Title: **PBMC Neutralization assay**

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Title: **PBMC Neutralization assay**

1. **Aim**

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. Two different protocols are in use for the PBMC neutralization assay, depending on whether viruses are tested for sensitivity to a polyclonal (serum) or monoclonal (antibody) neutralizing agent.

1.1. Definitions and abbreviations

IMDM : Iscove's Modified Dulbecco's Medium
PBMC : Peripheral Blood Mononuclear Cells
PBS : Phosphate Buffered Saline
TNC : Tri Sodium Citrate
PHA : Phytohaemagglutinine
FCS : Fetal Calf Serum
rIL2 : Recombinant interleukin-2
TCID50 : 50% Tissue culture infectious dose

2. **Preparation of frozen, pooled PBMCs**

PBMCs are isolated from buffycoats from 8 – 12 different HIV-negative healthy blood donors. PBMCs from each donor are isolated separately and are simultaneously tested for the presence of the CCR5 32bp deletion. PBMCs from donors homozygous for the 32bp deletion are excluded from the PBMCs pool.

- Transfer the buffycoat to a 250 ml flask and take a 200 µl sample for the CCR5 32bp deletion PCR. Add up the buffycoat to 200 ml with PBS supplemented with 10% TNC.
- Add 12.5 ml Ficoll to each of eight 50 ml tubes and add 25 ml of diluted buffycoat on top of the ficoll in each tube.
- Centrifuge the tubes for 20 min at 1000xg (acceleration 7, break 1) at RT.
- Remove the ring fraction on top of the ficoll with a pasteur pipet and add 2 rings together in 1 clean 50 ml tube. Supplement the ring fractions with PBS/ 10% TNC to 50 ml.
- Centrifuge the tubes for 15 at min 400xg (acceleration 9, break 7) at RT.
- Discard the supernatant and resuspend the cells in 50 ml PBS/ 10% TNC.
- Centrifuge the tubes for 10 min at 250xg (acceleration 9, break 9) at RT.
- Discard the supernatant and resuspend the cells in 50 ml PBS/ 10% TNC. Repeat the centrifugation and resuspension step until the supernatant is (almost) as clear as water.
- When many erythrocytes are present, these can be removed by cell lysis. Resuspend the cells in 1 ml ACK lysing buffer before the last wash step, agitate gently for 1 minute, add up to 50 ml PBS/ 10% TNC, and centrifuge the tubes for 10 min at 250xg (acceleration 9, break 9) at RT.
- PBMCs of different donors are pooled and frozen at a concentration of 50 million cells/ampoule (1.8 ml) in IMDM supplemented with 20% FCS and 10% DMSO.

3. **PHA stimulation of PBMCs**

- Thaw the PBMCs in IMDM supplemented with 20% FCS and centrifuge the cells for 10 min at 400xg.

- Resuspend the PBMCs in IMDM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), ciproxine (5 µg/ml) and PHA (200 ug/ml) to a concentration of 5 x 10^6 cells/ml.
- Incubate at 37ºC, 10% CO_2 for 3 days.

4. PBMC serum neutralization assay

Neutralization assays are performed in triplicate in 96-wells plates, using 20 TCID virus and 10^5 PBMCs per well, in IMDM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), ciproxine (5 µg/ml), rIL2 (20 units/ml), and polybrene (5 µg/ml). Two different protocols are in use, depending on the nature of the neutralizing agent (polyclonal or monoclonal). For polyclonal agents, smaller working volumes are used than for monoclonal agents. Moreover, a wash step is included in the polyclonal assay to reduce cytotoxic or other unwanted effects of the polyclonal reagent. In both protocols, virus production in culture supernatant at day 7 is analyzed by an in-house ELISA.

4.1.1. Polyclonal reagents

- Add 25 µl medium to rows B till H.
- Add 50 µl 2x neutralizing agent dilution to row A
- Prepare serial dilutions of the neutralizing agents by pipetting 25 µl from row A to row B, then mix, add 25 µl of row B to row C, mix, etc. up to row G from which 25 µl goes into waste.
- Add 25 µl of virus dilution (800 TCID/ml) to rows A till H.
- Incubate for 1 hour at 37ºC, 10% CO_2.
- Centrifuge the 3-day PHA stimulated PBMCs for 10 min at 400xg, resuspend to a concentration of 4 x 10^6 cells/ml and add 25 µl PBMC to rows A till H.
- Incubate for 4 hour at 37ºC, 10% CO_2.
- Centrifuge the PBMCs for 3 min at 400xg (acceleration 9, break 4), remove the medium and add 150µl PBS. Centrifuge the PBMCs 3 min 400g (acceleration 9 break 4), remove the PBS and add 150µl medium.
- Incubate at 37ºC, 10% CO_2.
- Analyse p24 production in culture supernatant at day 7 by ELISA.

4.1.2. Monoclonal reagents

- Add 50 µl medium to rows B till H.
- Add 100 µl 2x neutralizing agent dilution to row A
- Prepare serial dilutions of the neutralizing agents by pipetting 50 µl from row A to row B, then mix, add 50 µl of row B to row C, mix, etc. up to row G from which 50 µl goes into waste.
- Add 50 µl of virus dilution (400 TCID/ml) to rows A till H.
- Incubate for 1 hour at 37ºC, 10% CO_2.
- Centrifuge the 3-day PHA stimulated PBMCs for 10 min at 400xg, resuspend to a concentration of 2 x 10^6 cells/ml and add 50 µl PBMC to rows A till H.
- Incubate at 37ºC, 10% CO_2.
- Analyze p24 production in culture supernatant at day 7 by ELISA.