

Single cycle neutralization assay, using PBMC as cellular targets.

University Louis Pasteur, INSERM U544, France

Neutnet code: 8

Contact person: Christiane Moog; c.moog@viro-ulp.u-strasbg.fr

Wrote on: 28 April 2003	Name : Nadège LAGARDE	Function : Technician	
Adapted: 02 march 2005	Maryse PERESSIN		

MATERIAL AND PRODUCTS:

96 sterile flat bottom wells plates (COSTAR 3599).

96 non-sterile wells plates (COSTAR 3598).

Tubes in polypropylene with conical bottom 15 and 50mL BLUE MAX (Becton Dickinson)
Sterilin boxes.

Multichannel pipette 12 tips electronic multichannel pipette (BioHit) 8 tips.

BioHit tips proline 4 tips bands non-sterile (350µL), 1200µL sterile BioHit tips.

Eppendorf distributor with 25, 50 or 100µL Eppendorf Combitip syringes.

RPMI 1640 medium with glutamax and 25mM HEPES (GibcoBRL) complemented with 5mL penicillin (10000U/mL)-streptomycin (10000µg/mL) (GibcoBRL).

Fetal calf serum = FCS (GibcoBRL) heat inactivated at 56°C during 30 minutes.

Human Interleukin 2 = IL2 (R&D system) at 10µg/ml.

PBS with Ca and Mg without antibiotics (GibcoBRL).

Fixation and permeabilisation kit = Cytfix/cytoperm (Becton Dickinson).

RD1 or FITC anti-p24 antibody KC57 (Coulter Beckman).

PBS + 3% FCS.

Serum samples.

Virus samples.

PROTOCOL**D -1: Thawing of PHA-activated PBMC.**

- Prepare 35 to 40ml RPMI in a 50ml BLUE MAX tube for 5 PBMC containing cryotubes.
Attention: check the grade in oxygen in the nitrogen room, wear protection glasses and special gloves, and don't forget to replace the top of the container.
- Transports the cryotubes in a small nitrogen container, quickly thaw this PBMC in the 37°C water bath. Transfer them into 50mL tubes. Rinse the cryotubes with RPMI.
- Centrifuge the 50mL tubes 10 minutes at 270G.
- Eliminate the supernatant after centrifugation and dilute the pellet in RPMI 10 % FCS 0,1 % IL2 medium (at 10µg/mL).
- Culture the cells in the 5% CO₂ incubator at 37 ° C over night.

1. Neutralization protocol.**In L2 laboratory**

Day 0: Report the assay format with the name of the serums, the virus, the dilutions and the date on the 96 wells plate.

15 serums / 2 dilutions per serum

	Dilution 1			Dilution 2			Dilution 1			Dilution 2			
Serum 1													Serum 9
Serum 2													Serum 10
Serum 3													Serum 11
Serum 4													Serum 12
Serum 5													Serum 13
Serum 6													Serum 14
Serum 7													Serum 15
Serum 8													

control virus control cells

10 serums / 3 dilutions per serum

	Dilution 1			Dilution 2			Dilution 1			Dilution 2					
Serum 1													Dilution 1 } Dilution 2 } Dilution 3 }	} } }	Sérums 9
Serum 2															
Serum 3															
Serum 4													Dilution 1 } Dilution 2 } Dilution 3 }	} } }	Sérums 10
Serum 5															
Serum 6															
Serum 7															
Serum 8															

Témoin virus

Témoin cellules

Thaw of the serums or purified IgG.

Prepare the dilutions of the serum in 96 wells dilution plate (dilutions done in RPMI-10% FCS-0.1%IL2 medium) respecting the plate format. Change the tips for **each dilution**. Then, distribute with multichannel pipette:

- 25µL/well of each dilution in 2 separate wells in the 96 wells neutralization plate.
- Add 25µL/well of RPMI-10%FCS-0.1%IL2 medium without antibodies in the control virus wells
and 50µL/well of medium in the uninfected control wells (without antibodies and virus).

Transfer the plates and RPMI-10%FCS-0.1%IL2 medium into the L3.

In the L3

Prepare the virus dilutions in RPMI-10%FCS-10%IL2 medium.

Note: This dilution will allow obtaining 2 to 4% of infected PBMC after 24 hours.

Distribute 25µL of virus/wells except in the uninfected control wells.

Incubate for 1 hour at 37°C in the 5% CO2 incubator.

In the L2

Count the PHA-activated PBMC (pool of the 5 different donors). Dilute the cells at 10-20 millions cells/mL in RPMI-10%FCS-0.1%IL2 medium in a 50mL tube.

Transfer the tube into the L3 (cells can be maintained at +4°C if not used immediately).

In the PL

Distribute 25µL of cells per wells with the Eppendorf distributor.

Incubate 24 to 36 hours at 37°C into the 5% CO2 incubator.

2. p24 intracellular staining.

At +36 hours after infection:

In the L2

Prepare the dilution of the p24 antibody at 1/160 in PermWash 1X in a 15mL tube (you will need 10mL for 2 plates).

In the L3

Add PBS into the sterilin box.

Wash the cells by addition of 100µL of PBS/wells with the BioHit multichannel pipette and centrifuge 10 minutes at +4°C at 450G.

Remove **the totality** of the supernatant.

Add 50µL of cytofix per wells with the Eppendorf distributor (at this moment the cells can be kept several days before further staining).

Incubate 10 minutes at +4°C in the dark.

Wash the cells by addition of 150µL of PermWash 1X (diluted in PBS) with the Eppendorf distributor.

Centrifuge 10 minutes at +4°C at 2000rpm.

Remove **the totality** of the supernatant.

Switch of the light:

Add 50µL of anti-p24 antibody with the Eppendorf distributor.

Incubate 10 minutes at +4°C in the dark.

Wash the cells by addition of 150µL of PBS-3%FCS medium with the Eppendorf distributor

Centrifuge 10 minutes at +4°C at 2000rpm.

Remove **the totality** of the supernatant.

Add 200 to 300µL of PBS (depending on the number of cells) with the BioHit multichannel pipette.

Homogenize and transfer with a multichannel pipette in new non-sterile 96 wells plates out of the L3.

Scotch and leave at +4°C in the dark until measurement by the flow cytometry.

3. Flow cytometry

Follow the flow cytometry procedure.

4. Results

The neutralizing titer will be defined as the dilution of the antibody allowing the inhibition of 90% of the infected cells.

The calculations and the graph will be automatically performed with an Excel matrix.