical balance • ELISA reader • Elisa washer • Freezers -20^{0}C (± 3°C) • Micro plate incubator shakers at +370C (± °C) • Magnetic stirrer • pH meter • Refrigerated centrifuge • Refrigerators, • Vortex mixer • Water still/de-ionizer	• Multi-Channel pipettes (8 and12), 50, 300 µl • Single channel Micro-pipettes10, 20, 100, 1000µl • Pipette tips assorted volumes • U shaped polypropylene 96-well micro plates • Absorbent paper towels-or lint free clothe. • Coated plates, • Micro plate covers (Lid, aluminium foil or adhesive) • Cryovials, • Glassware (Pipettes, 5ml, 10ml, beakers, cylinders and flasks) • Laboratory-timer • Laboratory permanent marker pens. • Reagent troughs • Duran bottles	• MmmLc Antigen coated plates • Positive control (lyophilized) • Strong positive control (lyophilized) • Negative control (lyophilized) • Disinfectant for safe disposal of test sera (e.g, alcohol 70%) • Conjugate concentrate (100x) • Dilution buffer • TMB substrate • Stop solution • Wash concentrate • Detection solution (lyophilised) of monoclonal ant-MmmLc Antibody. • Distilled or deionized water

7.0 METHODOLOGY

7.1 TEST PRINCIPLE

Micro plates are coated with an MmmLc purified lysate. Samples to be tested are premixed with a specific monoclonal Antibody Mab 4/52 in a separate plate (preplate) and content of the "preplate" is transferred into the coated micro plate. Any MmmLc specific antibodies present in the sample will form an immune–complex with MmmLc antigen coated micro plate, competing with Mab 4/52 for the specific epitopes. After washing away unbound material, an anti-mouse antibody enzyme conjugate is added. In the presence of immune-complex (+ve reaction) between MmmLc antigen and antibodies from the sample, Mab 4/52 cannot bind to its specific epitopes and the

conjugate is free to bind to Mab 4/52. In a negative reaction, unbound conjugate is washed away and enzyme substrate is oxidized (TMB) if added. In the presence of enzyme, the Substrate is oxidized and develops a blue compound becoming yellow after stopping. Subsequent color development is inversely proportional to the amount of anti-MmmLc antibodies in the test sample

7.2 PREPARATION OF REAGENTS

7.2.1 Wash buffer

Dilute 100ml of wash concentrate (20x) in 1900ml of distilled water to make wash solution. If the buffer is not for immediate use, prepare the amount required for cleaning 1 plate i.e. 20ml "wash concentrate (20x) in 380mls of distilled water.

Guideline of wash buffer dilution 1:10 depending on the number of plates

No of plates	Wash concentrate (ml)	Distilled Water (ml)
1	20	380
2	40	760
3	60	1140
4	80	1520

7.2.2 Diluent buffer (PBST)

Use the supplied ready to use diluent buffer

7.2.3 Serum/monoclonal diluent buffer

Supplied freeze dried, require reconstitution with 1ml-distilled water. Working mAb is diluted 1/120 with dilution buffer, i.e. 100ul mAb in 11.9ml "Dilution buffer

Guideline of serum/monoclonal dilution 1:120 depending on the number of plates

No of plates	Monoclonal (µl)	Diluent buffer 24 (ml)
1	100	11.9
2	200	23.8
3	300	35.7
4	400	47.6

7.2.4 Conjugate

Dilute conjugate 1/100 in "dilution buffer 24"

Guideline of conjugate dilution 1:100 depending on the number of plates

No of plates	Conjugate (µl)	Diluent buffer 24 (ml)
1	100	9.9
2	200	19.8
3	300	29.7
4	400	39.6

7.2.5 Substrate buffer

Use the supplied ready to use solution from the kit

7.2.6 Stop Solution:

Use the supplied ready to use solution from the kit

7.3 TEST PROCEDURE.

Note: This test procedure is based on Idexx test kit.

Pre-plate dilution

1. Dispense 100µl of dilution buffer into each wells of the pre-plate (s);
2. Dispense another 110µl of dilution buffer into two appropriate wells (Conjugate control wells);
3. Dispense 11µl of undiluted strong positive into four appropriate wells (Strong positive control wells);
4. Dispense 11µl of undiluted positive control into two or four appropriate wells (Positive control wells);
5. Dispense 11µl of undiluted negative control into two appropriate wells (Negative control wells);
6. Dispense 110µl of dilution buffer into four appropriate wells (Mab Control wells);
7. Dispense 11µl of undiluted sample into each remaining wells of the pre- plate;
8. Dispense 110ul of dilution detection solution into each wells of the pre-plate except in Conjugate control (CC) and Mab control wells (already 110ul detection solution);

Testing

1. Transfer 100µl from each wells of the pre-plate (s) to the appropriate wells of the coated micro plate (s);
2. Cover the micro plate (s) with a seal and incubate for 1hour (±5min.) at 370c with gentle agitation;
3. Empty or aspire liquid from micro plate (s) wells and wash each wells with approximately 300µl of wash solution two times, discard the liquid contents of all wells after each wash;
4. Firmly tap residual wash fluid from each micro late onto absorbent paper towel, avoid drying of the micro plate between washes and prior to the addition of the next reagent;

5. Add 100µl of dilution conjugate into each well;
6. Cover the micro plate and incubate for 30minutes (±3min) at +370c (±30c) in a dark place;
7. Dispense 100µl of stop solution into each well;
8. Measure and record the absorbance value of samples and control at 450nm.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cc	Cc	S_1	S_9	S_{17}	S_{25}	S_{33}	S_{41}	S_{49}	S_{57}	S_{65}	S_{73}
B	CP++	CP++	S_2	S_{10}	S_{18}	S_{26}	S_{34}	S_{42}	S_{50}	S_{58}	S_{66}	S_{74}
C	CP++	CP++	S_3	S_{11}	S_{19}	S_{27}	S_{35}	S_{43}	S_{51}	S_{59}	S_{67}	S_{75}
D	CP+	CP+	S_4	S_{12}	S_{20}	S_{28}	S_{36}	S_{44}	S_{52}	S_{60}	S_{68}	S_{76}
E	CP+	CP+	S_5	S_{13}	S_{21}	S_{29}	S_{37}	S_{45}	S_{53}	S_{61}	S_{69}	S_{77}
F	Cm	Cm	S_6	S_{14}	S_{22}	S_{30}	S_{38}	S_{46}	S_{54}	S_{62}	S_{70}	S_{78}
G	Cm	Cm	S_7	S_{15}	S_{23}	S_{31}	S_{39}	S_{47}	S_{55}	S_{63}	S_{71}	S_{79}
H	CN	CN	S_8	S_{16}	S_{24}	S_{32}	S_{40}	S_{48}	S_{56}	S_{64}	S_{72}	S_{80}

Cc: Conjugate control (without serum, without Mab = 100% inhibition)
Cm: Monoclonal control (without serum = 0% inhibition)
CP++: Strong positive serum
CP+: Weak positive serum
CN: Negative serum
S1: Test serum No. 1
S2: Test serum No. 2

7.4 PROCEDURE NOTES

- Bring all the reagents, including the test sera, to room temperature (22-25°C) one hour before use.
- The tests should be done on dust free benches. Clean the benches with a disinfectant
- Reading may be performed up to 1 hour after the reaction has been stopped as long as the plates are kept in the dark.

7.5 QUALITY CONTROL

- The lyophilized controls must be reconstituted one day in advance with distilled water, aliquoted and kept at -20°C;
- Samples and controls are pre diluted on the pre plate (Uncoated);
- Refer to the kit in use.

7.6 QUALITY CONTROL MATERIALS

- The kit contains a strong and a weak positive control, Negative control, (mAb) control and a positive control, which can be replaced with an in house, control. The usefulness of these reagents as controls for the test is assured when the positive and negative controls produce results as indicated in the cELISA kit.

- For internal control, if supplied controls show abnormalities, the interpretation of the results is not valid.
- External quality control should always be done by participating in proficiency testing schemes from a certified scheme provider

7.7 Reading.

Read the Optical densities at 450nM (blank with air)

Calculate the percentage of inhibition (PI) for each serum as follows:

$$SPI\% = 100x\ (MabCx\text{-}SA450)/\ (MabCx\text{-}CCx)$$

Quality control results will be filed in the Quality Manager's Random Check Result File

7.8 Test validation

For the assay to be valid, all the following criteria should be fulfilled:

- Mab control mean absorbance (MabCX) is greater than or equal to 0.5 and less than or equal to 2.00 (best if close to 1)
- Conjugate control mean absorbance (CCX) is less than 0.3
- Negative control percentage inhibition (NCP) is less than or equal 35%.
- Positive control percentage of inhibition (PCPL) should be greater than or equal to 50% and less than or equal to 80%.
- Strong positive control percentage of inhibition (SPCPI) is greater than or equal to 60% and less than or equal to 90%.

7.9 CALIBRATOR.

N/A

7.10 CALIBRATION.

- The ELISA reader should be calibrated at least once a year or in accordance with the manufacturer's instructions.
- Calibration of pipettes and other equipment should be done regularly by certified engineers.

7.11 RESULTS.

- Sample with percentage of inhibition less than 40% are considered negative for the presence of MmmLc Antibody
- Sample between 40% and 50 % Inhibition are considered suspicious
- Sample with percentage of inhibition greater than or equal to 50% are considered positive for presence of MmmLc antibody

NB: Repeat the tests for all doubtful results.

8.0 REFERENCES

1. OIE MANUAL – 2014 Edition
2. ISO /IEC 17025 LMS Quality manual
3. Diagnosis and control of contagious caprine pleuropneumonia F. THIAUCOURT, G. BÖLSKE, B. LENEGUERSH, D. SMITH and H. WESONGA Rev. sci. tech. Off. int. Epiz., 1996, 15 (4), 1415-1429

8.0 APPENDICES:

8.1 Document change history

8.2 SOP Distribution Listing

8.3 S0P Training Log

8.1 Appendix 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **CCPP COMPETITIVE ELISA ASSAY**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

8.2 Appendix.2: SOP DISTRIBUTION LISTING:

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Section / Area	**SOP Manual Number**	**Date Issued**

8.3 Appendix 3: SOP TRAINING LOG

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: **CCPP COMPETITIVE ELISA ASSAY**		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.		

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

6. FOOT AND MOUTH DISEASE NSP cELISA

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: FOOT AND MOUTH DISEASE NSP cELISA			
	Name	Signature	Date
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE / INTRODUCTION:

1.1 PURPOSE

The purpose of this SOP is to describe one of the diagnostic tests for Foot and mouth disease (FMD). The SOP describes the indirect competitive ELISA (c-ELISA) used for the detection of serum antibodies against non-structural protein (NSP) of FMD virus (FMDV)

1.2 INTRODUCTION:

Foot and mouth disease (FMD) is a highly infectious disease caused by a virus in the genus Aphthovirus, family Picornaviridae. There are seven serotypes of FMD virus (FMDV) namely O, A, C, SAT 1, SAT 2, SAT 3 and Asia1 that affect cloven-hoofed animals, including domestic and wild bovids, ovines, caprines and porcines. In a susceptible population, morbidity approaches 100%. Exotic breeds are more susceptible to the disease than traditional breeds. While the disease is rarely fatal in adult animals, there is often high mortality in the young ones due to myocarditis or lack of milk when the dam is infected by the disease. FMD is characterized by fever and blister-like sores on the tongue and lips, in the mouth, on the teats and between the hooves. The disease causes severe production losses and while majority of affected animals recover, the disease often leaves them weakened and debilitated.

2.0 SCOPE AND RESPONSIBILITY

2.1 SCOPE

This SOP is for use by all the technical staff in the serology section involved in the laboratory diagnosis of FMD.

2.2 RESPONSIBILITY

2.2.1 It is the responsibility of the head of the FMD laboratory to ensure that this SOP is distributed to only the staff concerned with this test.

2.2.2 It is also the responsibility of the head of the FMD laboratory to ensure that all the staff using the SOP are trained and competent.

2.2.3 It is the responsibility of the head of the FMD laboratory to approve the test result.

3.0 DEFINITIONS AND ABBREVIATIONS

3.1 ABBREVIATIONS

Ab	antibody
oC	Degrees in Celsius
cELISA	Competitive enzyme-linked immunosorbent assay
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
HRP	Horse radish peroxidase
H2SO4	Sulphuric acid
mAb	Monoclonal antibodies
Nc	Negative Control
NSP	Non-structural proteins
OD	Optical Density
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffered Saline with Tween 20
Pc	Positive Control
PI	Percentage Inhibition
PPE	Personal Protective Equipment
rpm	revolution per minute
RT	Room temperature
SOP	Standard Operating Procedure
TMB	3, 3', 5, 5' tetramethyl benzidine

3.2 DEFINITIONS

Non-structural proteins (NSP) are highly conserved proteins of the FMDV and are not serotype specific.

4.0 SAFETY PRECAUTIONS

- In addition to this test-SOP all the safety-measures must be observed while carrying out the test
- Handle all Biological Material as potentially infectious.
- Wear personal protective equipment (PPE) when handling samples and reagents
- Stop solution contains H2SO4 (0.5M) that can cause serious burns if in contact with the skin, mucous membrane and the eyes.

- Decontaminate the work area and pipettes in 5% freshly prepared sodium hypochlorite solution for at least one hour.
- Control sera contain sodium azide that may be toxic if ingested.

5.0 SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
• Serum	• Whole blood is collected in plain vacutainer or clot activator tubes	• Whole blood is left to stand for 2 hours at 22-25^{0}C, then centrifuged at • 2000rpm for 10 minutes. The serum is collected in sterile vials, labeled and assigned a laboratory number and entered into the registry book. The serum can be stored at - • 20^{0}C. The serum is brought to 4^{0}C before use. Fresh serum can also be used directly after centrifugation and maintained at +4°C within 24 hours. • In cases where there is haemolysis or autolysis the technician has the discretion to call for a fresh sample

6.0 EQUIPMENT / MATERIALS/ REAGENTS

Equipment	Supplies	Reagents
• Analytical Balance • ELISA reader with software, computer and printer • Freezers -200C • Incubators at +370C • Micro titer plate-washer. • pH meter • Plate agitator • Centrifuge • Refrigerators • Shaker Incubator 37°C • Vortex mixer • Water distiller or deionizer system	• Antigen coated plate • Single channel pipettes10, 20, • 100, 1000µl. • Multi-Channel pipettes (8 and 12), • 50, 300 µl • Pipette tip filler (electrical or manual) • Pipette tips assorted volumes • Maxisorp flat bottom 96 well plate • Disposable absorbent paper towels or lint free cloth. • Micro plate covers (lids, or aluminium foil or adhesive)	• Enzyme Conjugate, • Control sera (positive and Negative) • Stop Solution (10x H2SO4.□ • Substrate;(TMB). • Wash solution (commercial); • Distilled or de- ionized water. • Disinfectant (Sodium hypochlorite)

Equipment	Supplies	Reagents
	• Glassware (Pipettes, 5ml, 10ml, beakers, cylinders and flasks) • Lab-timer • Reagent troughs • Personal Protective Equipment (PPE) • Racks	

7.0 METHODOLOGY

7.1 TEST PRINCIPLE

This test is based on the AniGen® FMD NSP Antibody ELISA kit. The assay is a Competitive Enzyme Linked Immunosorbent Assay for the qualitative detection of antibodies to NSP of the FMDV. The kit contains a microtitre plate, which is pre-coated with recombinant 3ABC antigen on the well. For testing, pre- coated plates are incubated with an equal mixture of serum and mAb HRP (1:100 dilutions in the conjugate diluent) for 90 minutes at 37°C.

During first incubation, if present in the test sample, the antibodies against 3ABC in the test serum and HRP conjugated monoclonal antibodies against 3ABC competitively bind to the antigens in the well. Following this incubation, all unbound material is removed by aspiration and washing before the addition of a substrate solution. The residual enzyme activity found in the well will thus be directly inverse proportional to the anti-3ABC antibodies in serum or plasma, and evidenced by incubating the solid-phase with a substrate solution. The reaction is stopped by addition of the stop solution and

colorimetric reading will be performed by using a spectrophotometer at 450nm and 620nm. The specially selected 3ABC antigens are used as capture material in the test. These enable the FMD NSP Ab ELISA to identify the FMDV outbreak antibodies in sera, with a high degree of accuracy.

7.2 PREPARATION OF REAGENTS

Allow all the reagents to equilibrate at room temperature (22-25°C) for 30 min before use.

7.2.1. Preparation of wash solution (10X concentrated):

The washing solution must be diluted 1 to 9 with distilled or de-ionised water before use, (e.g. dilute 50ml stock washing solution in 450ml of distilled or de-ionised water and mix thoroughly to give a 1 in 10 concentration).

Crystals in washing solution might appear if stored at cold temperatures; it's not a quality problem. Use the solution after dissolving crystals by placing the vials at 37°C for few minutes.

7.2.2. Dilutions of Wash Concentrate depending on the number of plates

No of plates	Wash concentrate (ml)	Distilled Water (ml)
1	20.16	181.44
2	40.32	362.88
3	60.48	544.32
4	80.64	725.76

7.2.3 Dilutions of Enzyme-Conjugate depending on the number of plates

No of plates	Conjugate (ml)
1	5
2	10
3	15
4	20

7.2.4 Dilutions of Substrate-buffer depending on the number of plates

No of plates	Substrate (ml)
1	10
2	20
3	30
4	40

7.2.5 Stop Solution:

Purchase ready to use solution from the kit

7.3 PROCEDURE

7.3.1 Pre-plate

1. Allow all the reagents and samples to equilibrate at room temperature (22-25 °C) for 30 min and shake them gently before use;
2. Prepare the strip wells for negative control, positive control and each of the samples;
3. Dispense 50µl of negative control in to three wells.
4. Dispense 50µl of Positive Control in to two wells;
5. Dispense 50µl of the sample in to appropriate wells;
6. Dispense 50µl of enzyme conjugate into corresponding wells;
7. Shake the plate (s) gently and cover the plate (s) with an adhesive plate sealer, shaking is very important to get the reproducible results;
8. Incubate the plate (s) for 90 min at 37 (+/-1)0C;
9. Aspirate the liquid contents of all wells, wash the plate (s) 6 times with 350µl of diluted washing solution, and tap the plate (s) firmly after the last washing;
10. Dispense 100µl of substrate in to each well right after removing washing solution, (if over 5 min. delayed, it might cause the OD values to decrease);

11. Incubate the wells for 15 min at (temperature18-25 0C) in the DARK;
12. Dispense 100µl of stopping solution in to each well;
13. Blank the spectrophotometer with air.
14. Measure and record the absorbance of the samples and controls at 450nm in a bichromatic spectrophotometer (with reference wavelength at 620nm) immediately after the end of assay, (within 30min.).
15. Calculate the results.as shown in section 7.7 of this SOP.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	S_4	S_{12}	S_{20}	S_{28}	S_{36}	S_{44}	S_{52}	S_{60}	S_{68}	S_{76}	S_{84}
B	NC	S_5	S_{13}	S_{21}	S_{29}	S_{37}	S_{45}	S_{53}	S_{61}	S_{69}	S_{77}	S_{85}
C	NC	S_6	S_{14}	S_{22}	S_{30}	S_{38}	S_{46}	S_{54}	S_{62}	S_{70}	S_{78}	S_{86}
D	PC	S_7	S_{15}	S_{23}	S_{31}	S_{39}	S_{47}	S_{55}	S_{63}	S_{71}	S_{79}	S_{87}
E	PC	S_8	S_{16}	S_{24}	S_{32}	S_{40}	S_{48}	S_{56}	S_{64}	S_{72}	S_{80}	S_{88}
F	S_1	S_9	S_{17}	S_{25}	S_{33}	S_{41}	S_{49}	S_{57}	S_{65}	S_{73}	S_{81}	S_{89}
G	S_2	S_{10}	S_{18}	S_{26}	S_{34}	S_{42}	S_{50}	S_{58}	S_{66}	S_{74}	S_{82}	S_{90}
H	S_3	S_{11}	S_{19}	S_{27}	S_{35}	S_{43}	S_{51}	S_{59}	S_{67}	S_{75}	S_{83}	S_{91}

EC: Enzyme Conjugate
PC: positive control serum NC: Negative control serum
S1:Test serum No. 1, S2:Test serum No. 2, etc.

7.4. PROCEDURE NOTES

NOTE: In order to obtain reproducible results, the following rules must be observed;

1. Use fresh samples. Hemolyzed or contaminated samples might cause false results.
2. Remove the blood corpuscles in sample before use for they may cause non specific reaction.
3. Use disposable gloves while handling potentially infectious materials and performing the assay, after assay, wash hands with sanitizers.
4. Store all reagents at 2-8°C in the dark, bring to 18-25°C before use, and return to 2-8°C after use.
5. Unused micro plate wells should be stored while sealed in plastic bags at 2-8°C. and should be used as soon as possible, do not reuse micro wells or pour reagents back in to their original bottles once dispensed,
6. Do not inter-mix components from kits with different batch numbers.
7. Substrate and stopping solutions can cause irritation or burns to the skin and eyes; in case of an accident, rinse immediately with fresh cold water.
8. Do not expose substrate solution directly to light or to any oxidizing agents, handle all substrate solutions with new clean glass or plastic ware.
9. Do not use reagents after expire dates.
10. Do not reuse containers and/or residual reagents, to avoid cross contaminations
11. Careful pipetting, timing and washing throughout this procedure are necessary to maintain precision and accuracy.

12. Dispose containers and residues safely in accordance with national regulations.
13. The ELISA kit should be stored at 2-8°C.
14. Working dilution of the washing solution once prepared must be stored at 18-25°C for one week
15. Optimal results will be obtained by strict adherence to this SOP.

7.5 QUALITY CONTROL

Not applicable

7.6 QUALITY CONTROL MATERIALS

- The kit contains a Positive and Negative control to ensure reliable results.
- As an internal control measure; if supplied controls show abnormalities, the results are invalid.
- External quality control should always be done by participating in proficiency tests

7.7 INTERPRETATION OF THE TEST

7.7.1-Test validation

The absorbance of the control sera should meet the following values;

- Mean negative control serum : above 0.800
- Mean positive control serum : below 0.400

Both PI values of the positive control must comply with the specification.

If these specifications are not met, the test is to be repeated.

7.7.2 PI value calculation and decision

Calculate the mean absorbance of the negative controls, and then calculate the PI (Percent Inhibition) value by each serum, using the following formula;

PI value = [1-(OD sample/mean OD negative)] x 100

Based on the PI value and animal species, the samples are interpreted as follows:

- **Positive: PI value of sample should be 50 and above (i.e. ≥50.0)**
- **Negative: PI value of sample should be below 50 (i.e. <50.0)**
- **Ambiguous results values of between 45-49**

For example,

- Mean OD negative: 1.251
- OD sample: 0.868
- PI value = [1-(0.868/1.251)] x 100 = 30.6 ☐ the sample is considered Negative.

7.7.3 Reading results and interpretation

- Read the Optical densities at 450nm wavelength (blank with air)

Calculate the NC mean obtained from the duplicate wells, then calculate PI value of each test sample using the following formula:

- PI value= {1-(mean OD450 of sample/NC mean)}*100
- Example -NC mean: 1.292, PC mean: 0.211, mean OD450 of sample: 1.250
- PI value of sample = {1-(1.250/1.291)}*100= 32
- The result of the sample is interpreted as Negative.

NB: repeat the test for ambiguous or doubtful results.

7.8 CALIBRATOR.

Not applicable

7.9 CALIBRATION.

7.9.1 The ELISA Reader should be calibrated at least once a year in accordance with the Manufacturer's instructions

7.9.2 Calibration of pipettes and other equipment done regularly by certified engineers.

8.0 REFERENCES

1. AniGen® FMD NSP Antibody ELISA test kit Protocol.
2. Bronsvoort BM, Toft N, Bergmann IE, Sorensen KJ, Anderson J, Malirat V, Tanya VN, Morgan KL. Evaluation of three 3ABC ELISAs for foot-and-mouth disease non-structural antibodies using latent class analysis, BMC Veterinary Research 2006 Oct.16.
3. ISO /IEC 17025 LMS Quality manual.
4. OIE MANUAL – 2009 Edition.

9.0 APPENDICES:

9.1 Document change history
9.2 SOP distribution listing
9.3 SOP training log

9. 1 APPENDIX 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **FOOT AND MOUTH DISEASE NSP cELISA**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

9.2 APPENDIX 2: SOP DISTRIBUTION LISTING:

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Section / Area	SOP Manual Number	Date Issued

9.3 APPENDIX 3: SOP TRAINING LOG

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**

Title: **FOOT AND MOUTH DISEASE NSP cELISA**

Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

6.1 DETECTION OF FOOT AND MOUTH DISEASE VIRUS ANTIBODIES USING THE VIRUS NEUTRALIZATION TEST (VNT)

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: DETECTION OF FOOT AND MOUTH DISEASE VIRUS ANTIBODIES USING THE VIRUS NEUTRALIZATION TEST (VNT)			
	Name	Signature	Date
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1. PURPOSE / INTRODUCTION:

1.1. PURPOSE

The purpose of this SOP is to provide a procedure for testing for Foot and mouth disease virus (FMDV) by using the Virus Neutralization Test. (VNT) The aim of the FMDV antibody detection VNT is to detect the FMDV serotype specific antibodies in the serum of susceptible animals.

1.2. INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious disease affecting all cloven hoofed animals and is widespread over the world. It is also the most important economic threat to the livestock industry. It is therefore vital to have guidelines on an activity that facilitates proper diagnosis.

2. SCOPE AND RESPONSIBILITY:

2.1. SCOPE:

This SOP applies to all serological samples from all species of cloven – hoofed animals suspected to be infected with FMDV. The SOP is applicable to all staff involved in the testing, analysis and reporting of results.

2.2. RESPONSIBILITY:

The Head of FMD Laboratory is responsible for the implementation of this procedure and for the compliance by other staff in the laboratory. The Head of FMD Laboratory is also responsible for ensuring that the staff using the SOP are appropriately qualified and trained to carry out the procedures.

3. DEFINITIONS AND ABBREVIATIONS:

3.1. ABBREVIATIONS

BHK-21	Baby Hamster Kidney culture 21 cell line
CPE	Cytopathic effect
DVS	Director of Veterinary Services
FCS	Foetal Calf Serum
FMDV	Foot-and-mouth disease virus
FMDV A	FMDV serotype A
FMDV C	FMDV serotype C
FMDV O	FMDV serotype O
FMDV SAT 1	FMDV Southern African Territories Type 1
FMDV SAT 2	FMDV Southern African Territories Type 2
IBRS-2	Swine kidney cell line
LAF-cab	Laminar air flow cabinet
OIE	World organization for Animal health
TCID50	Tissue culture infective dose
VNT	Virus Neutralization Test
MEM	Minimum Essential Media

3.2. DEFINITIONS

Serological sample – Any sample including blood samples from all species of FMD susceptible animals that is to undergo serological analysis for the presence of circulating antibodies.

4. SAMPLES

Recommended Samples	Collection Notes	Pre-Analytical Processing
Serum	N/A	See 7.2.1

5. EQUIPMENT / SUPPLIES/ REAGENTS:

Equipment	Supplies	Reagents
• Water purification system • Refrigerator • Freezer • Incubator (set at 37°C) • Compound light and inverted microscopes • Water bath • pH Meter • Vortex Mixer • Weighing Balance • BSL_2 LAF-cabinets	• Micro-pipettes, (variable volume ranges) • Multi channel pipettes (8 and 12 channels) • Sterile micro- pipette tips • Reagent troughs • Reagent reservoirs • Glassware / Plastic ware (Pipettes 5ml, beakers, cylinders, flasks) • Cryovials • Timer • Microscope slides and coverslips	• 0.2% (w/v) Citric Acid (for decontamination) • Phenol Red - pH indicator (pH 6.4-8.2) • Sterile water • FCS (Fetal calf serum) • MM I media with antibiotics (neomycin, streptomycin and penicillin) • IBRS 2 OR BHK cell suspension in RPMI medium (1.5 x 106 cells/ml) or any other appropriate cell line

Equipment	Supplies	Reagents
	• Haemocytometer • Cell counter • Bijoux bottles (5ml & 10ml) • Staining troughs • Disposable absorbent paper/cloth towels,(lint free). • Tissue culture plates, flat-bottom 96-well plates • Permanent lab. marker pens • Adhesive labels	• Negative control sera (normal bovine sera) • Positive control sera

6. SAFETY PRECAUTIONS

1. Follow universal safety practices (Good Laboratory Practices) when in the laboratory and when handling diagnostic specimens, reagents and chemicals.
2. All the work with live virus must be carried out in a room that is fitted with a LAF- cab.
3. Materials that were in contact with virus must be deposited in a container with 0.2% citric acid solution for decontamination.
4. The working area and the cabinet must be cleaned with citric acid followed by 70% ethanol, and left for 20 minutes before another virus can be handled in the same cabinet.
5. Sterile techniques must be observed in all steps.

7. METHODOLOGY

7.1. TEST PRINCIPLE

Virus neutralization test is an antigen-antibody based immunological test. Serial dilutions of heat inactivated test serum are incubated with known viral suspension of infectious virus. If antibodies to the virus are present, it binds to the virus, preventing its attachment to and subsequent infection of cells. Following this incubation, virus susceptible cells are added to the virus-serum mixture, and the final virus/serum/cell combination is incubated for a period of 2-3 days. After this incubation period the test is read by examining each well of the plate for the presence of viral infection by direct microscopy or staining of the test wells of the plate for evidence of viral cytopathic effect (CPE). Test sera, which contain antibodies to the virus being tested, are able to neutralize the aliquot of virus used in the test, thus preventing infection of the cells when they are added to the plate. Where high concentrations of antibody to the virus in question are present in the serum sample, virus neutralization will occur even at high serum dilutions. Conversely, where little or no antibody to the virus is present in the test sample, it will be unable to neutralize the aliquot of infectious virus at the first dilution used in the test. The result of the test is the point at which the serum sample has been diluted such that it is no longer able to neutralize the entire virus in the test. This dilution, or its log equivalent, is reported as the titre of the serum tested.

7.2. PROCEDURES

7.2.1. Sample reception and preparation

- Enter relevant details in the "Day Book" or daily records and designate a reference number to each sample.
- Inactivate the serum sample at 56oC for 30 minutes.
- Store the samples at +4oC for short term periods or at -200C for long-term storage
- Dispose the waste material in 0.2% citric acid.

7.2.2. Prepare disinfectant Solution (0.2% (w/v) citric acid)

- Dissolve 200gm of citric acid per 1litre of locally produced distilled / deionized water.
- Label and store at room temperature.

7.2.3. Titration of virus and choosing virus dilutions for the VNT

1. Prepare 1.0ml aliquots (up to 100) of virus stocks and keep at -70oC.
2. Titrate each new batch beforehand in micro-titre plates to determine its titre as follows:
3. Add 50 µl of MM2 media to all wells of columns 2-12.
4. Add 100 µl of virus suspension into column 1 wells and make a two-fold serial dilution of the virus from column 1-11 of the microtitre plate by transferring 50 µl across the columns and discarding 50 µl from column 11 with column 12 being the cell control.
5. Add 50 µl of MM2 media to all the wells of the microtitre plate to result into a total volume of 100 µl per well.
6. Add 50 µl of BHK-21 OR IBRS-2 cells at a concentration of 1.5 x 106 /ml.
7. Incubate the plate for 72 hours at 37oC and determine the titre as in 7.3.1 and 7.3.2.
8. Calculate the dilutions of virus to be used in the VNT thus: first subtract 2.0 logs from the virus titre to determine a dilution that will give 2 logs of virus. Then determine four values that are 0.5 apart from this value.
9. Example: For a virus titre of 106.3 /50µl, subtract 2.0, this gives a value of 104.3. Diluting the virus 10-4.3 will give 2.0 logs of challenge virus in the VNT. Test the positive control sera against virus dilutions of log: 3.5; 4.0; 4.5 and 5.0.
10. Include a back titration of the virus that will give the actual virus titre of each specific test.

    Mark the plates with the corresponding sera numbers.

    Mark the corresponding10ml bijoux with the sera numbers and aliquot 7ml MM2 medium in each bijoux. Prepare larger volumes of the four dilutions needed for the VNT.

    All serum samples are tested against the four virus dilutions. These are 0.5 log dilutions and it is chosen so that the 102 TCID50 doses will be included in one of the four dilutions.

7.2.4. Preparation of the microtitre plates

Add 50µl MM2 medium (with a multi-channel pipette) to all the wells except the first row (A).

7.2.5. Dilution of the serum samples

First prepare a 1/8 dilution of the control sera (e.g. add 1ml of undiluted serum to 7 ml of the MM2 medium) using a micro-pipette. Next, prepare a 1/8 dilution of test sera in10ml bijoux bottles.

7.2.6. Titration of the sera

- Add 50 µl of the pre-diluted serum, with a multi-pipette, to each well in row A.
- Transfer 50 µl of this dilution from row A to row B and carefully mix the 100 µl contents in the well by filling and emptying the multi-channel pipette tips several times, taking care not to introduce air bubbles as this will result in a double dilution.
- Transfer 50 µl from row B to row C and repeat the mixing procedure.
- Transfer 50 µl from row C to row D mixing carefully.
- Repeat this step down to row H.
- Finally discard 50µl of the dilution from row H to leave a final volume of 50 µl. This will result in a test sample dilution series from 1/16 to 1/2048 in 50µl volumes.
- For the next test sample, repeat the above procedure using new pipette tips.

7.2.7. Addition of the antigen dilutions to the test sera

The four virus dilutions as previously decided upon in the virus titration are added to the plates at this stage as follows;

- Add 50µl of the highest virus dilution in each well of the last three columns (10, 11 and 12). Use a multi-channel pipette with eight tips for this step.
- Add 50µl of the second highest virus dilution to all the wells in columns 7, 8 and 9.
- Repeat the above step for the other two dilutions.

The lowest virus dilution will be in column 1, 2 and 3. You may use the same tips for the above steps.

- Incubate plates for one hour at 37oC.

7.2.8. Addition of the cell suspension to the microtitre plates

- Add 50 µl of the cell suspension per well (1.5 x 106 /ml BHK-21 OR IBRS 2 cells in MM2 medium containing 5% FCS and with a multi-channel pipette.
- Incubated plates for 72 hours at 37oC.

7.3. RESULTS

7.3.1. Reading of plates

Read the microtitre plates after 72hours by checking for CPE either using an inverted microscope or directly by visualizing after staining using naphthalene black. Wells that show CPE are recorded as positive.

7.3.2. Calculation of virus titre

The titre of the virus is calculated as that dilution where 50% of the wells will show positive CPE. This is done according to the method of Kärber (1931) as follows: Total number of wells exhibiting 100% CPE divided by number of wells per dilution minus 0.5 (correction factor) multiplied by the log dilution interval. Add the highest dilution step with 100% CPE in all the wells.

7.3.3. Calculation of serum titre

With each VNT, a virus titration is included, so that the actual virus titre and virus doses for that experiment can be determined. For each dose of virus the corresponding serum titre is determined. Serum titres are expressed as the log10 reciprocal of the dilution, which has protected 50% of cultures from that dose of virus. The final endpoint titre of the serum is determined as the log reciprocal of the dilution, which protected 50% of cultures from 102 TCID50 of virus (Kärber, 1931). This is done by plotting the virus doses between 101.4 and 102.6 against their corresponding serum titre and calculating the final serum titre at 102 TCID50. In practice this is done by using the analysis presented for the determination of a regression line on a calculator.

7.3.4. Reporting, dispatch and feedback

- The head of the FMD laboratory authorizes the release of the results using appropriate approved formats and methods.

7.4. PROCEDURE NOTES

Not applicable

7.5. QUALITY CONTROL

7.5.1. In every test there must be a control plate in which different inputs of virus are run against titrated homologous sera. The titers are recorded and analyzed and should always be within the acceptable ranges.

7.5.2. The Quality Manager will perform random checks to ensure that this SOP is implemented as written.

7.5.3. All the deviations or variations against this SOP must be documented in the Quality Managers random check log. In such cases, Corrective and Preventive Action shall be initiated, implemented, and closed. Documentation will be done in the Non-conformance Corrective and Preventive Maintenance.

7.6. QUALITY CONTROL MATERIAL

Positive control sera (cattle sera) collected 2 weeks post second vaccination with FMDV vaccines prepared against the homologous virus strains e. g in Kenya, the following strains are currently in use;

- SAT 1 T155/71
- SAT 2 K52/84

- Type A K5/80
- Type O K77/78
- Type C K.267/67

Negative control bovine sera (Fetal Calf sera-commercially obtained)

7.7. CALIBRATOR
Not applicable

7.8. CALIBRATION
All equipment shall be calibrated annually and internal checks done on a regular basis. All records shall be filed with the QM.

7.9. QUALITY CONTROL RESULTS
Results for QC checks on implementation and compliance to this SOP will be filed in the Quality Manager's random check result file.

8. REFERENCES:

- Karber, G., 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Archiv fur experimentelle Pathologie und Pharmakologie, 162, 480-483
- Golding, S.M., Hedger, R.S., Talbot, P. & Watson, J. (1976). Radial immunodiffusions and serum neutralization techniques for the assay of antibodies to swine vesicular disease. Res. Vet. Sci., 20, 142–147.
- OIE manual 2012 chapter 2.1.5.

9. APPENDICES:
9.1. Document change history
9.2. SOP Distribution listing
9.3. SOP Training log

9.1 Appendix.1: DOCUMENT CHANGE HISTORY:
Version Table:

Original Title: **DETECTION OF FOOT AND MOUTH DISEASE VIRUS ANTIBODIES USING THE VIRUS NEUTRALIZATION TEST (VNT)**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1:Title	Dated:	SOP No.:	No. Pages:
Version 2:Title	Dated:	SOP No.:	No. Pages:
Version 3:Title	Dated:	SOP No.:	No. Pages:
Version 4:Title	Dated:	SOP No.:	No. Pages:
Version 5:Title	Dated:	SOP No.:	No. Pages:

9.2 Appendix 2: SOP DISTRIBUTION LISTING:

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9.3 Appendix 3: SOP TRAINING LOG

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**

Title: **DETECTION OF FOOT AND MOUTH DISEASE VIRUS ANTIBODIES USING THE VIRUS NEUTRALIZATION TEST (VNT)**

Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

6.2 INDIRECT SANDWICH ELISA FOR DETECTION OF FOOT AND MOUTH DISEASE VIRUS (FMDV) ANTIGENS

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: INDIRECT SANDWICH ELISA FOR DETECTION OF FOOT AND MOUTH DISEASE VIRUS (FMDV) ANTIGENS			
	Name	Signature	Date
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1. PURPOSE

The purpose of this SOP is to describe the diagnostic technique used for detection of all serotypes of FMDV antigens. The indirect sandwich ELISA is for the detection of FMDV antigens from test tissue samples (lesions, vesicles epithelium and oesophago-pharyngeal (OP)).

2. INTRODUCTION

Foot and mouth disease is a widespread, highly contagious disease of all cloven - hoofed animals caused by a virus belonging to the Picornaviridae family. It is characterized by a high fever for two or three days, followed by appearance of unruptured vesicles, blisters and sores in the mouth, tongue, teats and feet at the coronary band and inter-digital space; lameness, salivation, discharges from the nose and the mouth. There are seven immunologically and biochemically distinct serotypes of FMD virus, namely, O, A, C, Asia 1 and the Southern African Territories (SAT) 1, SAT 2 and SAT 3. Notwithstanding the aforementioned, FMD is currently the most important disease of trade, in some cases posing a threat to the livestock industry through a ban on livestock imports/exports.

3. SCOPE

This SOP is to be used by all personnel involved in FMDV antigen detection test in tissue samples.

4 RESPONSIBILITY

4.1 The head of the laboratory is responsible for ensuring the correct application of this procedure by suitably trained staff.

4.2 The head of the laboratory is also responsible for ensuring that the laboratory staff are appropriately qualified and trained to safely and properly handle specimens for the test.

4.3 The laboratory staff are responsible for ensuring that the proper procedures are followed according to the SOP.

5 DEFINITIONS AND ABBREVIATIONS

5.1 ABBREVIATIONS

BSL Biosafety level
ELISA Enzyme Linked Immunosorbent Assay
FMDV Foot and Mouth Disease Virus
LQM Laboratory Quality Management
OD Optical Density
OP Oesophago-pharyngeal
OPD Ortho-Phenylene diamine
PBS Phosphate Buffered Saline
PPE Personal Protective Equipment
SOP Standard Operating Procedure

5.1 DEFINITIONS

Not Applicable

6.0 SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
Tissues from lesions, epithelia, vesicles and OP.	Ensure equipment used for processing samples are sterile.	• Bring all reagents including the test samples to room temperature. Start work after 1 hour of attaining 18-25°C. • Weigh approximately 1gramme of the sample and place in a mortar. • Grind the tissue with a small amount of sterile sand and add sample buffer to make a 10%(w/v) suspension. • Transfer the ground suspension to a suitable tube and centrifuge at 1000 rpm for 10 minutes. • Remove 3mls of the supernatant for testing. Store the remainder in 0.04M PBS at -900C to -50°C for future use.

7.0 EQUIPMENT / SUPPLIES/ REAGENTS:

Equipment	Supplies	Reagents
• ELISA plate Washer • ELISA reader (with 492 nm filter) • Reader software, computer printer. • Orbital Shaker incubator (37oC) • Centrifuge • Biosafety cabinet with HEPA filter) • Refrigerator • Freezer • Water purification system (distiller/de- ionizer.	• Wash buffer • Nunc-Maxisorp flat bottom 96 well ELISA plates • Multichannel and single pipettes of different volumes (10, 20, 50,1000uL. • Various pipette tips. • Aluminum foil, plate covers, disposal containers, absorbent paper towels • Sterile sand • Reagent troughs • Glassware/plastic ware (measuring cylinders, beakers, reagent tubes, etc) • Scissors • Forceps • PPE • Timer • Centrifuge tubes • Mortar • Pestle • PH meter	• ELISA wash buffer • Tween 20 • Sterile distilled/de- ionized water) • Positive control antigens • Negative controls • Disinfectant(Virkon®or 0.2% w/v Citric acid or 4%Na2CO3) • Dilution buffer • 0.04M Phosphate Buffered Saline (PBS) • Rabbit antisera (trapping) • Guinea pig antisera (detection) • Carbonate bicarbonate buffer • Conjugate • OPD substrate • Hydrogen peroxide, • Stop solution (1.25M Sulphuric acid)

8 SAFETY PRECAUTIONS

- Wear appropriate PPEs such as gloves, lab coat, etc.
- Treat all samples as potentially infected.
- Work under BSL 3 or BSL 2 plus laboratory and a biosafety cabinet with HEPA filters.
- Dispose of all waste generated in a biohazard bag while sharps should be disposed of in a sharps container.

9.0 METHODOLOGY:

9.1 TEST PRINCIPLE

This ELISA involves sandwiching the antigen between two antibodies (primary (trapping) antibody and a labeled enzyme-linked secondary (detecting antibody)). In a positive situation, the reaction between the enzyme and substrate elicits a chromogenic (fluorescent) signal measured as Optical Density.

10 PROCEDURES:

10.1 Reagents preparation

10.1.1 Test sample

Suspensions of10% original sample in Phosphate Buffered Saline (PBS) or undiluted clarified cell culture supernatant fluid).

10.1.2 Dilution of rabbit (trapping) anti-sera

Prepare working dilutions of 1/1000 of rabbit antisera in coating buffer.

Volumes required for the specified number of plates and samples is as shown in the table below;

		TRAPPING ANTIBODY (rabbit) (per serotype)	
No. of plates	**Max no. of samples**	**Coating buffer(ml)**	**Rabbit antisera (µl)**
1	3	1	1
2	8	2	2
3	13	3	3
4	18	3	3
5	23	4	4
6	28	4	4
7	33	5	5
8	38	6	6
9	43	6	6
10	48	7	7

10.1.3 Reagent volumes required for other steps of the procedure

Volumes required for other reagents to be prepared in the subsequent steps are as calculated in the table below;

		Trapping antibody (rabbit) (per serotype)		Detecting antibody (guinea pig) (per serotype)		Conjugate		Chromogen + Substrate	
No. of plates	**Max no. of samples**	**Coating buffer (ml)**	**Serum stock (µl)**	**Diluent Buffer B (ml)**	**Serum stock (µl)**	**Diluent Buffer B (ml)**	**Conjugate stock (µl)**	**OPD (ml)**	**H2O2 (µl)**
1	3	1	1	1	10	6	30	6	30
2	8	2	2	1.5	15	11	55	12	60
3	13	3	3	2	20	16	80	18	90
4	18	3	3	3	30	21	105	24	120
5	23	4	4	3.5	35	26	130	30	150
6	28	4	4	4	40	31	155	36	180
7	33	5	5	4.5	45	36	180	36	180
8	38	6	6	5	50	41	205	42	210
9	43	6	6	6	60	46	230	48	240
10	48	7	7	6.5	65	51	255	54	270

10.1.4 Preparation of Diluent Buffer A

0.01 M Phosphate Buffered Saline, pH 7.4 ± 0.20 plus 0.05% (v/v) Tween 20 Dissolve five PBS tablets per 1 litre of distilled or deionized water. Add 500 µl of Tween 20 per litre and mix well. Label and store at +4oC for not longer than two weeks.

10.1.5 Diluent buffer B preparation

0.01 M PBS, pH 7.4 ± 0.2 plus 0.05% Tween 20 plus 5 % (w/v) skimmed milk powder. On the day of testing, take 5gm of skimmed milk powder to 100ml of Diluent A to form a final concentration of 5% (w/v) to the required volume of Diluent Buffer A.

10.1.6 Preparation of Blocked Guinea Pig detection serum (Detecting antibody)

Before the end of the sample and control antigen incubation period, prepare working dilutions of "blocked" guinea pig detection sera in Diluent Buffer B in the appropriate order in a 8-well reservoir. 1/100 dilutions of each antiserum are used in sufficient volume for all the plates being run. Compartments A to H receive respectively antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and Normal, non-immune serum. Return the remainder of the detecting antibody stocks to the fridge at + 1oC to +8oC.

10.1.7 Preparation of Conjugate

Immediately before the end of detecting antibody incubation period, prepare a working dilution (1/200) of the conjugate in Diluent Buffer B in a volume sufficient for all microplates – see 10.1.3 above. Both the conjugate stock and working dilution should be handled with care; agitation should be gentle but thorough. Return the remainder of the conjugate stock to -30oC.

10.2 Procedure

10.2.1 Procedure notes.

- Ensure that tissues are properly ground using sterile sand.
- Return the test samples to appropriate storage temperature and the remainder of the control antigen stocks to -30oC to 5oC for further use.
- Discard used pipette tips into a disinfectant
- Let the OPD chromogen come to room temperature IN THE DARK. Always wear gloves when handling OPD chromogen.
- Turn on the microplate reader 15 minutes before reading to allow the reader to warm up.

10.2.2 Coating of microplates

1. Label the plates correctly.
2. Gently agitate contents of rabbit trapping antibody (FMDV serotypes O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1; plus normal non immune rabbit serum).
3. Dispense 50µl of antisera to FMDV serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and normal non immune serum to wells A, B, C, D, E, F, G, and H, respectively.

4. Transfer 50µl volumes of the trapping sera dilutions from 8 well reservoir to each microtitre plate well and tap the plate to ensure even distribution in the wells. Rows A to H receive respectively antisera to serotypes O, A, C, SAT 1 SAT 2, SAT 3, Asia 1 and normal non-immune serum.
5. Cover the plates with a lid(s) and incubate in a pre warmed orbital shaker at 37oC for 1 hour. OR Alternatively, the plates can be left to coat overnight at 4 oC without agitation.
6. Wash the ELISA plates with wash buffer (PBS) 3 times in ELISA plate washer or manually by inverting the microtitre plate and using an abrupt downward motion, discharge the contents of all antibody coated micro-plate into a sink or reservoir. Fill all 96 wells of the micro-plate with wash buffer. Discharge the contents of the micro-plate by downward motion. Repeat with two more cycles of filling and emptying and slap the inverted micro -plate on to a lint-free absorbent towel to remove all residual contents.

10.2.3 Addition of Test Samples and Control Antigens

1. Plate 1:

- Add 50µl of diluent buffer A to all wells of column 1 - 6.
- Dispense 12.5 µl of control antigens of the 7 serotypes as follows:

FMD type O	into well A1
FMD type A	into well B1
FMD type C	into well C1
FMD SAT 1	into well D1
FMD SAT 2	into well E1
FMD SAT 3	into well F1
FMD Asia 1	into well G1
Negative control antigen	into well H1

2. Ensure that contents of wells 1 of rows A – H are well-mixed before making the serial dilutions in step 9.
3. Carry out serial dilutions by transferring 12.5 µl from well 1 to well 2, repeat the same and transfer 12.5 µl from wells 2 to 3; wells 3 to 4. Mix and discard 12.5 µl from well 4 (rows A-H) to obtain a five-fold dilution series of each control antigen.

- Add 50µl of sample 1 to wells 7 and 8 of rows A to H,
- Add 50µl of sample 2 to wells 9 and 10 of rows A to H,
- Add 50µl of sample 3 to wells 11 and 12 of rows A to H,

Plate layout No. 1

		1	2	3	4	5	6	7	8	9	10	11	12
A	O	1/5	1/25	1/125	1/625			Sample1	Sample1	Sample2	Sample2	Sample3	Sample3
B	A												
C	C												
D	SAT1												
E	SAT2												
F	SAT3												
G	Asia1												
H	Neg.control												

1. If more than three samples are involved in the same test, run, the second plate (see plate lay-out No.2 below) and subsequent microplates are arranged as follows:

- Dispense 50µl of the Diluent buffer A (PBST) to the wells (rows A to H) of columns 5 and 6 (buffer control columns).
- Five test samples may be added in 50µl volumes in duplicates to rows A to H to columns 1-2, 3-4, 7-8, 9-10 and 11-12 respectively.

Plate layout No. 2

		1	2	3	4	5	6	7	8	9	10	11	12
A	O	Sample 4		Sample 5		Buffer		Sample 6		Sample 7		Sample 8	
B	A												
C	C												
D	SAT1												
E	SAT2												
F	SAT3												
G	Asia1												
H	Neg.control												

1. Tap the sides of the microplates to ensure that the contents are evenly distributed over the bottom of each well.
2. Cover with lids and place on the orbital shaker at +35oC to +39oC for 1 hour.
3. Wash plates by flooding with wash buffer (PBS) three times as before and empty residual wash fluid. Blot the plates dry.
4. Transfer 50µl of rabbit anti-guinea-pig immunoglobulin to each well in appropriate order ie rows A to H receive respectively antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1.and normal, non-immune serum. Tap the sides of the plates to ensure the contents are evenly distributed over the bottom of each well.
5. Cover with lids and place on the orbital shaker at +35oC to +39oC for 1 hour.

6. Wash plates by flooding with wash buffer three times as before and empty residual wash fluid. Blot the plates dry.
7. Add 50µl of conjugate to all wells of rows A to H. Tap the sides of the plates to ensure the conjugate working dilution is evenly distributed over the bottom of each well.
8. Cover with lids and place on the orbital shaker at +35°C to +39°C for 45 minutes.
9. Wash microplates as described previously. Ensure that all 96 wells of each microplates have been completely flooded with wash buffer to eliminate unreacted conjugate.
10. Add 50µl of substrate chromogen solution to the wells of microplate starting with first column of blanking plate followed by all the wells. Begin timing after filling the first wells and incubate at 18-25oC for 15 minutes without shaking in the dark.
11. Add 50µl of stop solution (1.25M Sulphuric acid), starting with the first column of the blanking plate.

10.2.4 Reading plates

Read the plates at 492 nm wavelength on a spectrophotometer (ELISA reader) linked to a computer and record results as optical density (OD). Read the microplate starting with the blanking plate. Follow this by the test plate. Repeat blanking for each microplate.

10.2.5 Interpretation of results

- Check that there has been colour development intensity (from very strong to very weak) resulting from five-fold dilutions of each of the control inactivated antigens on plate1, rows A to H columns 1 to 4 to indicate that the plates have been correctly coated with anti sera and that the test is valid.
- Calculate the mean background reactions for each plate by adding the OD values of 5 & 6 wells of each row (serotype) and dividing by two.
- These OD values are due to reagents and not specific reaction between antigen and antisera.
- Subtract each mean background OD for each serotype from the actual OD for that serotype to obtain a corrected OD value. "For example for type O row A, add the OD values for wells A5 & A6 and divide by two which is equal to mean background OD of row A. subtract this figure from all actual OD values in row A. Repeat for each row (serotype)."
- A mean of each group of 2 wells for each test sample can then be derived to give a mean corrected OD figure for each test sample against each antiserum serotype.
- A mean corrected OD value of > 0.1 above background indicates a positive result.

11 QUALITY CONTROL.

- Make sure that the serotype sequence is followed as directed or guided in the given wells.
- All freshly prepared reagents should conform to guidelines as stipulated in the instruction manual.

11.1 QUALITY CONTROL MATERIALS.

Descriptions of reference materials is contained in the list of reagents.

11.2 CALIBRATOR.

A qualified technical person for the equipment and supplies or the supplier of the equipment.

11.3 CALIBRATION.

pH meter and ELISA reader ought to be calibrated regularly to ensure reliable results.

11.4 QUALITY CONTROL RESULTS.

A mean corrected OD value of > 0.1 above background indicates a positive result.

12 REFERENCES:

1. FERRIS, N.P and DAWSON, M. (1988). Routine application of ELISA in comparison with complement fixation for the diagnosis of foo-an-mouth and swine vesicular diseases. Veterinary Microbiology 16, 201-209.
2. ROEDER, P.L. and Le BLANC SMITH, P.M. (1987). Detection and typing of foot-and- mouth disease virus by ELISA: a sensitive, rapid and reliable technique for primary diagnosis. Research in Veterinary Science 43, 225-232.
3. OIE Manual of diagnostic tests and vaccines for terrestrial animals 2013. Chapter 2.1.5. Foot and mouth disease. Found at; http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.05_FMD.pdf.
4. IZLER ELISA kit instruction manual.

13 APPENDICES

13.1 APPENDIX 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **INDIRECT SANDWICH ELISA FOR DETECTION OF FOOT AND MOUTH DISEASE VIRUS (FMDV) ANTIGENS**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

13.2 APPENDIX .2: SOP DISTRIBUTION LISTING

This section is to be completed by the Document Coordinator in consultation with the Document Initiator and Laboratory Management indicating the Section / stations where controlled copies of this document shall be circulated.

Section / Area	SOP Manual Number	Date Issued

13.3 APPENDIX 3: SOP TRAINING LOG

This section is to be used to document training of the SOP.

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: **INDIRECT SANDWICH ELISA FOR DETECTION OF FOOT AND MOUTH DISEASE VIRUS (FMDV) ANTIGENS**		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.		

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

7. HAEMAGGLUTINATION / HAEMAGGLUTINATION INHIBITION TESTS FOR DIAGNOSIS OF NEWCASTLE DISEASE VIRUS (NDV) BY ANTIBODY DETECTION.

Standard Operating Procedure APPROVED	SOP No: Version: Original Supersedes: None Effective Date: Review Date:

Title: HAEMAGGLUTINATION / HAEMAGGLUTINATION INHIBITION TESTS FOR DIAGNOSIS OF NEWCASTLE DISEASE VIRUS (NDV) BY ANTIBODY DETECTION.			
	Name	**Signature**	**Date**
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1 PURPOSE/INTRODUCTION:

1.1 PURPOSE

This SOP provides instructions for diagnosis of NDV in Avian samples.

1.2 INTRODUCTION

Newcastle disease (ND) is a highly infectious disease of poultry. It is caused by the virus of the family paramyxoviridae, genus Avulavirus. It usually spreads rapidly resulting in serious, socio-economic consequences and major trade implications. Newcastle disease is of major importance in both commercial poultry flocks and backyard chicken flocks where it may cause outbreaks with up to 100% mortality.

2 SCOPE/RESPONSIBILITY

2.1 SCOPE

The scope of this procedure is to provide instructions on how to perform the HA-HI test for NDV in Avian Samples.

2.2 RESPONSIBILITY

2.2.1 The head of the laboratory is responsible for ensuring the correct application of this procedure by suitably trained staff.

2.2.2 The head of the laboratory is also responsible for ensuring that the laboratory staff are appropriately qualified and trained to safely and properly handle specimens for molecular analysis.

2.2.3 The laboratory staff are responsible for ensuring that the proper procedures are followed according to the SOP.

3.0 DEFINITIONS AND ABBREVIATIONS:

3.1 ABBREVIATIONS

°C Degree Celcius
HA Haemagglutination
HI Haemagglutination Inhibition
N/A Not Applicable
ND Newcastle Disease
NDV Newcastle Disease Virus
PBS Phosphate Buffered Saline
PPE Personal Protective Equipment
PCV Packed Cells Volume
RBCs Red Blood Cells
rpm Revolution Per Minute
SOP Standard Operating Procedure
vNDV Virulent Newcastle Disease Virus

3.2 DEFINITIONS

Not applicable

4.0 SPECIMEN

RecommendedSpecimens	Collection Notes	Pre-Analytical Processing
Serum, Whole blood, cloacal, tracheal swabs, long bone, intestines, spleen, liver	Collect whole blood from the wing vein of the bird with 2ml syringe and needle (23 gauge).	Process samples accordance with SOP for sample collection, packaging, processing transportation and storage.

5. EQUIPMENT / SUPPLIES/ REAGENTS

Equipment	Supplies	Reagents
• Refrigerator 4 – 8°C • Freezer -20°C/ – 40°C • Water distiller / deionizer • Vortex • Magnetic stirrer • Roller mixer • Bench top Centrifuge • Autoclave • Hematocrit centrifuge • Magnetic stirrer • pH meter • Water bath	• PPE • Various sizes of test tube racks • V bottomed microtitre plates with covers • Glass Slides • Hematocrit reader • Pipette Tips (20 – 200 µl, 1000 µl) • Reagent troughs • 250ml Flasks • Measuring cylinder • U-bottom shaped microtitre plates	• PBS • 1% Chicken Red Blood Cells • Newcastle disease virus antigen • Newcastle disease virus (NDV) specific antiserum • Distilled water • Alsever's solution; components • Glucose • Sodium chloride • Trisodium citrate Potassium chloride

Equipment	Supplies	Reagents
	• Capillary tubes • Single and multichannel micro pipettes • Timer • Hematocytometer • Heparinised capillary tubes • plasticin • Cryovials • 23 gauge needle • Absorbent paper towels • 2ml Syringe	• Citric acid • di-sodium hydrogen orthophosphate • Potassium di-hydrogen orthophosphate • Negative and Positive control sera

6 SAFETY PRECAUTIONS

- Wear appropriate PPE.
- Environmental conditions: Dust the benches that will be used to do the tests (e.g. clean and disinfected with 70% alcohol and at room temperature (18-25oC)

7 METHODOLOGY:

7.1 TEST PRINCIPLE

7.1.1 HEMAGGLUTINATION

All strains of Newcastle disease virus and avian influenza can agglutinate chicken red blood cells. This is the result of the haemagglutinin part of the haemagglutinin viral protein binding to receptors on the membrane of red blood cells. The linking together of the red blood cells by the viral particles results in clumping. Haemagglutination is visible macroscopically and is the basis of haemagglutination tests to detect the presence of viral particles.

7.1.2 HEMAGGLUTINATION INHIBITION

Haemagglutination Inhibition test method is based on a reaction between the virus and the specific antiserum. When the antiserum reacts with the virus, it binds to the epitopes that are responsible for haemagglutination; thus, these epitopes will not be available to bind to RBCs. If the antiserum is not specific for the virus, haemagglutination will occur, indicating non-identity between the two reagents

7.2 Procedure for preparation of 1% Chicken Red Blood Cells

7.2.1 Preparation of Phosphate buffer saline - PBS

1	Sodium Chloride (NaCl) Potassium Chloride (KCL) Disodium Hydrogen orthophosphate Na2HPO4	8.00g 0.20g 1.14g
2	Potassium di-hydrogen orthophosphate KH2PO4 Distilled Water up to 1000 ml	0.20g
3	Adjust pH to 7.2-7.4	
4	Aliquot into 500 ml bottles.	
5	Autoclave at 121°C for 15 minutes	

ALTERNATIVELY, if the PBS is inform of tablets or sachets, reconstitute according to manufacturer's instructions OR use ready to use liquid PBS.

1	Glucose	20.5g
2	Sodium chloride	4.2g
3	Tri-sodium citrate (2H2O (2-hydrate) Citric acid	8.0g 0.55g
4	Distilled water	1000 ml

- Dispense into 10 ml or 50 ml amounts
- Autoclave at 121°C for 15 min

7.2.3 Preparation of erythrocytes (RBC)

7.2.3.1 Prepare the RBC stock solution as follows:

1. Place 5 ml of Alsever's solution into each of two syringes.
2. Use the 2 syringes with Alsever's solution from (a) to draw 5 ml whole blood from each of 2 birds to give a total mixture volume of 10 ml in each of the 2 syringes.
3. Pool the 2 mixtures of blood and Alsever's in the 2 syringes into a universal bottle to give a total volume of 20ml and mix gently. This will be your stock solution.
4. Store the stock solution at 4°C±2°C. The stock solution can be stored for up to 5 days provided there is no hemolysis.
5. Cells for immediate use (working solution) are prepared freshly each day from the stock solution.

7.2.3.2 Prepare the working RBC solution from the stock solution as follows:

1. Mix the stock solution cells gently and aliquot 1 ml per test plate.
2. Centrifuge the aliquoted cells at 2200 rpm for five minutes and discard the supernatant to remove the Alsever's solution.
3. Add PBS (double the volume of RBCs) and centrifuge at 2200 rpm for 5 min and remove supernatant. Repeat this step twice.

7.2.3.3 Determine the packed cell volume (PCV) of the prepared working solution as follows:

1. Fill two capillary tubes with the blood from step 7.2.3.2(c) above and centrifuge in a haematocrit for 3 minutes.
2. Read off the PCV from the haematocrit reader. The PCV should be in the range of 55%- 70%. If the PCV is less than 55%, the cells must be re-centrifuged and excess PBS removed and if more than 70% dilute with more PBS.
3. Prepare 1% cells from the PCV determined working solution according to the following formula:

EITHER; C1 x V1 = C2 x V2

Where:
- C1 is the original PCV
- C2 is the concentration required (i.e. 1%)
- V1 is volume of Packed Cells
- V2 is the Volume of the 1% cells to be prepared

OR
Mix 1 part RBC with 99 parts PBS

7.3 PROCEDURE FOR HAEMAGGLUTINATION

- Prepare a 1% suspension of red blood cells from the 10% suspension by taking one part (1ml) of the suspension and mixing with nine parts (9 ml) of PBS
- Using the multichannel pipette, dispense 25 µl of PBS into each well of two (sample in duplicate) or three (sample in triplicate) rows of the microtitre plate.
- Place 25 µl of virus suspension (for example, allantoic fluid) into the first well of each row. (This is a 1:2 dilution.)
- Mix well and make two-fold dilutions of the suspension across the row by transferring 25 µl of fluid from one well to the next. Discard 25 µl from the last well of each row so that the volumes in each well are the same.
- Dispense 25 µl PBS into each well of a control row. This row will show the normal settling patterns of the red blood cells in suspension.
- Add 25 µl PBS to each well (including the control wells).
- Add 25 µl of 1% red blood cell suspension to each well.
- Mix gently, cover the plate and allow to stand at room temperature for 45 minutes.
- Read the results and record.
    - Positive: a thin film of red blood cells — indicates the presence of haemagglutinin (+)
    - Negative: a sharp button of red blood cells at bottom of well — indicates the absence of haemagglutinin (–)

NOTE:
Red blood cell control wells should always be included in the test. These wells contain 50 µl PBS and 25 µl 1% red blood cells, but no virus. At the end of the reaction time, these wells should show no haemagglutination that is, there should be a button of red blood cells at the base of the well. The wells show the normal settling patterns and time of red blood cells in suspension.

It is important that the test results are read as soon as possible after the 45 minutes incubation period has ended. After some time, the virus elutes, that is, the agglutinated cells disassociate (separate) and red blood cells will roll to form a button. Thus, a positive sample may not show haemagglutination and could appear as a negative sample. Remember that different strains of ND virus elute at different rates and elution of the

virus occurs more rapidly at higher temperatures.

7.4 PROCEDURE FOR HAEMAGGLUTINATION INHIBITION (HI) TEST

7.4.1 Preparation of 4 HA units of NDV antigen suspension for control NDV and test samples

The standard amount of Newcastle disease virus used in the haemagglutination inhibition (HI) test is 4HA units. It is necessary to prepare and test a suspension of Newcastle disease virus containing 4HA units in order to carry out the HI test. This involves a series of the following steps.

1. Using the quantitative HA test (see section 7.3), titrate the standard and test ND virus antigen suspension and calculate the HA titre.
2. Calculate the dilution factor required to produce 4 HA units. A simple way is to divide the HA titre by 4
3. Worked example:

If HA titre of the antigen titrated is 128;

- Calculation of dilution factor to prepare 4 HA units: 128/4 = 32
- Calculation of volume of 4 HA unit dilution of antigen required: (1 ml of the original suspension diluted in 31 ml of PBS).
- Allow 2.5 ml per plate; total volume required = 2.5 ml
- Apply dilution factor = 2.5 ml/32 = 0.0781 ml = 78.1 µl
- For 1 plate: mix 78.1 µl of original virus suspension with 2.4219 ml of diluent. PBS is a suitable diluent.
- For 10 plates: mix 781 µl of original virus suspension with 24.219 ml of diluent. PBS is a suitable diluent.

7.4.2.1 Back titration of diluted antigen suspension

- Using the HA test procedure, (see section 7.3), titrate the diluted (4HA) suspension of virus.
- Read HA titre. It should equal 4 HA units. If not adjust the dilution and titrate again.

7.4.3 Confirmation of titre of standard NDV positive and negative sera)

- Using the HI test (see procedure below), titrate known positive and negative sera. Use the 4 HA unit dilution of antigen in an HI test to test the standard positive and negative serum. The HI titre of the laboratory standard positive serum should equal the predetermined titre.
- The results of the back titration of the diluted antigen and the HI titre of the standard positive are both used to confirm the antigen has been diluted to a concentration equivalent to the standard 4 HA units.
- If the HI titre of the positive control serum is less than the standard titre, the antigen is too concentrated. Prepare a new dilution and test again.
- Conversely if the HI titre of the positive control serum is too high the antigen is too dilute. Prepare a new dilution and test again

7.4.4 Run the Test Sample.

1. Calculate the number of plates required and number each row (sample number) with a marker pen. If there is sufficient specific ND positive serum, it is good to do the tests in duplicate.
2. Add 25 µl PBS to each well of a 96-well V-bottomed microtitre plate.
3. Shake specific ND positive serum gently to mix. Add 25 µl of specific ND positive serum to wells A1 to H1 (control) well.
4. Using a multichannel pipette, transfer 25 µl of fluid from one well to the next. Stop at the second last well
5. Discard 25 µl of fluid from the second last well of the row. Do not dilute the last (control, twelfth) well of each row.
6. Add 25 µl of 4 HA units sample to each well in corresponding marked rows (not to the control twelfth well).
7. Tap the side of the plate gently to mix, cover and allow to stand at 220-250C for 30 minutes.
8. Add 25 µl of a 1% suspension of red blood cells to each well.
9. Tap the side of the plate gently to mix, cover and allow to stand at 22-25oC for 45 minutes.
10. Read the agglutination pattern.
11. Determine the endpoint. This is the well that shows complete inhibition of haemagglutination

7.5 READING AND INTERPRETATION OF TEST RESULTS

7.5.1 Reading the Test Results

Record the antibody titre for each sample. The titre is recorded as the highest dilution of test sample that causes complete inhibition of haemagglutination. HI results from individual birds are usually expressed as the reciprocal (positive value) of the end point serum dilution.

Wells with complete haemagglutination are recorded as "+" (positive HA); wells with a distinct button formation are recorded as "-" (negative HA); wells with partial button formation (fuzzy margin, or donut-like appearance) are recorded as "I" (incomplete HA). When interpretation between complete and incomplete inhibition is doubtful, tilt the microtiter plate at about 45 degree angle for 20-30 seconds and look for "tear drop" appearance of erythrocytes in the wells with complete inhibition.

(Note: wells with complete inhibition should form a "tear drop" at the same rate as positive control wells. Wells with complete or incomplete inhibition that show delayed "tear drop" as compared to the positive control should not be interpreted as inhibition.)

7.5.2 Interpretation of the Test Results:

- An isolate is identified as specific NDV if the endpoint of inhibition (HI endpoint titer) is within 2-well dilutions (4-fold difference) from the homologous NDV positive control.

- A test is considered valid if:
  - » NDV positive reference antigen and its homologous antiserum demonstrates the expected HI titre; and
  - » The back titration demonstrates presence of 4 HAUs.
  - » If these conditions are not met, the test should be repeated.

7.6 QUALITY CONTROL

7.6.1 INTERNAL QUALITY CONTROL

- Bench protocol must strictly be followed to ensure standard level of assay performance.
- Controls should always be included in the HA/HI test:
- Read the control wells first.

7.6.2 EXTERNAL QUALITY CONTROL

Participate in proficiency testing and inter-laboratory comparisons at least once a year.

7.6.3 QUALITY CONTROL MATERIAL

Use the reference materials provided with the kit.

7.6.4 CALIBRATION

- All the refrigerators, freezers and micropipettes must be calibrated by a certified company at least once a year.

8 REFERENCES

- OIE Diagnostic manual Diagnostic virology
- Reynolds DL1, Maraqa AD: A rapid virus neutralization assay for Newcastle disease virus with the swine testicular continuous cell line Avian Dis. 1999 Jul-Sep;43 (3):564-71.
- .OIE MANUAL – 2009 Edition

9. APPENDICES

9.1 Appendix .1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **HAEMAGGLUTINATION/ HAEMAGGLUTIN ATION INHIBITION TESTS FOR DIAGNOSIS OF NEWCASTLE DISEASE VIRUS (NDV).**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

9.2 Appendix 2: SOP DISTRIBUTION LISTING:

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Section / Area	SOP Manual Number	Date Issued

9.3 Appendix 3: SOP TRAINING LOG

This section is to be used to document training of the SOP.

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: **HAEMAGGLUTINATION/HAEMAGGLUTINATION INHIBITION TESTS FOR DIAGNOSIS OF NEWCASTLE DISEASE VIRUS.**		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.		

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

DETECTION OF NEWCASTLE DISEASE VIRUS IN CLINICAL SAMPLES BY REALTIME RT-PCR

Standard Operating Procedure APPROVED	SOP No: Version: Original Supersedes: None Effective Date: Review Date:

Title: DETECTION OF NEWCASTLE DISEASE VIRUS IN CLINICAL SAMPLES BY REALTIME RT-PCR			
	Name	**Signature**	**Date**
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE/INTRODUCTION:

1.1 PURPOSE

This SOP describes the procedure used to detect Newcastle disease virus. The real-time reverse transcriptase- polymerase chain reaction (qRT-PCR) technique was developed to assist in the rapid diagnosis of Newcastle virus infections in poultry.

1.2 INTRODUCTION

Newcastle disease (ND) is a highly infectious disease of domestic poultry and numerous species of wild and captive birds. The disease is caused by Avian paramyxovirus serotype-1 (APMV-1) of the family paramyxoviridae, genus Avulavirus which is an RNA virus. It can spread rapidly resulting in serious socio-economic consequences and major trade implications. Newcastle disease is of major importance in both commercial and backyard chicken flocks where it may cause outbreaks with up to 100% mortality.

2.0 SCOPE AND RESPONSIBILITY

2.1 SCOPE

This SOP is applicable to diagnostic specimens or samples referred to the Molecular Laboratory for detection of Newcastle Disease Virus (NDV).

2.2 RESPONSIBILITY

- It is the responsibility of the head of the laboratory to ensure that the staffs running the test are adequately trained and competent.
- It is also the responsibility of the head of the laboratory to ensure that the SOP is adhered to.

3.0 DEFINITIONS AND ABBREVIATIONS:

3.1 ABBREVIATIONS

APMV-1 Avian paramyxovirus serotype-1
BSC Biosafety Cabinet
Ct Cycle threshold
CRM Certified reference material
N/A Not Applicable
ND Newcastle disease
NDV Newcastle Disease Virus
qRT-PCR Real time Reverse Transcriptase Polymerase Chain Reaction
RNA Ribonucleic acid
RT-PCR Reverse Transcriptase Polymerase Chain Reaction
SOP Standard Operating Procedure

3.2 DEFINITIONS

Not applicable

4.0 SPECIMEN

Recommended Specimens	Collection Notes	Pre-Analytical Processing
Extracted RNA from Cloacal and tracheal swabs, Tissue sample (spleen, kidney, liver brain, intestine, trachea, lung)	Keep the RNA at -80oC until tested.	Bring all the reagents and template RNA on ice except for the enzyme that must be kept frozen until time of use.

5.0 EQUIPMENT / SUPPLIES/ REAGENTS:

Equipment	Supplies	Reagents
• Class II biosafety cabinets (BSC) • PCR work station • Refrigerator (4°C ±2) • Deep freezers (-20°C ±3) • Ultra low temperature deep freezer (80°C ±5) • Micro centrifuge (refrigerated) • Vortex mixer • Real-Time PCR System with its computer and its software • Ice flake maker	• Powder free Gloves e.g. Nitrile. • Cryogloves • Cryovials • Self- sealing biohazard plastic bags • Sealing tape • Permanent lab marker pen/ sample labels • Absorbent paper towel • Ice flakes • Cool box • Biohazard label • Water proof envelopes • Eppendorff tube racks	• Molecular grade nuclease-free sterile distilled water • In vitro transcribed vNDV matrix-fusion gene positive control RNA • Absolute Ethanol, molecular grade • One-Step rRT-PCR (Cat.# 210210 or 210212 Qiagen® ValenciaC. A) orAgPath-ID™ or One step RT-PCR kit Applied BiosystemsAmbion® (P/N AM1005 4387424, 4387391) • Hydrolysis probes and primers (oligonucleotides) (Table 1) for the detection of NDV. (Biosearch technologies).

Equipment	Supplies	Reagents
	• Calibrated pipettors (0.5, 20, 200 and 1000 µl) three sets: one for extraction room, second to master mix room and a third for DNA amplification room. • Sterile assorted size of aerosol resistant tips (10, 20, 200 and 1000 µl) • 1.5 ml sterile micro centrifuge tubes • PCR reaction tubes/plates • Dedicated lab coats	• RNase Inhibitor,40 units/µl (Promegacatalog# N2511 or N2515, Madison, WI • MgCl2, 25mM (Promegacatalog # A3511o r 43513,Madison, WI) • TE buffer pH 8.0, 1x, molecular grade (Promegacatalog #V 6231 or V6232, Madison, WI) • RNase away/or equivalent for cleaning of PCR work station/BSC

6.0 SAFETY PRECAUTIONS

- Follow universal safety practices when in the laboratory and handling diagnostic specimens.
- Wipe work surfaces, pipettes, and centrifuge with RNase away to remove any potential RNase contamination and 10% bleach to remove any DNA/RNA contaminants.
- Turn on the thermo cycler and computer CPU and allow warming up for approximately 10 minutes prior to use.

6.1 REAGENTS PREPARATION (work in the clean room)

- Keep all reagents on ice during assay set up.
- Mix the RT- PCR buffer by inversion (DO NOT VORTEX).
- Vortex all primers for 5 seconds.
- Mix all probes by inversion (do not vortex).
- Place primers and probes on ice
- Ensure that the probe has been kept away from light prior to usage

7.0 METHODOLOGY:

7.1 TEST PRINCIPLE

The One-step real time reverse transcriptase Polymerase Chain Reaction or quantitative RT-PCR (qRT-PCR) method involves reverse transcription of RNA and PCR amplification in a single test tube. The starting material for this method is RNA (intact, high quality RNA). RNA is first transcribed by reverse transcriptase from total RNA or messenger RNA (mRNA) into complementary DNA (cDNA). The cDNA is then used as the template for the Real-Time PCR (qPCR) reaction in which sequence specific primers are used to amplify the DNA of the desired genome. A fluorogenic hydrolysis/Taqman probe is used to monitor the PCR product formation at each cycle during the PCR. The probes are labelled at the 5' end with a reporter dye (e.g. FAM) and a quencher dye (e.g. Blackhole quencher [BHQ-1] at the 3' end. The proximally located quencher dye absorbs the emission of the reporter dye as long as the probe

is intact and not hybridized to the target. When the probe is hybridized to the target, the 5' exonuclease activity of Taq-polymerase will cause hydrolysis of the probe, separating the quencher from the reporter dye. This separation results in an increase in fluorescence emission of the reporter dye, which is detected spectrophotometrically and recorded. The amount of fluorescence recorded is proportional to the amount of target template in the samples.

7.2 vNDV Real Time RT-PCR Probe and Primer Sequences

Specificity and target gene	Primer probe name – genomic target	Sequence
APMV-1 (Matrix)	M+4100**(Forward Primer)	5'-AGTGATGTGCTCGACCTTC-3'
	M+4169** (Matrix Probe)	5'-FAMTTCCTCTAGCAGTGGGACAGCCTGC [BHQ]-3'
	M-4220** (Reverse Primer)	5' -CCTGAGGAGAGGCATTTGCTA-3'
vNDV (fusion)*	F+4829** (Forward Primer)	5'-GGTGAGTCTATCCGGARGATACAAG-3'
	F+4894** Probe-1 (VFP-1) (virulent fusion)	5` [FAM]AAGCGTTTCTGTCTCCTTCCTCCA [BQH]-3'
	F4939**(Reverse primer)	5'-AGCTGTTGCAACCCCAAG-3'

Note: It is strongly recommended that the probes and primers be purified to a high level to reduce nonspecific reactions.

7.3 PROCEDURE PRECAUTION

- All procedures should follow the unidirectional PCR set up with the room temperature (22-25oC). The PCR rooms should be clean and the operator should follow all the procedures in accordance with this SOP.
- Before the beginning of the RT-PCR test, placing the clean pipettors, racks, tips e.t.c into the clean hood and exposure to UV germicidal light for 3 hours or overnight is beneficial for the degradation of contaminating RNA from pipettors and other equipment; Clean the PCR work station with RNase away or similar product to avoid any degradation of RNA.
- Change gloves at every step
- Allow sufficient time for the UV radiation to clear from the biosafety cabinet.

7.3.1 REVERSE-TRANSCRIPTION AND PCR

- Two work areas are required for this procedure: a "clean" room with a dedicated BSC, freezer and supplies, and amplification room.
- Never introduce RNA/DNA material into the "clean" area and always change gloves before entering the "clean" area.
- In the "clean" hood, prepare a master mix of the following reagents sufficient for the number of samples being tested.
- The amount given in the table is per sample and may vary depending on the kit being used.

- Information on setting-up and programming the Thermal cycler can be found in the respective machines user manuals.

7.3.2 Real-time RT-PCR reaction mix volumes and conditions for NDV with APMV-I (matrix) primer/ probe sets.

7.3.3 PREPARING REACTION MIX (MASTER MIX)

Master mix preparation for one reaction

Reagent	Volume required per reaction (µl)	Final Concentration
Nuclease free H2O	6.95	
5x buffer	5	
1x 25mM MgCl2	1.25	3.75mM*
dNTP's (10 mM each)	0.8	320 µM each dNTP's
Forward Primer (20 pMol/µl)	0.5	10pmol/25µl
Reverse Primer (20pMol/µl)	0.5	10pmol/25µl
RNase Inhibitor (13.3 units/ µl)	0.5	0.266 units/µl
Enzyme Mix	1	(6pmol/ µl)
Probe	0.5	0.12 µM
Master mix per reaction	17	
Template (cDNA)	8	
Total volume reaction mix	**25**	

- The real-time RT-PCR reaction should be prepared with the following components and volumes using the appropriate primer and probe set and cycling conditions. Set up the reactions with the reaction tubes in the cooling block and use aerosol resistant pipette-tips.
- Make sure that the components of master mix are in ice flake including the template RNA and controls before commencing the actual testing.
- Prepare the reaction mix (everything except the template) by pipeting: RNase free H2O, kit supplied 5X reaction buffer, kit supplied dNTP's, 25mM MgCl2 and forward and reverse primers, into a nuclease free micro centrifuge tube using the volumes per reaction for each reagent given. Frozen reagents, including MgCl2 should be briefly vortexed and pulsed centrifuged prior to pipeting. Next add the RNase inhibitor and enzyme. Add the probe last. Mix reagents and centrifuge briefly. Once the probe has been added to the reaction mix, minimize exposure to light.
- Move the master mix to the RNA transfer hood and add the reaction mix (17µl) to the Smart Cycler tubes (add the mix to the bottom of the reaction tube).
- Add 8 µl of purified test sample RNA to the reaction tube using a pipettors designated for RNA transfer. Close the lid of the tube and number the reaction tubes according to test worksheet. Transfer 8 µl of diluted transcribed RNA into

the positive control reaction tube and 8 µl of clean RNase free water into negative control reaction tube. The transcribed RNA should be diluted by the user to a working dilution that will have a cycle threshold of approximately 25.0

- Centrifuge reaction tubes briefly to remove any air bubbles from the reading window of the PCR tubes.

7.3.3 RT step thermo cycling for Qiagen® one-step RT-PCR Kit.

RT Step	1 cycle	30 min	50°C
		15 min	95°C

7.3.4 Thermocycling conditions for gene specific probe and primer sets

Probe/Primer set	Number of cycles	Step	Time	Temp (in °C)
APMV-1 matrix	40 cycles	Denaturation	10 secs	94°C
		Annealing	30 secs	60°C
		Extension	10 secs	72°C
vNDV/VFP-1	40 cycles	Denaturation	10 secs	95 °C
		Annealing	30 secs	58°C
		Extension	10 secs	72°C

Note: The fluorescence is detected at the Annealing step.

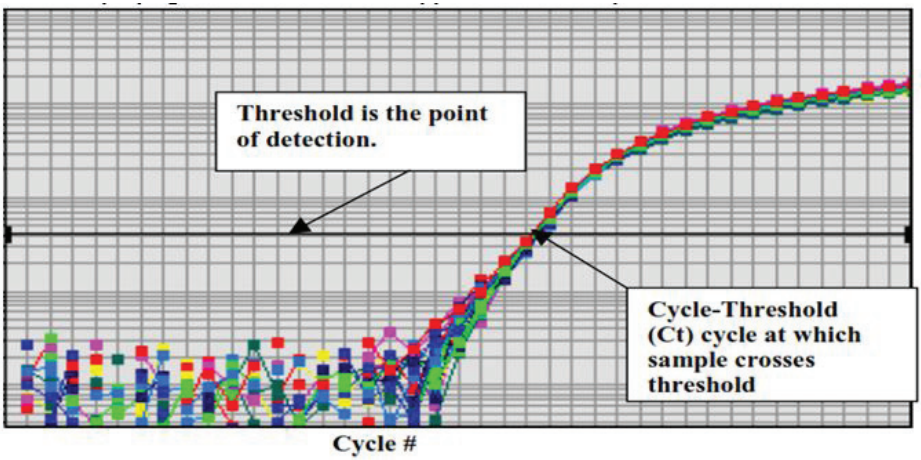
Bubbles in the reaction portion of the tube may be an indication of insufficient volume of RT-PCR master mix or the absence of sample RNA.

7.4 READING AND INTERPRETATION OF TEST RESULTS

Click:

- On results icon on the thermocycler software on the computer,
- Amplification plot
- Choose Analysis settings: Auto Ct or manual Ct After run is complete:
- Click on Analysis
- Click on Analyze
- Results
- File
- Print

Chart displaying how the results will appear on the computer screen



TEST VALIDATION CRITERIA

- The cycle threshold (Ct), the cycle number corresponding to an increase of the fluorescence over a threshold, is calculated automatically.
- The point where the fluorescence measurement is above the background signal and goes into detectable level is called the cycle threshold (Ct).
- This will be the starting fluorescence point to consider a sample as positive.
- The Ct value is inversely proportional to the starting amount of DNA template present in the reaction mixture that means in the analyzed sample.
- The procedure will be valid if both extraction and reaction positive controls give a Ct value of 22±2, and both extraction and reaction negative controls do not show any Ct value.
- In a positive sample, a sigmoid-shaped amplification curve will be obtained, indicating the cycle's number versus read fluorescence level, where the Ct value will be under 35.0.
- A negative sample will maintain the fluorescence profile under background fluorescence level and the equipment will not report any Ct value.
- Ct value >36 should be considered as doubtful result if a sigmoidal plot is observed and the analysis should be repeated for confirmation.
- Ct value >36 should be considered as negative if the amplification plot has a linear shape. These latter plots may represent spurious probe degradation or non-specific fluorescence.

7.5 TEST RECORDS

Computer printout filed according to filing procedures.

7.6 QUALITY CONTROL

Use CRM for quality control purposes if possible otherwise we can use lab developed ones

7.7.1 INTERNAL QUALITY CONTROL

Use positive and negative control samples in each test run.

7.7.2 EXTERNAL QUALITY CONTROL

To assure the quality of results, it is recommended that the laboratory participate in Proficiency Test or ring trial at least once annually.

7.8 CALIBRATOR.

Not applicable.

7.9 CALIBRATION.

We need to calibrate all testing and measuring equipment used in the testing of our samples.

7.10 REPORTING RESULTS.

Use appropriate format for reporting test results which should signed by the person who did the test and approved by the lab head.

7.11 QUALITY CONTROL RESULTS.

Results for QC checks on implementation and compliance to this SOP will be filed in the Quality Manager's Random Check Result File.

8 REFERENCES

1. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2015. Chapter 2.3.14:http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.14 NEWCASTLE_DIS.pdf.
2. Joyce C (2002). "Quantitative RT-PCR. A review of current methodologies". Methods Mol. Biol. 193: 83–92.doi:10.1385/1-59259-283-X:083. PMID 12325527
3. Biosearch Technologies (http://blackholequenchers.com and Operon - http://oliges.qiasen.com/). Suggested primer sources: Integrated DNA Technologies, (http://www.idtdna.com) or Operon (http://oligos.qiagen.com/). Other companies can be used to order both primers and probes.

9. APPENDICES

9.1 Appendix 1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **DETECTION OF VIRULENT NEWCASTLE DISEASE VIRUS IN CLINICAL SAMPLES BY REALTIME RT-PCR**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

9.2 Appendix.2: SOP DISTRIBUTION LISTING:

This section is to be completed by the Document Coordinator in consultation with the Document Initiator and Laboratory Management indicating the Section / stations where controlled copies of this document shall be circulated.

Section / Area	SOP Manual Number	Date Issued

9.3 Appendix 3: SOP TRAINING LOG

This section is to be used to document user training on the SOP.

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: **DETECTION OF NEWCASTLE DISEASE VIRUS IN CLINICAL SAMPLES BY REALTIME RT-PCR**		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.		

DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

8. COMPETITIVE ELISA FOR PESTE DES PETITS RUMINANTS (PPR)

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: COMPETITIVE ELISA FOR PESTE DES PETITS RUMINANTS (PPR)			
	Name	Signature	Date
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE/INTRODUCTION

1.1 PURPOSE

The purpose of this SOP is to guide laboratory staff in carrying out diagnostic tests on samples from animals suspected of PPR disease using competitive ELISA technique.

1.2 INTRODUCTION

Peste des Petits Ruminants (PPR) is a highly contagious, widespread, virulent and devastating viral disease of domestic and wild small ruminants caused by a morbillivirus closely related to Rinderpest Virus (RP). Clinically it is characterized by fever, occulo-nasal discharges, stomatitis (inflammation of the mucous membrane of the respiratory and digestive tracts), profuse diarrhoea and pneumonia with foul offensive breath.

2.0 SCOPE/RESPONSIBILTIES

2.1 SCOPE

The scope of this SOP is to provide the standard procedures for running cELISA techniques in the laboratory, and therefore it must be used by laboratory personnel assigned to carry out this technique.

2.2 RESPONSIBILITIES

2.2.1 The head of the laboratory is responsible for ensuring the correct application of this procedure by suitably trained staff.

2.2.2 The head of the laboratory is also responsible for ensuring that the laboratory staff are appropriately qualified and trained to safely and properly handle specimens.

2.2.3 The laboratory staff are responsible for ensuring that the proper procedures are followed according to the SOP.

3.0 DEFINITIONS AND ABBREVIATIONS

3.1 ABBREVIATIONS

Ab	Antibody.
Ag	Antigen
cELISA	Competitive Enzyme-linked immuno-sorbent assay
DPBST	Diluents phosphate buffer saline Tween-20
GLP	Good laboratory practice
HRP	Horseradish peroxidase
mAb	Monoclonal antibody
NP	Nucleocapsid Protein
Po	Peroxidase
PPE	Personal Protective Equipment
PPR	Peste des Petits Ruminants
QMS	Quality Management System
SOP	Standard Operating Procedure
TMB	3,3',5,5' tetramethyl benzidine

DEFINITIONS

Not applicable (N/A)

4.0 SAFETY PRECAUTIONS

- The laboratory personnel should wear appropriate personal protective equipment (PPE) while handling kit reagents or specimens; wash hands thoroughly afterwards.
- Chromogens are mutagenic and carcinogenic; therefore gloves and facemasks must be used all the time when running the ELISA test.
- Reagents should be stored safely and be inaccessible to unauthorized person.
- When preparing laboratory solutions, always use acid to water, never water to acid.
- Every reagent and equipment should be handled in the manner recommended by the manufacturer. Follow the established Good Laboratory Practice (GLP). Use the approved forms, manuals, SOPs, test methods accurately at all times.
- Pipettes should not be placed on the bench when doing the test. Use pipette holders.
- Decontaminate the work area and pipettes in 5% freshly prepared sodium hypochlorite solution for at least 1hour or 1% Virkon as a disinfectant.
- Control sera contain sodium azide that may be toxic if ingested.
- Do not pipette the samples with your mouth.

In addition to this test-SOP, all the safety-measures must be observed while carrying out the test

5.0 SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
Serum	Whole blood is collected from the jugular vein of the Sheep or Goats using plain vacutainer tubes	Blood is left to stand for 2 hours at room temperature then centrifuged at 2000rpm for 10 minutes.The serum is collected in sterile vials, labeled and assigned a laboratory number. The serum can be stored at -200 C. The serum is brought to 40 C before use. Allow all the reagents to come to room temperature 210C (+/-50C) before use. Homogenize all reagents by inversion or vortex

6.0 EQUIPMENT / SUPPLIES/ REAGENTS

Equipment	Supplies	Reagents
ELISA reader • Computer with printer • Incubator/shaker • Micropipettes • Centrifuge • Balance • Bi-distiller/de-ionizer • Freezer (-200C to -800C) • Refrigerator +20C to +80C • Magnetic stirrers • pH meter (indicators) • Vortex mixer • Water Conductivity Meter	• Aluminum foils • Disposable transfer pipettes • Timers • Flasks • Graduated cylinders • Graduated pipettes 1-10mL, (pi-pump pipette: 1mL, 5mL, 10mL, a piece of each) • Multichannel and single pipettes • Laboratory permanent marker • Micro-pre-dilution plates 96-wells (U shaped) poly propylene • Micro plates 96-wells polystyrene (preferably flat shaped) for ELISA plus adhesives • Racks • Absorbent paper towels • PPE • Reagent troughs • Cryovials • Sterile universal bottles • Sealing tape	• Absolute alcohol (C_2H_2OH) • Antigen, • Carbonate /bicarbonate buffer (HCO_3) • Conjugate, Control sera (C++, C+ and C-) Substrate, • Detergents • Hydrogen peroxide: (H_2O_2) • Sodium azide (NaN_3) or Stop Solution (commercial) • Sulfuric acid: (H_2SO_4) • Tween 20 ($C_{58}H_{114}O_{26}$) • Wash solution (commercial) • Distilled water/De-ionized water

7.0 METHODOLOGY:

7.1 TEST PRINCIPLE

The test depends on inhibition of the binding of a mouse monoclonal antibody (mAb) raised against a PPR-specific epitope in the presence of positive serum (sera containing antibodies against PPR nucleoprotein). Inhibition is detected as a reduction in the OD reading obtained with the mAb alone following the addition of peroxidase labelled anti-mouse conjugate and substrate/chromogen mixture. To achieve this inhibition, 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 that masks the NP epitopes. An anti-NP-peroxidase (Po) conjugate is added to the microwells. It fixes to the remaining free NP epitopes, forming an antigen-conjugate-peroxidase 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 test plate is read at 450nm.

7.2 PREPARATION OF REAGENTS

7.2.1 Wash Buffer

Either

Use the wash buffer in the kit

Dilute 100ml of wash concentrate (20x) in 1900 ml of distilled water. If it is not to be used immediately then prepare for 1 plate i.e 9 ml "wash concentrate (20x) in 171ml of distilled water.

Guideline for preparation of wash buffer according to number of plates

No of plates	Wash concentrate 20x (ml)	Distilled Water (ml)
1	9	171
2	18	342
3	27	513
4	81	684

OR

Use this when the one in the kit is finished

Dissolve one PBS tablet in 1 litre of distilled water as recommended by the manufacturer, add 2.5 ml of Tween 20 and mix well. Transfer this to wash fluid container with a tap to which tubing may be attached. Further dilute with addition of 4 litres of distilled water, mix well label and store at room temperature for not more than one week.

OR

Dissolve 1 PBS satchet in 1 liter of distilled water to give a buffer of 0.01M pH 7.4, add 2.5 ml of Tween 20 and mix well. Transfer this to wash fluid container with a tap to which tubing may be attached. Further dilute with addition of 4 liters of distilled water, mix well label and store at room temperature for not more than one week.

7.2.2 Diluents Buffer

Supplied as ready to use

7.2.3 Conjugate

Dilute conjugate to 1/10 for short incubation or to 1/20 for overnight incubation in dilution buffer 4"

Guideline for dilution of conjugate dilution 1:10 according to number of plates

No of plates	Conjugate (ml)	Diluent buffer 24 (ml)
1	1	9
2	2	18
3	3	27
4	4	36

7.2.4 Substrate buffer

Supplied as ready to use 'TMB"

7.2.5 Stopping Solution

Supplied as ready to use

7.3 PROCEDURE

Note: This test procedure is based on IDVET test kit

1. Dispense 40 µl of **Dilution Buffer** 13 to each well.
2. Dispense 10 µl of the **positive control** to wells A1 and B1.
3. Dispense 10 µl of the **negative control** to wells C1 and D1.
4. Dispense 10 µl of each sample to be tested to the remaining wells.
5. Incubate for up to 45 mins (+/-4 mins), at 370C (+/-30C).
6. Wash each well 3 times with approximately 300 µl of the **Wash Solution**. Avoid drying of the wells between washings.
7. Add 100 µl of the **Conjugate 1**X to each well.
8. Incubate for **30 mins (+/- 3 mins)** at 210C (+/-50C).
9. Wash each well 3 times with approximately 300 µl of the **Wash Solution**. Avoid drying of the wells between washings.
10. Add 100 µl of the **Substrate Solution** to each well.
11. Incubate for **15 mins (+/- 2 mins)** at 210C (+/-50C) in the dark.
12. Add 100 µl of the **Stop Solution** to each well in order to stop the reaction.

Plate layout for cELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	CP	S_5	S_{13}	S_{21}	S_{29}	S_{37}	S_{45}	S_{53}	S_{61}	S_{69}	S_{77}	S_{85}
B	CP	S_6	S_{14}	S_{22}	S_{30}	S_{38}	S_{46}	S_{54}	S_{62}	S_{70}	S_{78}	S_{86}
C	CN	S_7	S_{15}	S_{23}	S_{31}	S_{39}	S_{47}	S_{55}	S_{63}	S_{71}	S_{79}	S_{87}
D	CN	S_8	S_{16}	S_{24}	S_{32}	S_{40}	S_{48}	S_{56}	S_{64}	S_{72}	S_{80}	S_{88}
E	S_1	S_9	S_{17}	S_{25}	S_{33}	S_{41}	S_{49}	S_{57}	S_{65}	S_{73}	S_{81}	S_{99}
F	S_2	S_{10}	S_{18}	S_{26}	S_{34}	S_{42}	S_{50}	S_{58}	S_{66}	S_{74}	S_{82}	S_{100}
G	S_3	S_{11}	S_{19}	S_{27}	S_{35}	S_{43}	S_{51}	S_{59}	S_{67}	S_{75}	S_{83}	S_{101}
H	S_4	S_{12}	S_{20}	S_{28}	S_{36}	S_{44}	S_{52}	S_{60}	S_{68}	S_{76}	S_{84}	S_{102}

Key for plate layout
CP: Positive Control
CN: Negative Control
S1:Test serum No. 1
S2:Test serum No. 2

13. Gently shake the plate until the coloured solution is homogenize
14. Wipe carefully the bottom of the plate
15. Read and record the OD at 450nm
16. Interpret and record the results.

7.4 PROCEDURE NOTES

Bring all the reagents including the test sera to room temperature 30 min before use. TMB Substrate and Wash solutions can cause eye irritation; so take appropriate precaution.

7.5 QUALITY CONTROL.

Homogenize all reagents by inversion or vortex. Also take note of the manufacturer's instruction on the ELISA Kits.

7.6 QUALITY CONTROL MATERIAL.

Use the material provided by manufacturer.

7.7 CALIBRATOR.

Not applicable.

7.8 CALIBRATION

The ELISA Reader should be calibrated at least once a year or in accordance with the manufacturer's instructions.

7.9 VALIDATION CRITERIA

Read the Optical densities at 450nm (blank with air)
Calculate the Competition percentage for each sample

$$S/P = \frac{ODSample}{(ODNC)} \times 100$$

OD_S =Test sample OD,
OD_{PC} =Positive control OD,
OD_{NC} =Negative control OD

- Sera with less than or equal to 35 % are considered positive for PPR
- Sera greater than 35% and less than or equal to 45% are considered doubtful.
- Sera with greater than 50 % are considered Negative for PPR

7.10 RESULTS.

Interpretations of results are done according to the ELISA kit manufacturer's instruction. Samples presenting competition percentage

- Less than or equal to 50% are considered **Positive for PPR**
- Greater than 50% and less than or equal to 60% are considered **doubtful**
- Greater than 60% are considered **negative**

NB: Repeat the test for doubtful results.

Result	Interpretation
S/N% ≤ 50%	Positive
50% < S/P% <= 60%	Doubtful
S/P% >60%	Negative

7.11 QUALITY CONTROL RESULTS.

- QC results is calculated according to the ELISA kit manufacturer's instruction. The test) is valid if mean value of the Negative Control OD (ODNC is greater than 0.700
  ODNC > 0.700
- The test is valid if the mean value of the Positive controls (ODPC) is less than 30% of the ODNC
  ODPC / ODNC < 0.3

8.0 REFERENCES:

- IDVET KIT PROOCOL
- OIE TERRESTRIAL MANUAL 2009
- J. Anderson, J.A. McKay, R.N. Butcher: The use of monoclonal antibodies in competitive ELISA for the detection of Antibodies to rinderpest and peste des petits ruminants viruses. IAEA-TECDOC-623. The sero-monitoring of rinderpest throughout Africa; Phase one. Proceedings of a Final Research Co-ordination Meeting of the FAO/IAEA/SIDA/OAU/IBAR/PARC Co-ordinated Research Programme organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture and held in Bingerville, Côte d'Ivoire, 19-23 November 1990

9. APPENDICES

9.1 APPENDIX .1: DOCUMENT CHANGE HISTORY:

Version Table:

Original Title: **COMPETITIVE ELISA FOR PESTE PETIT DES RUMINANT (PPR)**	**Dated:**	**SOP No.: SOP/SER/002**	**No. Pages:**
Version 1: Title	Dated:	SOP No.:	No. Pages:
Version 2: Title	Dated:	SOP No.:	No. Pages:
Version 3: Title	Dated:	SOP No.:	No. Pages:
Version 4: Title	Dated:	SOP No.:	No. Pages:
Version 5: Title	Dated:	SOP No.:	No. Pages:

9.2 APPENDIX.2: SOP DISTRIBUTION LISTING:

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9.3 APPENDIX 3: SOP TRAINING LOG

This section is to be used to document training of the SOP.

Standard Operating Procedures (SOPs) Insert SOP code (Regional/country/lab/number)		**SOP No:** **Version:** Original **Supersedes:** None **Effective Date:** **Review Date:**
Title: COMPETITIVE ELISA FOR PESTE PETIT DES RUMINANT (PPR)		
Statement: I have read and I understand this SOP and will follow the instruction within. Any change, variation or breach of the procedure within the document will be notified to my line manager immediately. I understand that it is a disciplinary offence not to follow the procedure documented in this SOP.		

DATE	**TRAINEE NAME**	**SIGNATURE**	**TRAINER NAME**	**SIGNATURE**

Name of training approval authority_____ Signature:_____Date: _____

8.1 TAQMAN RT-PCR FOR DIAGNOSIS OF PPR VIRUS

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: TAQMAN RT-PCR FOR DIAGNOSIS OF PPR VIRUS			
	Name	Signature	Date
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE / INTRODUCTION.

1.1 PURPOSE

The purpose of this SOP is to describe the Real-Time PCR molecular diagnostic test used for detection of Peste des Petits Ruminants (PPR) virus.

1.2 INTRODUCTION

Peste des Petits ruminant is an acute viral disease of small ruminants characterized by fever, occulo-nasal discharges, stomatitis, diarrhoea and pneumonia with foul offensive breath. PPRV is in the genus Morbillivirus, family Paramyxoviridae. Virus members of this group have six structural proteins: the nucleocapsid protein (Np), the phosphoprotein (P), polymerase (L for large protein) protein, the matrix (M) protein, the fusion (F) protein and the haemagglutinin (H) protein. PPR virus is transmitted mainly by aerosols between animals living in close contact. This disease affects mainly goats and sheep, but it is usually more severe in goats where it causes heavy losses and is only occasionally severe in sheep while cattle can only be infected sub-clinically.

Real Time PCR is a sensitive and specific molecular assay that uses structural specific primers. The technique can detect very minute target viral sequences which are amplified many times. Real time RT -PCR for PPR Virus is based on the amplification of part of the N gene which is located at the most 3' end of the genome. It is the most expressed gene due to a transcriptional gradient from the 3' to the 5' end of the genome. The primers and the probe are designed in the 3'end variable nucleotide sequence of the N gene that is a well conserved.

2.0 SCOPE / RESPONSIBILITY

2.1 SCOPE

This SOP is to be used by all personnel involved in molecular testing of PPR samples in all small ruminant species.

2.2 RESPONSIBILITY

- It is the responsibility of the Laboratory head to ensure that all staff using this SOP are trained and competent to carry out the test.
- It is the responsibility of the Laboratory head to ensure that this SOP is distributed in all appropriate areas of the laboratory

3.0 DEFINITIONS AND ABBREVIATIONS

3.1 ABBREVIATIONS

CRM	Certified Reference Material
CT	Cycle Threshold
LQM	Laboratory Quality Management
PCR	Polymerase Chain Reaction
PPR	Paste des Petit Ruminants
PPE	Personal Protective equipment
RNA	Ribonucleic Acid
RT	Reverse Transcription
SOP	Standard Operating Procedure
Taq	Thermus aquatica polymerase enzyme

3.2 DEFINITIONS

Cycle Threshold (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold and is used to verify amplification quality.

4.0 SAFETY PRECAUTIONS

- All good laboratory practices must be followed while working.
- Appropriate safety procedures must be adhered to while handling all potential biological pathogens.
- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for sample preparation, PCR setup, PCR amplification, Analysis of PCR products.
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully.
- Try not to splash or spray PCR samples.
- Keep reactions components capped as much as possible
- Use aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution.

5.0 SPECIMEN

Recommended Specimens	Collection Notes	Pre-Analytical Processing
RNA extracted from blood, lung tissue or ocular/nasal swabs	Extracted RNA should be clear	Place all reagents and materials to be used into the biosafety cabinet or in close proximity. All preparations must be maintained at 4°C

6.0 EQUIPMENT / MATERIALS/ REAGENTS.

Equipment	Supplies	Reagents
• Biosafety cabinet Class 2 • PCR work station • Refrigerated Micro centrifuge • Real Time PCR machine with its software and computer • Refrigerator • Freezer (-20°C and -70°C or below) • Ice maker	• Filter micro pipette tips • Micro-Pipettes (various volumes) • Ice cubes • Ice buckets • Eppendorff tubes or equivalent • Disposable powder free gloves e.g. Nitrile gloves • Cryogenic gloves • Micro Amp Optical 96Well PCR Reaction Plate or optical tubes • Micro Amp Optical Caps or Optical caps Adhesive Covers • Micro Amp 96-well Tray/ Retainer Set • Cold block	• 2X one-step RT-PCR buffer • PPR forward and reverse Primers • Taqman probe • RNase/ Nuclease free water free water • Extracted RNA • Superscript 111 RT/ Platinum Taq mix • Disinfectant (Sodium Hypochlorite (10% bleach), 70% alcohol etc) • RNase easy or equivalent

7.0 METHODOLOGY:

7.1 TEST PRINCIPLE:

The One-step Real Time Reverse Transcriptase Polymerase Chain Reaction or quantitative RT-PCR (qRT-PCR) method involves reverse transcription of RNA and PCR amplification in a single test tube. The starting material for this method is RNA (intact, high quality RNA). RNA is first transcribed by reverse transcriptase from total RNA or messenger RNA (mRNA) into complementary DNA (cDNA). The cDNA is then used as the template for the Real-Time PCR (qPCR) reaction in which sequence specific primers are used to amplify the DNA of the desired genome. A fluorogenic hydrolysis/Taqman probe is used to monitor the PCR product formation at each cycle during the PCR. The probes are labelled at the 5' end with a reporter dye (e.g. FAM) and a quencher dye (e.g. Blackhole quencher [BHQ-1] at the 3' end. The proximally located quencher dye absorbs the emission of the reporter dye as long as the probe is intact and not hybridized to the target. When the probe is hybridized to the target, the 5' exonuclease activity of Taq-polymerase will cause hydrolysis of the probe, separating the quencher from the reporter dye. This separation results in an increase

in fluorescence emission of the reporter dye, which is detected spectrophotometrically and recorded. The amount of fluorescence recorded is proportional to the amount of target template in the samples.

7.2 REAGENTS PREPARATION (work in the clean room)

- Prepare Reagents as indicated in the Taqman kit
- Keep all reagents on ice during assay set up.
- Mix the RT- PCR buffer by inversion (DO NOT VORTEX).
- Vortex all primers for 5 seconds.
- Mix all probes by inversion (do not vortex).
- Place primers and probes on ice
- Ensure that the probe has been kept away from light prior to usage

7.3 PROCEDURE

7.3.1 RNA EXTRACTION

Refer to the extraction SOP

7.3.2 PRIMERS/PROBE SEQUENCES

- PPR Forward Primer: 5'- AGA GTT CAA TAT GTT RTT AGC CTC CAT-3'
- PPR Reverse Primer: 5'-TTC CCC ART CAC TCT YCT TTG T-3'
- Probe: FAM-CAC CGG AYA CKG CAG CTG ACT CAG AA-TAMRA

It is strongly recommended that the probes and primers be purified to a high level to reduce nonspecific reactions.

7.3.3 Composition of reaction/Master Mix for One-Step RT-PCR

Reagent	Volume (µl)	Concentration
2x one-step RT-PCR buffer	12.5	
Forward primer	1.0	10 pmol
Reverse primer	1.0	10 pmol
Probe	0.5	5 pmol
RNase/ nuclease free water	6	
Superscript III RT/Platinum Taq mix	0.5	
RNA template	3.0	
Total volume	25	

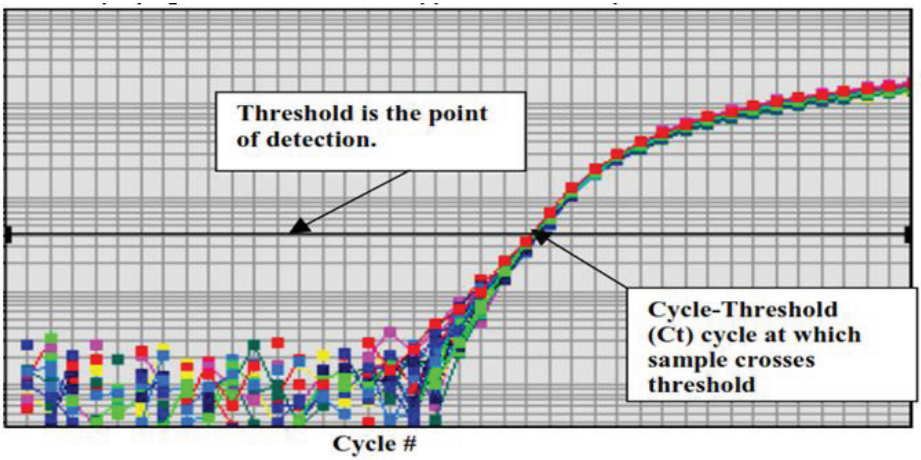
- The real-time RT-PCR reaction should be prepared with the following components and volumes using the appropriate primer and probe set and cycling conditions. Set-up the reactions with the reaction tubes in the cooling block and use aerosol resistant pipette-tips.
- Make sure that the components of master mix are on ice flake including the template RNA and controls before commencing the actual testing.

- Prepare the reaction mix (everything except the template) by pipeting: RNase free H_2O, kit supplied 5X reaction buffer, kit supplied dNTP's, 25mM MgCl2 and forward and reverse primers into a nuclease free micro centrifuge tube using the volumes per reaction for each reagent given.
- Frozen reagents, including MgCl2 should be briefly vortexed and pulsed centrifuged prior to pipeting.
- Next add the RNase inhibitor and enzyme.
- Add the probe last.
- Mix reagents and centrifuge briefly. Once the probe has been added to the reaction mix, minimize exposure to light.
- Move the master mix to the RNA transfer hood and add the reaction mix (25µl) to the Smart Cycler tubes (add the mix to the bottom of the reaction tube).
- Add 8 µl of purified test sample RNA to the reaction tube using pipettors designated for RNA transfer.
- Close the lid of the tube and number the reaction tubes according to test worksheet.
- Transfer 3 µl of diluted transcribed RNA into the positive control reaction tube and 8 µl of clean RNase free water into negative control reaction tube.
- The transcribed RNA should be diluted by the user to a working dilution that will have a cycle threshold of approximately 25.0
- Centrifuge reaction tubes briefly to remove any air bubbles from the reading window of the PCR tubes.

cycle	step	temp	time	
Cycle 1	reverse transcription	55oC	30 min	
		45oC	10 min	
Cycle 2	RT Inactivation/Taq activation	95oC	10 min	40 cycles
Cycle 3	PCR	95 oC	15 min	
	Hold	60oC	45 sec	

7.4 PROCEDURE NOTES

- Carry out sample and reagent preparation in different clean benches.
- Always use filter tips to prevent cross-contamination between samples.
- Use disposable gloves
- Use pipettes designated for master mix laboratory only.
- Work under a biosafety cabinet.
- Close each tube before opening another one.
- Do not expose master mix to UV light.
- Isothermal amplification reaction is very sensitive to cross contamination. Even minute amounts of amplification products in the reaction could cause false positive result.



7.5 QUALITY CONTROL

Include negative and positive controls in each set of assay

7.6 QUALITY CONTROL MATERIALS.

- Positive control (CRM positive obtained from positive sample): Known PPR viral RNA
- Negative control: nuclease free Water instead of RNA template

7.7 CALIBRATOR.

Not applicable

7.8 CALIBRATION.

All equipment must be calibrated according to the manufacturer's instructions

7.9 INTERPRETATION OF RESULTS.

Cycle threshold values are indirectly proportional to the concentration of the viral load. Low CT values are considered positive and High CT values are considered negative, however, the cut-off varies.

7.10 QUALITY CONTROL AND VALIDATION CRITERIA

Results are valid if the controls are positive with low Ct value and the negatives are negative with no Ct value. Cut off point for positivity or negativity varies in accordance with the standard curve derived from serial dilutions.
Note. Enzymes to be kept frozen until time of use

8.0 REFERENCES:

- OIE terrestrial Chapter 2.7.11. manual Peste des petits ruminants (NB: Version adopted in May 2013)

- Quantitative one-step real-time RT-PCR for the fast detection of the four genotypes of PPRV Olivier Kwiateka, Djénéba Keitaa, Patricia Gil a, Jovita Fernández-Pinerob, Miguel Angel Jimenez Claverob, Emmanuel Albinaa, Genevieve Libeaua,☐ Jan 2010.

9.0 APPENDICES

9.1 APPENDIX 1: DOCUMENT CHANGE HISTORY:

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DATE	**TRAINEE NAME**	**SIGNATURE**	**TRAINER NAME**	**SIGNATURE**

Name of training approval authority_____ Signature:_____Date: _____

9. COMPETITIVE ELISA FOR THE DETECTION OF ANTI-RVFV-NUCLEOPROTEIN IgG ANTIBODIES (SERUM OR PLASMA)

Standard Operating Procedure APPROVED			SOP No: Version: Original Supersedes: None Effective Date: Review Date:
Title: COMPETITIVE ELISA FOR THE DETECTION OF ANTI-RVFV-NUCLEOPROTEIN IgG ANTIBODIES (SERUM OR PLASMA)			
	Name	Signature	Date
Prepared By			
Reviewed By			
Quality Management Unit Authority			
Approval Authority			

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1.0 PURPOSE/INTRODUCTION

1.1 PURPOSE

The purpose of this SOP is to guide laboratory staff in carrying out diagnostic tests on samples from animals suspected with Rift Valley Fever Virus (RVFV) using competitive ELISA technique.

1.2 INTRODUCTION

Rift Valley Fever (RVF) is a peracute or acute zoonotic disease of domestic ruminants in Africa caused by a bunyavirus that is transmitted by mosquitoes. The disease is characterized by high mortality rate in young animals, abortion, nasal discharge and diarrhea, often hemorrhagic, and dysgalactia following heavy rainfall with floods. The impact is greatest in sheep, goats, cattle and camels. The disease is transmitted to human beings through mosquito bites and contact with infected material, particularly slaughter fluids of infected animals. RVF has caused serious disease in laboratory workers and must be handled with high levels of biosafety and biosecurity.

2.0 SCOPE/RESPONSIBILTIES

2.1 SCOPE

This SOP is designed to detect antibodies directed against RVFV nucleoprotein (NP) in serum or plasma. The detection of anti-nucleoprotein IgG antibodies by ELISA method indicates exposure to the virus by natural infection or by vaccination. Therefore laboratory personnel assigned to carry out diagnosis of RVF in the laboratory shall use this SOP.

2.2 RESPONSIBILITIES

2.2.1 The head of the laboratory is responsible for ensuring the correct application of this procedure by suitably trained staff.

2.2.2 The head of the laboratory is also responsible for ensuring that the laboratory staff are appropriately qualified and trained to safely and properly handle specimen.

2.2.3 The laboratory staff are responsible for ensuring that the proper procedures are followed according to the SOP.

3.0 DEFINITIONS AND ABBREVIATIONS

3.1 ABBREVIATIONS

Ab	Antibody
Ag	Antigen
cELISA	Competitive Enzyme-linked immunosorbent assay
DPBST	Diluents phosphate buffer saline Tween-20
GLP	Good laboratory practice
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
NC	Negative control
NP	Nucleoprotein
PC	Positive Control
PPE	Personal Protective Equipment
QAM	Quality Assurance Manager
QMS	Quality Management System
RVF	Rift Valley Fever
RVFV	Rift Valley Fever Virus
SOP	Standard Operating Procedures
TMB	3,3',5,5' tetramethylbenzidine

3.2 DEFINITIONS

Not applicable

4.0 SAFETY PRECAUTIONS

- The laboratory personnel should wear appropriate personal protective equipment (PPE) while handling kit reagents or specimens; wash hands thoroughly after working in the laboratory.
- Chromogens are mutagenic and carcinogenic; therefore gloves and facemasks must be used all the time when running the ELISA test.
- Reagents should be stored safely and be inaccessible to unauthorized persons.
- When preparing laboratory solutions, always add acid to water, never water to acid.
- Every reagent and equipment should be handled according to the instructions given in the fact sheets of the manufacturer.

- Follow the Good Laboratory Practices (GLP). Use the approved forms, manuals, and SOPs accurately at all times.

5.0 SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
Plasma Serum	Whole blood is collected from livestock in plain vacutainer tube or with clot activator tube Heparinized tubes for plasma	Blood for serum separation is left to stand for 2 hours at room temperature, then centrifuged at 2000rpm for 10 minnutes. The serum is collected in sterile vials, labeled and assigned a laboratory number. The serum can be stored at -20^{0}C. The serum is brought to 4^{0}C before use. Fresh serum can also be used directly after centrifugation. Heparinized blood should be centrifuged at 2000rpm for 10 minutes and plasma is kept at -20 ^{0}C.

7.0 METHODOLOGY:

7.1 TEST PRINCIPLE

The test depends on inhibition of the binding of a mouse monoclonal antibody (mAb) raised against a RVF Nucleoprotein (NP) specific epitope in the presence of positive serum (sera containing antibodies against NP protein). To achieve this inhibition, the micro plate wells are coated with purified recombinant RVF-NP. When the samples containing RVF-NP specific antibodies are added into the micro wells, they form an antibody-antigen complex, which masks the NP epitopes. After washing, an anti-NP-peroxidase (Po) conjugate is added to the micro wells. It fixes to the remaining free NP epitopes, forming an antigen-conjugate-peroxidase complex. After another series of washing to eliminate the excess conjugate, the substrate solution 3,3',5,5' tetramethylbenzidine (TMB) is added. The resulting coloration depends on the quantity of antibodies present in the test sera. In the absence of antibodies against RVF-NP, a blue solution forms which becomes yellow after addition of stop solution. In the presence of antibodies no coloration appears.

7.2 PREPARATION OF REAGENTS

7.2.1 Wash Buffer

Either use the wash buffer in the kit

OR

Dilute 100ml of wash concentrate (20x) in 1900 ml of distilled water. If not for immediate use then prepare for 1 plate i.e 9 ml "wash concentrate (20x) in 171ml of distilled water.

Guidelines for preparation of wash buffer depending on number of plates

Number of plates	Wash concentrate 20x(ml)	Distilled water(ml)
1	9	171
2	18	342
3	27	513
4	81	684

OR

Use this when the one in the kit is finished

Dissolve one PBS tablet in 1 litre of distilled water as recommended by the manufacturer, add 2.5 ml of Tween 20 and mix well. Transfer this to wash fluid container with a tap to which tubing may be attached. Further dilute with addition of 4 litres of distilled water, mix well, label and store at room temperature for not more than one week.

OR

Dissolve 1 sachet in 1 litre of distilled water to give you a buffer of 0.01M solution of pH 7.4 and add 2.5 ml of Tween 20 and mix well. Transfer this to wash fluid container with a tap to which tubing may be attached. Further dilute with addition of 4 litres of distilled water, mix well label and store at room temperature for not more than one week.

7.2.2 Diluent Buffer

Supplied as ready to use

7.2.3 Conjugate

Prepare the Anti-RVF-NP Conjugate 1X by diluting the Anti-RVF-NP-Po conjugate 10X to 1/10 in dilution Buffer.

Guidelines for diluting conjugate at 1:10 depending on number of plates

No of plates	Conjugate (ml)	Diluent buffer 19 (ml)
1	1	9
2	2	18
3	3	27
4	4	36

7.2.4 Substrate buffer

Supplied as ready to use 'TMB"

7.2.5 Stopping Solution

Supplied as ready to use

7.3 PROCEDURE

This procedure is based on IDVET test kit protocol.

1. Dispense 50 µl of Dilution Buffer 19 to each well.
2. Add 50 µl of the Positive Control to wells A1 and B1.
3. Add 50 µl of the Negative Control to wells C1 and D1
4. Add 50 µl of each sample to be tested to the remaining wells.
5. Incubate for up to 45 mins (±3mins), at 370C (±20C).

Plate layout for Competitive ELISA for RVFV IgG

	1	2	3	4	5	6	7	8	9	10	11	12
A	CP	S_5	S_{13}	S_{21}	S_{29}	S_{37}	S_{45}	S_{53}	S_{61}	S_{69}	S_{77}	S_{85}
B	CP	S_6	S_{14}	S_{22}	S_{30}	S_{38}	S_{46}	S_{54}	S_{62}	S_{70}	S_{78}	S_{86}
C	CN	S_7	S_{15}	S_{23}	S_{31}	S_{39}	S_{47}	S_{55}	S_{63}	S_{71}	S_{79}	S_{87}
D	CN	S_8	S_{16}	S_{24}	S_{32}	S_{40}	S_{48}	S_{56}	S_{64}	S_{72}	S_{80}	S_{88}
E	S_1	S_9	S_{17}	S_{25}	S_{33}	S_{41}	S_{49}	S_{57}	S_{65}	S_{73}	S_{81}	S_{99}
F	S_2	S_{10}	S_{18}	S_{26}	S_{34}	S_{42}	S_{50}	S_{58}	S_{66}	S_{74}	S_{82}	S_{100}
G	S_3	S_{11}	S_{19}	S_{27}	S_{35}	S_{43}	S_{51}	S_{59}	S_{67}	S_{75}	S_{83}	S_{101}
H	S_4	S_{12}	S_{20}	S_{28}	S_{36}	S_{44}	S_{52}	S_{60}	S_{68}	S_{76}	S_{84}	S_{102}

6. Empty the wells. Wash each well 3 times with approximately 300 µl of the wash solution. Avoid drying of the wells between washings.
7. Add 100 µl of the conjugate working dilution to each well.
8. Incubate for 30 mins (± 3 mins) at 210C ± 50C).
9. Empty the wells. Wash each well 3 times with approximately 300 µl of the wash solution. Avoid drying of the wells between washings.
10. Add 100 µl of the substrate solution to each well.
11. Incubate for 15 mins (± 2 mins) at 210C (± 50C) in the dark.
12. Add 100 µl of the stop solution to each well in order to stop the reaction.
13. Read and record the OD at 450nm
14. Interpret and record the results.

7.3 PROCEDURE NOTES

- Allow all the reagents to come to room temperature, 22-250C before use.
- TMB substrate and wash solutions can cause eye irritation; so take appropriate precaution.

7.4 QUALITY CONTROL.

Homogenize all reagents by inversion or vortex. Also take note of the manufacturer's instruction on the ELISA Kits.

7.5 QUALITY CONTROL MATERIAL.

The kit contains a positive and negative control that should be aliquoted and stored at -20°C

7.6 CALIBRATOR.

Not applicable.

7.7 CALIBRATION

The ELISA Reader should be calibrated at least once a year or in accordance with the manufacturer's instructions

Pipettes should be calibrated according to the institutional calibration schedule.

Reading

Read the Optical densities at 450nm (blank with air)

Compute the sample positivity (S/P) by calculating the competition percentage for each sample as follows

$$S/P = \frac{ODSample}{(ODNC)} \times 100$$

OD_S =Test sample OD,
OD_{PC} =Positive control OD,
OD_{NC} =Negative control OD

7.8 TEST VALIDATION

QC results is calculated and verified according to the ELISA kit manufacturer's instruction

- The test is valid if mean value of the Negative Control OD (ODNC is greater than 0.700
  ODNC > 0.700
- The test is valid if the mean value of the Positive controls (ODPC) is less than 30% of the ODNC
  ODPC / ODNC < 0.3

7.9 RESULTS.

Samples presenting competition percentage

- ≤50% are considered Positive
- >50% ≤60% are considered doubtful
- Greater than 60% are considered Negative

(Interpretations of results are done according to the ELISA kit manufacturer's instruction).

8.0 REFERENCES:

- OIE Terrestrial manual 2014
- Hyun-Joo Kim, et al., Young-Joon Ko Competitive ELISA for the Detection of Antibodies to Rift Valley Fever Virus in Goats and Cattle. Journal of Veterinary Medical Science. 74(3):321-7

9 APPENDICES

9.1 APPENDIX .1: DOCUMENT CHANGE HISTORY:

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DATE	TRAINEE NAME	SIGNATURE	TRAINER NAME	SIGNATURE

Name of training approval authority_____ Signature:_____Date: _____

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