HIV-1 ISOLATION FROM INFECTED PERIPHERAL BLOOD MONONUCLEAR CELLS

All the reagents should be warmed at 37ºC before use.

Reagents and equipment

1. Whole blood from an HIV-1-seropositive individual.
2. Buffy coat from a healthy donor.
3. Phosphate-buffered saline without calcium and magnesium (PBS 1X)
4. Lymphocyte separation solution (Ficoll)
5. Phytohemagglutinine (PHA).
6. Recombinant interleukin 2 (IL-2).
7. RPMI-1640 with HEPES and L-glutamine
8. Penicillin plus streptomycin (P/S)
9. Fetal bovine serum (FBS)
10. Sterile pipettes (25 ml/10 ml/5 ml)
11. Micropipettes and tips.
12. Pasteur pipettes
13. Conical tubes (50 ml/15 ml)
14. Eppendorf tubes
15. Electric pipettor
16. Humidified 37ºC incubator with 5% CO_2
17. Sterile flask.
18. Cell counter/Neubauer chamber
19. Centrifuge
20. **Stimulation medium with PHA for PBMCs from healthy blood donor's buffy coat:** RPMI-1640 with HEPES and L-glutamine; 20% FBS (heat-inactivated at 56ºC for 30 minutes); 2 % P/S; PHA (5 µg/ml); IL-2 (2.5 ng/ml final concentration)
21. **Coculture medium for HIV-1 isolation:** RPMI 1640 with HEPES and L-glutamine; 20% FBS (heat-inactivated); 2% P/S; IL-2 (5 ng/ml final concentration)
22. **Storage medium:** 95% FBS + 5% DMSO

Methods

A. Isolation and stimulation of PBMCs from healthy blood donor's buffy coat.

1. Dilute the buffy coat with PBS (2 volume of buffy coat and 1 of PBS)
2. Prepare 50 ml conical tubes and add 10 ml of Ficoll per tube.
3. Add carefully over the Ficoll 20 ml of the diluted buffy to each tube.
4. Centrifuge the tubes at 1800 rpm for 40 minutes without break (accelerator 9-brake 1).
5. Remove the ring fraction with the PBMCs on top of the Ficoll with a sterile pipette and put it in a conical tube and supplement with 30 ml of PBS.
6. Centrifuge at 1500 rpm for 10 minutes (accelerator 9-brake 9).
7. Discard the supernatant and resuspend the cells in 20 ml of PBS.

8. Centrifuge at 1500 rpm for 10 minutes (accelerator 9-brake 9).
9. Discard the supernatant and resuspend the cells in 10 ml of RPMI-1640. Count the cells and adjust sample with stimulation medium to achieve a concentration of 2 million cells per ml.
10. Transfer 20 x 10^6 PBMCs in 10 ml of stimulation medium with PHA to a 75 ml flask.
11. Incubate at 37ºC, 5% CO2 for a maximum of three days.

B. Isolation of PBMCs from HIV-1-infected patient.

1. Centrifuge anticoagulated blood at 1360 rpm for 10 minutes to separate the plasma, which should be aliquoted and frozen.
2. Dilute the blood with PBS (2 volume of blood and 1 of PBS).
3. Prepare 50 ml conical tubes and add 10 ml of Ficoll per tube.
4. Add carefully over the Ficoll 20 ml of the diluted blood to each tube.
5. Centrifuge the tube at 1800 rpm for 40 minutes without break (accelerator 9-brake 1).
6. Remove the ring fraction with the PBMCs on top of the Ficoll with a sterile pipette, put it in a conical tube and supplement with 30 ml of PBS.
7. Centrifuge at 1200 rpm for 10 minutes (accelerator 9-brake 9)
8. Discard the supernatant and resuspend the cells in 20 ml of PBS.
9. Centrifuge at 1200 rpm for 10 minutes (accelerator 9-brake 9)
10. Discard the supernatant and resuspend the cells in 10 ml of RPMI-1640. Count the cells and adjust sample with coculture medium to achieve a concentration of 2 million cells per ml.

C. Coculture of PBMCs from HIV-1-infected patient and from healthy blood donor

1. Transfer 10x10^6 PBMCs from the patient in 5 ml of coculture medium to a 75 ml flask
2. Remove very carefully 6 ml of the supernatant of the flask with 1 to 3 days-stimulated PBMCs from healthy donor, leaving 4 ml medium with 20 x 10^6 PBMCs. Mix cells carefully.
3. Add 2 ml (10x10^6) stimulated PBMCs from healthy blood to the flask containing 10x10^6 PBMCs from patient and mix carefully.
4. Incubate at 37ºC, 5% CO2 for 3 hours.
5. Add coculture medium up to 10 ml and mix carefully.
6. Incubate at 37ºC, 5% CO_2.
7. On day 4, remove half the volume of supernatant, avoiding disturbing cells, and replace with fresh coculture medium.
8. On day 7, (a) mix supernatant and cells of the flask, put 5 ml containing 10x10^6 cells in a 15 ml. conical tube and centrifuge at 1200 rpm for 10 min; (b) save the supernatant at -80ºC in 1 ml aliquots; add 1 ml of storage medium to the pellet and store the cells at -80ºC during 24 hours and thenin liquid nitrogen; (c) remove half (2.5 ml) of the remaining supernatant of the flask (this should be done very carefully, in order to avoid disturbing cells at the bottom; (d) remove very carefully 6 ml of the supernatant of the flask with 1 to 3 days-stimulated PBMCs from healthy donor, leaving 4 ml medium with 20 x 10^6 PBMCs. Mix cells carefully; (e) add 2 ml (10x10^6) stimulated PBMCs from healthy blood to the flask; (f) add coculture medium up to 10 ml and mix carefully.
9. Incubate at 37ºC, 5% CO_2.

10. Repeat steps 6 and 7 during 28 days
11. Assay supernatants of days 7, 14, 21 and 28 for HIV-1 p24 antigen to assess HIV-1 replication

References

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