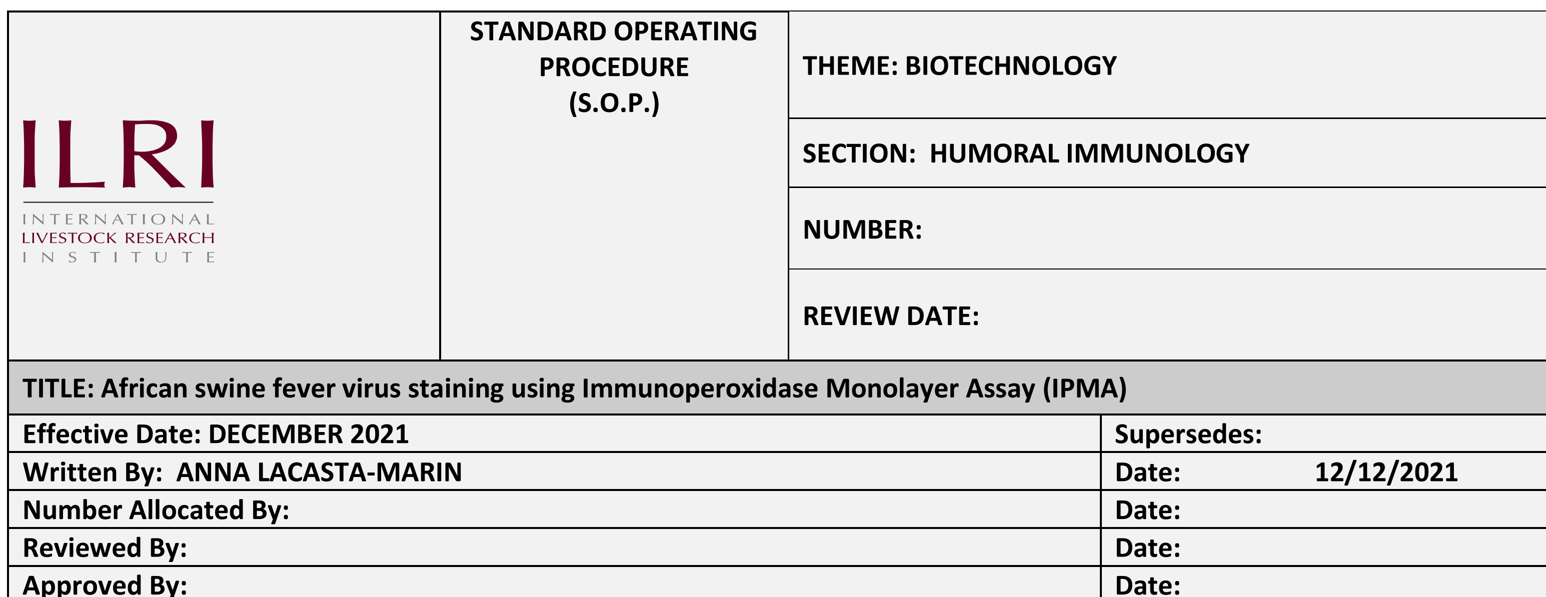

ILRI INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE	STANDARD OPERATING PROCEDURE (S.O.P.)	THEME: BIOTECHNOLOGY
		SECTION: HUMORAL IMMUNOLOGY
		NUMBER:
		REVIEW DATE:

TITLE: African swine fever virus staining using Immunoperoxidase Monolayer Assay (IPMA)	
Effective Date: DECEMBER 2021	Supersedes:
Written By: ANNA LACASTA-MARIN	Date: 12/12/2021
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1.0 PURPOSE

To describe the Immunoperoxidase Monolayer Assay to detect ASFV infection in WSL cells.

2.0 SCOPE

This protocol describes the Immunoperoxidase Monolayer Assay to detect African swine fever virus (ASFV) infection in WSL cells.

3.0 RESPONSIBILITIES

It is the responsibility of the technicians and scientists to ensure that the procedure is followed.

4.0 PROCEDURE

IPMA is a six-step procedure:

1) Culture the WSL cells
2) Infect the cells with ASFV
3) Incubate the infected cells for 5 days
4) Virus detection with rabbit anti-ASFV
5) Development of the reaction
6) Reading the plates

IPMA can be performed in any multi-well culture plate size (6-well, 24-well, 49-well, 96-well), the number of cells and the quantity of virus used will need to be adjusted. In the present protocol, 96-well plates will be the choice for IPMA. Cells are cultures at day -1 in a cell culture incubator to get a confluence of 80% the day after. The following day cells are infected at an MOI of 1 (1 virus: 1 cell) and incubated for 5 days at 37 °C 5% CO_2 in a cell culture incubator. After fixing the cells with absolute ethanol for 20 minutes at -20 °C, the endogenous peroxidase is inhibited using hydrogen peroxide for 15 minutes at room temperature in the darkness. Cells are then washed with 0.05%PBS-Tween20 and the ASFV immune sera is added. After 1h incubation at 37 °C the cells are washed, and the secondary

antibody added (anti-pig: HRP) and incubated for an extra 1 h at 37 °C. The reaction is developed with AEC reagents and plates are observed under the microscope to identify the infected cells.

This protocol should be followed carefully to ensure reproducibility.

MATERIALS

Item name	Brand	Catalogue number
96-well plate flat bottom	Corning	3598
RPMI 1640 medium powder	Merk	R4130-10L
Foetal Calf Serum	Gibco	10270106
L-Glutamine	Carl-Roth	HN08.2
Penicillin/Streptomycin	Carl-Roth	HP10.1
Gentamycin	Carl-Roth	HN09.2
b-mercapthethanol	Merk	4227.3
Absolute ethanol	Merk	E7023-500ML
Methanol-H_2O_2	Merk	H1009
Anti-pig: HRP	Sigma	A5670-1ML
AEC substrate buffer	Merk	152226-50ML
AEC reagent	Merk	152224-10ML
Phosphate buffered saline (PBS)	Merk	P3813-10PAK
Tween 20	Merk	822184
Anti-ASFV, anti-p30 and anti-p54 from IACUC2021-05	N/A (In-house reagent)	N/A (In-house reagent)
Anti-rabbit: HRP	Invitrogen	65-6120

1. Culture media complete RPMI (cRPMI)

Reagent	Volume (ml)
RPMI 1640 cell culture medium	439
foetal bovine serum heat inactivated	50
L-Glutamine 200 mM	5
Penicillin/Streptomycin 100x	5
2-Mercapto-ethanol (Stock at $5x10^{-2}$ M)	0.5
Gentamycin (50mg/ml)	0.5

Store at 4 °C. Use within 6 months.

2. Washing Buffer (WB) *(PBS-Tween 20)*

Materials

KH_2PO_4, Na_2HPO_4, NaCl, Tween 20, deioinsed water

Method

Combine the following:

KH_2PO_4	2.4 g
Na_2HPO_4	13.8 g
NaCl	96 g
Tween 20	12 ml
Deionized water	to 12 litres

The pH should be between 7.2 and 7.4. If the pH is not within this range, then it can be adjusted with HCl or NaOH.

Store at room temperature. Use within 6 months.

METHOD

Work inside the biosafety cabinet for all the steps, we need to keep all the reagents sterile. Only the washes can be done outside the biosafety cabinet.

DAY -1

(A) **Seed the cells into 96-well plates**

Cells	Number of cells	Cell culture media
PAMS	$5x10^5$cells/well	1% RPMI, 1% L-Glutamine, 1% Pen/Strept, 10% FBS
WSL	$2x10^4$ cells/well	1% DMEM, 1% L-Glutamine, 1% Pen/Strept, 10% FBS

DAY 0

(B) Virus preparation ASFV1033-GFP-A238LKO. Dilute the virus to get an moi of 1 depending on the type of cell to infect. See table below:

Cells	Number of virus	Cell culture media
PAMS	$5 x10^5$cells/well	1% RPMI, 1% L-Glutamin, 1% Pen/Strept, 10% FBS
WSL	$4 x10^4$ cells/well	1% DMEM, 1% L-Glutamin, 1% Pen/Strept, 5% FBS

(C) Virus infection.

1) Discard the media form the cells.
2) Dispense 100 µl/well of the pre-diluted virus. For virus titration use at least 6 wells form the plate.
3) Incubate for 5 days at 37 °C 5% CO2 in a cell culture incubator.

DAY 5

(D) Immunoperoxidase Monolayer Assay (IPMA)

1) Fix the plates with **absolute ethanol** (100 µl/well) and put them at -20 °C for at least 20 minutes.
Plates can be stored with absolute ethanol at -20 °C *for weeks*
2) Take out the plates from -20 °C, gently discard the ethanol by flicking the plate and then blotting once on clean paper.
3) Inhibition of endogenous peroxidase by hydrogen peroxide (without previous washing step): add 100 µl/well of **methanol-H_2O_2 solution**. Incubate 15 minutes at RT in darkness.
4) Wash the plate with 150 µl/well of PBS-T. Discard the solution by flicking the plate and then blotting once on clean paper. Repeat washing step for a total of 3 times.
5) Add 150 µl/well of **ASFV immune sera** (diluted 1:200 in PBS-T). Incubate 1 hour at 37 °C. ASFV immune sera: anti-ASFV, anti-p30 or anti-p54 from IACUC2021-05.
6) Wash the plate with 150 µl/well of PBS-T. Discard the solution by flicking the plate and then blotting once on clean paper. Repeat washing step for a total of 3 times.
7) Add 50 µl/well of **Protein A-Peroxidase** (diluted 1:750 in PBS-T). Incubate 1 hour at 37 °C.
8) Wash the plate with 150 µl/well of PBS-T. Discard the solution by flicking the plate and then blotting once on clean paper. Repeat washing step for a total of 3 times.
9) Add 50 µl/well of **AEC working solution** (see table 2). Incubate 10 minutes in darkness.
10) Wash the plate with 100 µl/well of PBS-T. Discard the solution by flicking the plate and then blotting once on clean paper. Repeat washing step for a total of 2 times.
11) Add 100 µl PBS/well to analyse. Keep the plate covered with aluminium foil and check under the microscope.

Table 2. Preparation of AEC working solution

AEC working solution
300µl Stock AEC solution (4 °C)
5 ml Acetate Buffer
3µl H_2O_2

5.0 DEFINITIONS

PBS: Phosphate-Buffered Saline
PBS-T20: PBS 1X pH 7.4 with 0,1% Tween20.
RT: room temperature

6.0 REFERENCES

None.

7.0 ATTACHMENTS

REVISION HISTORY

Revision No.	Supercedes	Reason

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