PBMC-based Neutralization Assay

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This document describes the isolation of Peripheral Blood Mononuclear Cells (PBMCs) from buffycoat, the stimulation of PBMCs by PHA, and the determination of the neutralization sensitivity of HIV-1 strains using a PBMC neutralization assay.

MATERIAL:

Biological safety cabinet class II
Humidified 37°C incubator with 5% CO_2
Centrifuge
Electric Pipettor
Adjustable pipetters
Multichannel pipetters
Twelve-channel manual ELISA aspirator (NUNC)
ELISA reader Berthold Mitras luminometer
Microscope
1-200µl pipette tips
15 and 50mL Polystyrene conical tubes
96 sterile round bottom wells plates with covers
24 sterile flat bottom wells plates with covers
Sterile reagent reservoirs
Sterile flasks
Reagents:
Interleukin-2 (Chiron)
PBS-Dulbecco's Phosphate-buffered saline without calcium and magnesium (Invitrogen)
PHA-Phytohaemagglutinine (final concentration 5mg/ml) (Sigma Aldrich)
RPMI (Invitrogen)
Fetal Calf Serum (Lonza)
Penicillin Streptavidine (Pen Strep, Lonza)

Lympholyte-H (Cederlane)
Target cells: Lympholyte gradient separated PBMC (Peripheral Blood Mononuclear Cell) from two healthy blood donors.

Medium should be warmed to 37˚C before use in all procedures described below.

PROCEDURE:

1. Preparation of PBMC

PBMCs are isolated from buffycoats from 2 or more different healthy blood donors. PBMCs from each donor are isolated and stimulated separately.

- Prepare two 50 ml tubes for each buffycoat and transfer 20 ml of buffycoat in each tubes. Dilute the buffycoats 1:1 with PBS.
- Add 10 ml of Lympholyte-H to each of four 50 ml tubes and add the diluted buffycoat on top of the Lympholyte-H in each tube.
- Centrifuge the tubes for 30 min at 1800 rpm without brake at RT.
- Remove the ring fraction on top of the Lympholyte-H with a sterile pipet and add 2 rings together in 1 clean 50 ml tube. Supplement the ring fractions with PBS to 50 ml.
- Centrifuge the tubes for 10 min at 1500 rpm at RT with brake.
- Discard the supernatant and resuspend the cells in 50 ml PBS.
- Centrifuge the tubes for 10 min at 1200 rpm at RT with brake.
- Discard the supernatant and resuspend the cells in 50 ml PBS.
- Centrifuge the tubes for 10 min at 900 rpm at RT with brake.
- When many erythrocytes are present, these can be removed by cell lysis. Resuspend the cells in 1 ml of sterile water before the last wash step, agitate gently for 20 seconds, add up to 50 ml PBS, and complete the washing.
- Discard the supernatant and resuspend the cells in 30 ml RPMI + 10% FCS + 1% PenStrep.

2. PHA stimulation of PBMCs

- Count the cells and transfer $1x10^6$ PBMC/ml in 75ml Flask with RPMI + 10% FCS + 1% PenStrep + 5 μg/ml PHA.
- Incubate at 37ºC, 5% CO_2 for 3-4 days.

3. Virus titration (ID50)

The medium used is RPMI 1640 supplemented with 10% FCS, 1% PenStrep and 200 units/ml recombinant interleukin-2 (IL2-medium).

Day 0

- Count the PHA-(3-4 days) stimulated PBMC.
- Prepapare virus dilution in IL2-medium in a sterile 24-flat bottom wells plates: five or six fivefold dilutions starting with a 1:5 dilution:
  First well: 400 µl medium + 100 µl virus supernatant
  Second to sixth well: 400 µl medium
  Take over 100 µl of supernantant from the first to the second well, and so on.
  Mix repeatedly at each passage
- Add 75µl of each virus dilution to five wells of a round-bottom 96-well culture plate.
- Add 10^5 PHA-stimulated PBMCs of minimum 2 donors diluted in 150 µl IL2-medium to each well.

Controls:

The negative virus controls consist in 3 wells virus (at the lowest dilution) but without cells and in 2 wells with cells but no virus.

Incubate at 37°C.

Day 1

Change 180 µl supernatant with fresh IL2-medium (in case of neutralization assay with polyclonal sera two washes of 180 µl each should be performed. First change supernatant, then centrifuge the plate and change supernatant again,).

Incubate at 37°C.

Day 3

Change 180 µl supernatant with fresh medium.

Incubate at 37°C.

Day 7

Harvest 160 µl of supernatant of each well for HIV-1 p24 Antigen determination.

4. HIV-1 p24 antigen ELISA assay

Analyze a sample from each well in an in house HIV-1 p24 antigen ELISA assay. See protocpol at www.aaltobioreagents.ie.

5. Calculation of the infected dose 50 (ID50)

The ID-50 is defined as the reciprocal of the virus dilution resulting in 50% positive wells by Reed-Muench calculation.

6. Neutralization assay

In each test, the ID50 must be repeated as to confirm the titration. In addition five replicate wells for each virus dilution used as TCIDs for the test should be included.

Day 0

- Count the PHA-stimulated PBMC.
- Prior to use, ensure that all test sera and control sera have been complement depleted by heat inactivation for 60 minutes at 56°C. Purified antibodies or peptide reagents do not need to be heat inactivated.
- Dilute antibody or sera directly in a 96-well round bottom plate in a total volume of 75µl medium. For sera use four steps of fourfold or six steps of twofold dilutions starting with a 1:10 dilution (final 1:20 dilution when virus is added).
- Each antibody/serum is tested in duplicate for each virus dilution.
- Each antibody/serum is tested with two virus dilutions corresponding to 20 and 40 TCID50, based on the ID50 performed.

Preparation of sera dilutions:

4-fold dilutions

First well: 90µl medium + 10µl serum

Second to fourth well: 75µl medium

Take over 25µl of supernatant from the first to the second well, and so on.

Mix repeatedly at each passage.

2-fold dilutions

First well: 135µl medium + 15µl serum

Second to sixth well: 75µl medium

Take over 75µl of supernatant from the first to the second well, and so on.

Mix repeatedly at each passage.

Controls:

One or two sera with high neutralizing titres are used as positive serum controls.

Preparation of virus dilutions:

- Calculate total volume of virus needed for each dilution to be used (Consider 75μl of virus dilution for each wells for each TCID50 tested).
- Prepare tubes for each virus dilution and add IL-2-medium.
- Thaw virus supernatant (not in water bath).
- As soon as supernatant is liquid, add adequate volume to the first tube.
- Mix well and transfer adequate volume to the second tube and so on.
- Add 75μl virus dilution in the corresponding wells.

Incubate the plate for 1 hour at 37°C.

Add 10^5 PHA-stimulated PBMCs of 2 or more different donors, diluted in 75μl of medium/well and incubate the plate at 37°C.

Day 1 and 3 change the medium as described for the virus titration assay.

Day 7

Harvest the samples and analyse a sample from each well in the HIV-1 p24-antigen ELISA assay (See point 4).

Advices:

To avoid evaporation and “microclima” problems:

Do not use the outer rows of wells of the plate, but fill those with some RPMI medium.

Wrap the culture plates with some transparent plastic paper or put them in plastic bags.

Result:

The neutralizing titer of a serum is usually defined as the reciprocal of the highest serum dilution giving a 90% reduction of infection.