PBMC based HIV-1 Neutralization Assay

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1. AIM:

Detection of neutralizing activity of monoclonal antibodies (mAb) as well as sera/plasma obtained from HIV infected individuals using a PBMC assay. In this assay we use an extended (24 hr) incubation period (Davis et al. J. Med. Virol. (2003); 71:331-342 and Vaccine (2004); 22:747-754) of virus and neutralizing agent prior adding the PHA/IL-2 stimulated PBMC. The absorption phase is terminated after one hour by three washing steps and cells are cultured at 37°C, 5% CO_2 during 14 days. Here after a reduction in virus infected titer, as mediated by exposure to the antibody is assessed by measuring the p24 in the culture supernatant using an 'in-house' quantitative ELISA.

2. MATERIALS AND EQUIPMENT

- Biological safety cabinet class II
- Humidified 37°C incubator with 5% CO_2.
- Centrifuge equipped with microplate carriers
- Electric pipettor
- Adjustable pipetters
- Multichannel pipetters
- ELISA plate reader (BioRad) with Microplate manager software
- 96 well plate washer
- Vacuum trap suction
- Microscope
- Disposable plastic pipettes
- 1-250µl pipette tips
- 50ml conical centrifuge tube
- 96-well flat bottom plates with lid
- Microtiter plate sealers
- Sterile reagent reservoirs
- IL-2 medium should be warmed to 37°C before use
- Lymphoprep separated PBMC from an HIV-1-seronegative donor.
- HIV-1 p24 in-house antigen ELISA

- Pooled normal human plasma (NHP)
- Control serum/plasma from HIV-1 positive individuals
- Trypan blue: 0.4 %
- 70 % ethanol

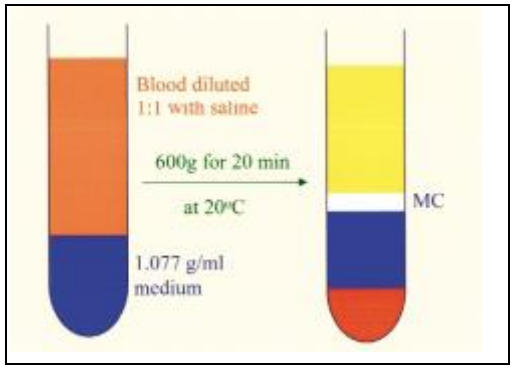
3. PROCEDURES

3.1 Preparation of donor PBMCs

a) Separation of donor PBMCs

- Lymphocytes are separated by centrifugation on a Lymphoprep-gradient.
- Take care for contamination if you open the buffy-coat bag. Disinfect the plastic tubing of the bag and the scissors with alcohol (70 %).
- The blood from one buffy-coat is diluted with basic RPMI 1640 medium to a total volume of 200 ml in a medium culture flask.
- Put 15 ml of Lymphoprep into 4 conic Falcon tubes of 50ml.
- Dispense carefully and slowly 30 ml of blood on the Lymphoprep. Avoid mixing of blood and separation fluid. Cap the tube to prevent the formation of aerosols.
- Centrifuge for 20 minutes at 2200 rpm (1400 g) at room temperature in a swing-out rotor.
- After centrifugation the mononuclear cells form a distinct band at the sample/medium interface, as shown in the figure below.
- The cells are removed from the interface using a pipette without removing the upper layer (the RPMI).
- Transfer the harvested fractions to 2 new conical tubes (Falcon 50ml) and resuspend the cells in basic RPMI 1640-medium to a final volume of 50ml.

Fig: Isolation of human mononuclear cells (MC) using Lymphoprep ™



b) Washing

- Centrifuge the cell suspensions 10 minutes at 2000 rpm (900 g).
- Pour off the supernatant into a waste bottle.
- Resuspend the cells of the 2 tubes in 10ml basic RPMI 1640 medium,

bring the 2 tubes together and add basic RPMI medium up to 50 ml. Take one drop to count the cells
- Centrifuge the cell suspension 10 minutes at 2000 rpm (900 g).
- Pour off the supernatant into a waste bottle.

c) Stimulation of the donor PBMCs

- Resuspend the cells at 1.10^6/ml in complete RPMI 1640 medium supplemented with 0.5µg/ml phytohaemagglutinin (PHA).
  The volume of the culture will decide the type of culture flask used:
  < 15ml : 25cm^2 flask (50ml)
  15-50ml : 81cm^2 flask (200ml)
  100-250ml : 175cm^2 flask (800ml)
  From one buffy-coat ± 600 -800.10^6 cells can be obtained.
- Incubate horizontally for 2-4 days at 37°C in a 5% CO_2 atmosphere.

- After 2-4 days PHA stimulation we bring the cell-containing culture suspension from the culture flask into 50ml tubes (Falcon).
- Centrifuge 10 minutes at 1500 rpm (500 g).
- Pour off the supernatant into a waste bottle.
- Resuspend the cells of one buffy-coat into one 50ml tube (Falcon) in complete RPMI 1640 medium supplemented with Interleukin 2 (IL-2). Add medium to a total volume of 50ml.
- Take one drop to count the cells.
- Bring the cell suspension into a culture flask and add complete RPMI 1640 medium supplemented with Interleukin 2 (IL-2) to the calculated volume, so that you become a cell concentration of 1.10^6/ml.

General remarks:
- When harvesting the cells from the gradient, carry along as little as possible of the Lymphoprep solution.
- It is important to thoroughly wash the cells. These washes dilute out the Lymphoprep solution and decrease the number of contaminating platelets.
- It is usually helpful to resuspend the pelleted cells in a small volume (± 10ml) before filling the tube. This guarantees that the added cells will be well dispersed and can, therefore, be thoroughly washed.

3.2 Neutralization protocol

Day -1:

- Prepare 6 serial 5 fold dilutions of the virus starting from 1/2 up to 1/6250.
- Prepare 6 serial 2 fold dilutions of the antibodies (TriMab, 4E10 and 447-52D) starting from 500µg/ml up to 15.625µg/ml.
- For sCD4 six serial 2 fold dilutions starting from 200µg/ml up to 6.25µg/ml are made in IL-2 medium

Mix into a sterile polypropylene tube 90 µl of a virus dilution (e.g. 1/2 VD) with 5µl NHP and 5µl of a mAb dilution (e.g. 500µg/ml). In this example this results in a final conc. of 25µg/ml.

This is done for each virus dilution in combination with each mAb dilution.

- A negative control is used for each virus and hereto the mAb is replaced by IL-2 medium.

  Incubate 24 hr at 37°C

- Put the required amount of PHA stimulated PBMCs on IL-2, at a concentration of 1.10^6 cells/ml

Note:

When working with sera/plasma the neutralizations are set-up by mixing 190µl of VD with 10µl of plasma (resulting in a 1/20 dilution). As controls a HIV negative plasma sample as well as a sample with a high neutralizing titer is used.

All test sera/plasma and control sera/plasma has been complement depleted by heat inactivation for 30 min at 56°C.

Day 0:

1. Transfer 20µl Virus / mAb mixture in 4 wells of a 96-well flat bottom plate.
2. Add 100µl of PHA-stimulated PBMC, resuspended at $7.5x10^5$ cells/ml to each well. Cover the plate and incubate 1 hr at 37˚C, 5%CO_2.
3. Wash the cells by filling wells to 200µl with IL-2 medium. Cover the plates and centrifuge for 10 minutes at 1200 rpm.
4. After each wash aspirate supernatants from each well with an aspirator set to leave ~20µl / well. Bring the volume up to 200µl in each well and then centrifuge again. Repeat for a total of 3 washes.
5. After the last wash add 200ml of IL-2 medium to each well. Incubate at 37˚C, 5%CO_2 for 7 days.

Day 7:

Remove 125µl of the culture supernatant and replace with 125µl of IL-2 medium. Incubate for another 7 days at 37°C, 5% CO_2.

Day 14:

Take 200µl of the culture SN and measure p24 antigen using an 'in-house' ELISA.

The Reed-Muench method is used for TCID50 calculations.

Calculate percent neutralization and determine the IC50, 75 and 90.

4. REAGENTS:

4.1 Basic RPMI 1640 medium

- 500ml RPMI 1640 with 25mmol HEPES (commercially available)
- Add 5ml 3% L-glutamine (commercially available)
- Add 0.5ml gentamycine (50mg/ml) (commercially available)

Store at 37°C, use at 37°C.

4.2 Complete RPMI 1640 medium supplemented with Interleukin 2 (IL-2)

- 420ml basic RPMI 1640 medium
- Add 75ml Fetal Calf Serum or Fetal Bovine Serum (final conc. 15%)
- Add 5ml polybrene (stock: 200µg/ml) (final conc. 2µg/ml)
- Add 250 µl hydrocortisone (stock: 10mg/ml) (final conc. 5µg/ml)

Store at 37°C

- Before use, add 10^5 U Interleukin-2 (final conc. 200 U/ml)

Store at 4°C, use at 37°C.

4.3 Complete RPMI 1640 medium supplemented with phytohaemagglutinin (PHA)

- 500ml basic RPMI 1640 medium
- Add 90ml Fetal Calf Serum or Fetal Bovine Serum (final conc. 15%)
- Add 6ml polybrene (stock: 200µg/ml) (final conc. 2µg/ml)

Store at 37°C

- Before use, add 12 ml phytohaemagglutinin (PHA) (stock: 25 µg/ml) (final conc. 0.5µg/ml)

Store at 4°C, use at 37°C.

RPMI 1640 medium with 25mmol Hepes and with L-glutamine

- Commercially available in bottles of 500ml (LONZA).
- Store at 4-8°C.

L-glutamine

- Commercially available in bottles of 100ml (LONZA).
- Store at 4-8°C.

Gentamycine (50mg/ml)

- Commercially available in bottles of 20ml (LONZA).
- Store at 4-8°C

Fetal Calf Serum or Fetal Bovine Serum

- Commercially available in bottles of 500ml (LONZA).
- Store at -20°C.
- Before use, serum must be decomplemented for 30 minutes at 56°C (in a water bath). The decomplemented serum can be stored at 4-8°C.

Polybrene (200 µg/ml)

- 20 mg polybrene (Sigma) is reconstituted in 100ml RPMI 1640 medium.
- Sterilize by filtration.
- Store at -20°C.

Hydrocortisone (10 mg/ml)

- 100mg of hydrocortisone (Merck) is reconstituted in 10ml ethanol.
- Store at 4-8°C.

Phytohaemagglutinin (PHA) (25 µg/ml)

- 2mg phytohaemagglutinin (Biotrading) is reconstituted in 80ml RPMI 1640 medium.
- Sterilize by filtration.
- Distribute in 4 ml aliquots.
- Store at -20°C.

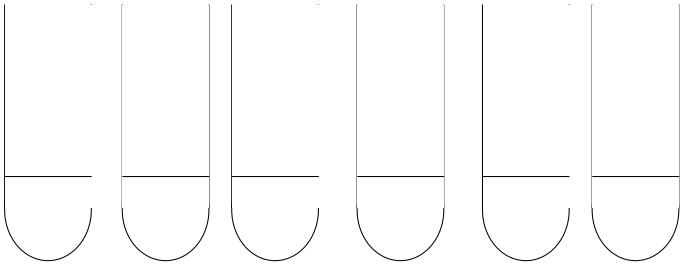
Interleukin 2 (IL-2) (recombinant)

- Commercially available (GENTAUR).
- Store at -80°C.

Simplified schematic overview of the Neutralization protocol:

• Add into each tube 90µl of virus dilution (VD):

VD: 1/2 1/10 1/50 1/250 1/1250 1/6250



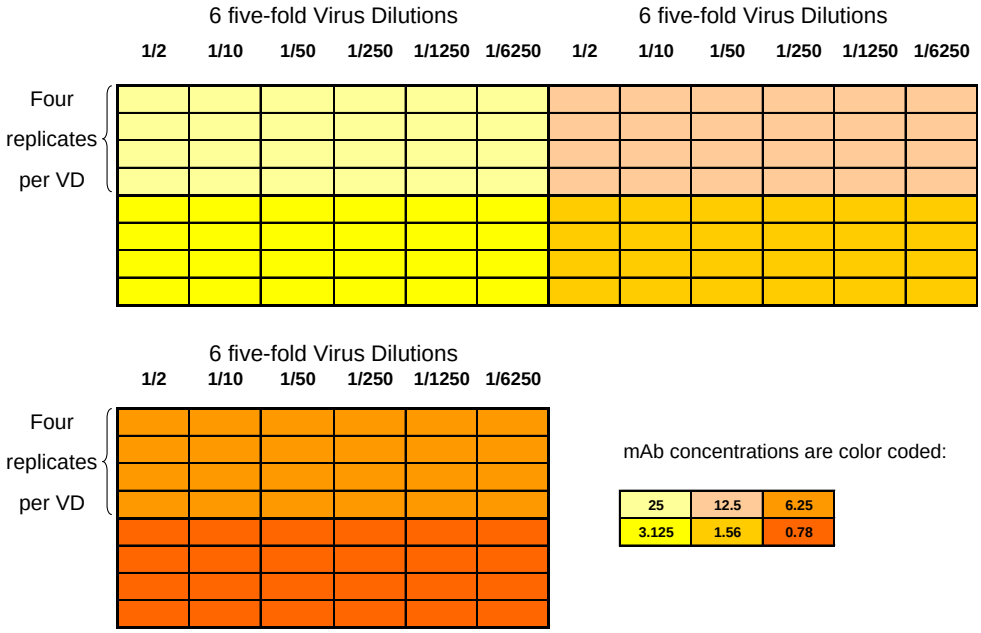
• Add to each tube: 5µl NHP and 5µl mAb (e.g. 500µg/ml)

→ 1/20 dilution of mAb (in this example resulting in a final cc of 25µg/ml)

Do this for all mAb dilutions (6/mAb) → *36 tubes / virus / mAb*

Incubate 24 hr at 37°C

• Transfer 20µl of each virus/mAb mixture in 4 wells of a flat bottom 96 well plate: → *1½ plate needed for each mAb*



- Add 100µl (75,000 cells) PHA/IL-2 stimulated PBMC per well

Incubate 1hr at 37°C

- Wash (3x) with IL-2 medium
- Add 200µl of IL-2 medium
- Incubate 14 days (37°C) changing half of the medium at d 7
- p24 antigen in-house ELISA is done on day 14
- Calculate IC50, EC75 and IC90 values