ILRI INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE	**STANDARD OPERATING PROCEDURE (S.O.P.)**	**THEME: BIOTECHNOLOGY**
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TITLE: *Theileria parva* sporozoite seroneutralization assay	
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1.0 PURPOSE

To describe the *Theileria parva* sporozoite seroneutralization assay.

2.0 Scope

This protocol describes the protocol to be able to perform the *T. parva* sporozoite seroneutralization assay (from now on Sporozoite-SNA) independently.

3.0 RESPONSIBILITIES

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

4.0 PROCEDURE

Sporozoite-SNA is a 7-step procedure:

(A) Preparation of the Peripheral Blood Mononuclear Cell (PBMC) culture
(B) Cell counting and dilution
(C) Antibody dilution (control and test)
(D) Sporozoite dilution and neutralization
(E) Infection
(F) Cell staining and FACS analysis

Sporozoite-SNA are performed in 96-well plates, enabling high throughput screening. Cells from the freezer are cultured overnight at 37°C. The next day the antibodies are diluted at an appropriate dilution and then mixed with the sporozoites. The mix is incubated for 30 minutes at room temperature, allowing the antibodies to bind/neutralize the sporozoites. Afterwards the cultured cells are added at a fix dilution and the infection is incubated for 11 days at 37°C and 5% CO_2. The read-out of the assay is done by means of FACS analysis and the results are presented as the percentage the infection is reduced in the presence of antibodies comparing with the infection control.

This protocol should be followed carefully to ensure reproducibility.

MATERIALS

1. Complete Roswell Park Memorial Institute medium (cRPMI)

In 500ml of RPMI 1640 (filtered)

- 1% L-Glutamine
- 2% Penicillin + Streptomycin
- 10% Foetal Calf Serum (FCS)
- 2-Mercaptoethanol (dilute 100µl of 2-mercaptoethanol with 13,5 ml RPMI and add 0,25ml of this solution to the medium)

Please keep sterility at all times when preparing this culture media.

2. Phosphate buffered saline (PBS)

Materials

KH_2PO_4, Na_2HPO_4, NaCl, deionized water

Method

Combine the following:

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

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

Sterilize the solution autoclaving.

3. 1% Paraformaldehyde (1% PFA)

Materials

Paraformaldehyde powder
PBS

Method

Combine the following:

Paraformaldehyde powder	1g
PBS	100ml

Dissolve using a magnetic stirrer and filter using a cell culture media filter.

4. 2% Saponin (2% SAP)

Materials

Saponin
PBS

Method

Combine the following:

Paraformaldehyde powder	2 g
PBS	100ml

Dissolve using a magnetic stirrer and filter using a cell culture media filter.

5. Control purified antibodies

3 different antibodies:

- Purified bovine anti-p67C polyclonal antibodies
- Purified AR22.7 mAb
- Purified 1A7 mAb

6. Test antibodies

The test antibodies will be diluted in cRPMI.

7. Sporozoites

The dilution and number of sporozoite will need to be assessed for every batch. The % of infected cells after 11 days incubation should be around 50%, using FACS analysis as read-out (explained in Method section F). E.g. Batch 10/17 is used at 1/20 to get to a 50% infection (approx.).

METHOD

Work inside the biosafety cabinet for all the steps, we need to keep all the reagents sterile. Only the staining if infected cells for FACS analysis can be done outside the biosafety cabinet.

DAY 1

(A) Preparation of the Peripheral Blood Mononuclear Cell (PBMC) culture.

1) Collect form the liquid nitrogen tank the number of vials needed for the number of samples planning to analyse.
2) Thaw the vials as fast as possible using a 37°C water bath.
3) Collect all the content in the vials and place the volume in a 50 ml Falcon containing cRPMI (37°C). Dilute 1/10 in cRPMI at least.
4) Centrifuge at room temperature at 650g for 10 min and discard the supernatant.
5) Resuspend every 107 cells in 1 ml of cRPMI (37°C).
6) Place the cells in a flask or in plate to incubate overnight at 37°C 5% CO_2 in a humidity chamber.

DAY 2

(B) Cell counting and dilution

1) Collect all the cultured PBMC in a Falcon tube.
2) Centrifuge at room temperature at 650g for 10 min and discard the supernatant.
3) Resuspend every 10^7 cells in 2 ml of cRPMI (37°C).
4) Count the cells using a Neubauer chamber.
5) Dilute or concentrate the PBMC at 10^6 cells/ml (=10^5 cells/100ul (well))
6) Keep the cells at room temperature until needed.

(C) Antibody dilution (control and test)

1) Label all the round-bottom 96-well plates (Corning, 3799) as needed. Minimum information should be the assay and the date. Label bottom and top or make a cross including bottom and top to know which belong to which.
2) Control antibodies dilution: purified AR22.7, 1A7 (all monoclonal Ab, mAb) and purified bovine anti-p67C polyclonal antibody. All of them diluted at 1 ug/ml in cRPMI. The volume needed are 150ul in total (50ul/well, triplicates), however 200ul/antibody will be prepared.
3) Test antibodies dilution: the test antibodies will not be diluted less than 1/100 (when the concentration of antibodies is not known, since the presence of bovine sera interferes with the assay). The recommended dilutions to start the analysis will be 1/100 and 1/1,000 per sample. For quantified samples the concentrations used are 10 and 1 ug/ml. Same dilutions/concentrations when mixing antibodies looking for synergies.

4) The volume needed are 150ul in total (50ul/well, triplicates), however 200ul/sample will be prepared.
5) Dispense 50ul/well of the diluted antibodies in triplicates following the plate layout.

(D) Sporozoite dilution and neutralization

1) Collect the frozen sporozoites from the liquid nitrogen tanks. Keep in liquid nitrogen until ready for dilution.
2) Prepare a Falcon tube with the appropriate volume of cRPMI for dilution. E.g. Batch 10/17 should be diluted 1/20, in every vial contains 100ul of sporozoites. Thus, every vial should be diluted in 2ml of cRPMI, enough for 40 wells (50ul/well).
3) Thaw the sporozoites as fast as possible and place all the volume in the already prepared media.
4) Dispense 50ul/well and mix gently.
5) Incubate for 30 min at room temperature.

Note: remember to include 6 wells with infection control (no-antibodies, media+sporozoites only).

(E) Infection

1) Dispense 100ul/well of PBMC at 10^6 cells/ml (10^5 cells/well).
2) Mix gently.
3) Incubate for 11 days at 37 °C 5% CO_2 in a humidity chamber.

(F) Cell staining and FACS analysis

1) Centrifuge the plates at room temperature, 650 g for 5 minutes and discard supernatant.
2) Gently resuspend the cells with the help of a vortex.
3) **Fixing**. Resuspend in 100 ul/well of 1% PFA buffer and incubate 15 min at room temperature.
4) Centrifuge the plates at room temperature, 650 g for 5 minutes and discard supernatant.
5) Gently resuspend the cells with the help of a vortex.
6) **Permeabilization.** Resuspend in 200 ul/well of 2% SAP buffer and incubate for 30 min at room temperature.
7) Centrifuge the plates at room temperature, 650 g for 5 minutes and discard supernatant.
8) Gently resuspend the cells with the help of a vortex.
9) **Primary antibody.** Resuspend the in 100 ul/well of anti-PIM (ILS40.2) diluted at 0.200 ug/ml (stock at 0.200 mg/ml), 1/1,000 dilution. Incubate for 30 minutes at 4 °C.
10) Centrifuge the plates at room temperature, 650 g for 5 minutes and discard supernatant.
11) Gently resuspend the cells with the help of a vortex.
12) Resuspend in 200 ul/well of 2% SAP buffer.
13) Centrifuge the plates at room temperature, 650 g for 5 minutes and discard supernatant.
14) Gently resuspend the cells with the help of a vortex.
15) **Secondary antibody.** Resuspend the cells in 100 ul/well of goat anti-mouse:FITC (BioLegend clone Poly4053, catalogue number 405305), diluted 1/500 in 2% SAP buffer. Incubate for 30 min at 4°C.
16) Centrifuge the plates at room temperature, 650 g for 5 minutes and discard supernatant.
17) Gently resuspend the cells with the help of a vortex.
18) Resuspend in 200 ul/well of FACS buffer (PBS supplemented with 2% FCS) and transfer into FACS tubes individually (no pooling wells).
19) **FACS analysis.**

Note: remember to include TpM cells in the FACS staining and analysis as positive controls.

PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	Serum 1 dil 1			Serum 5 dil 1			Serum 9 dil 1			AR22.7 mAb (1ug/ml)		
B	Serum 1 dil 2			Serum 5 dil 2			Serum 9 dil 2			1A7 mAb (1ug/ml)		
C	Serum 2 dil 1			Serum 6 dil 1			Serum 10 dil 1			Bovine anti-p67C (1ug/ml)		
D	Serum 2 dil 2			Serum 6 dil 2			Serum 10 dil 2			PBMC (non-infected)		
E	Serum 3 dil 1			Serum 7 dil 1			Serum 11 dil 1			Infection control (no antibodies)		
F	Serum 3 dil 2			Serum 7 dil 2			Serum 11 dil 2					
G	Serum 4 dil 1			Serum 8 dil 1			Serum 12 dil 1			TpM (no-stain)	TpM (secondary)	TpM (prim+sec)
H	Serum 4 dil 2			Serum 8 dil 2			Serum 12 dil 2					

In grey FACS staining controls using *Theileria parva* Muguga infected cell line (TpM).

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