

PBMC Neutralization assay

National HIV Repository and Bioinformatic Center, Thailand

Neutnet code: 11

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1. Materials

RPMI 1640 powder	10	packs
Fetal bovine serum	10	bottles
P24 ELISA kit (Organon Teknika)	10	packs
QIAamp viral RNA mini kit (250 tests)	1	pack
HIV RG RT PCR (Artus) (24 tests)	10	packs
IL-2, PHA, Penicillin, Streptomycin		
96 well microtiter plate		
LightCycler Capillaries	3	packs
15 ml test tubes		

2. Methods

2.1 Preparation of donor PBMCs

The buffy coat is diluted with equal volume of PBS and then 30 ml of cell suspension is gently placed on the top of 15 ml Ficoll-hypaque in a 50 ml centrifuge tube. Centrifuge at 1,500 rpm for 20 min. The PBMCs at interface are collected and transferred to new tube and wash with RPMI 1640. After that, the PBMCs are added with PHA medium for adjust to 1×10^6 cells/ml. The PBMCs are maintained in tissue culture flask at 37 °C, 5% CO₂. After 2-3 days, the PHA medium is removed and replaced with IL-2 medium for 1 day before adding to HIV-1 co-culture or TCID₅₀ testing or neutralization test.

2.2 HIV-1 propagation and co-cultivation

Virus stock is prepared by infection of PHA stimulated PBMCs with 200 µl of HIV-1 positive supernatant. After overnight incubation, the infected PBMCs are washed with RPMI 1640. Then 4 ml of IL-2 medium are added and cultured in tissue

culture flask at 37°C, 5% CO₂. The p24 antigen is detected by ELISA kit (Organon Teknika) every week. The culture medium is harvested as soon as the p24 positive in high titer. Then TCID₅₀ is determined and stored the supernatant at -80 °C for neutralization test.

2.3 Tissue culture infectious dose (TCID₅₀)

200 µl of supernatant from HIV-1 co-culture is diluted by 5 fold dilution. The dilution is performed in 6 dilutions per one virus. Then 100 µl of each dilution are incubated with 100 µl of PHA stimulated PBMCs in 15 ml test tube at 37 °C, 5% CO₂ for overnight. Then wash with RPMI 1640 for 3 times and add 1 ml of IL-2 medium to each tube. After that, 200 µl of cell suspension are transferred to each 5 wells of 96-well microtiter plate and incubated at 37 °C, 5% CO₂. On day 4, the medium is changed and day 8, the culture medium is collected to measure p24 antigen. TCID₅₀ is calculated according to the

Spearman-Karber formula.

$$1/TCID_{50} = B^e, \quad e = E + d(0.5 - N/r)$$

e = the exponent for reciprocal titer

B = the base for reciprocal titer, B is the fold-dilution used in the dilution series, usually 5.

r = the number of repetitions (number of wells) for each dilution, usually 5.

E = the (-) highest exponent used in the dilution series

N = the summation of negative wells in the titration

d = the spacing or distance between dilutions, usually 1

2.4 Neutralization test (Infectivity reduction assay calculated by RNA quantitation measured by real time RT-PCR)

This method estimates neutralization of HIV-1 positive serum or plasma by fixing antibodies in serial dilutions of virus. Then the viral load of each dilution is obtained at day 2 by using real time RT-PCR kit (Artus). The ratio of TCID₅₀ of seronegative serum and test serum is calculated and reported.

This method for detection one virus and one antibody

1. Heat inactivated serum for 30 min at 56°C.
2. Dilute seronegative serum and test serum to 1:30, or antibody with IL-2 medium.

3. Dilute virus HIV-1 from culture to 100 TCID₅₀ with IL-2 medium for 1,400 µl. Then dilute virus HIV-1 by serial 2 fold dilution from 100 to 6.25 TCID₅₀.
4. Incubate 75 µl of various virus HIV-1 dilutions with 75 µl of seronegative serum or positive control serum or test serum/antibody in test tubes at 37°C, 5% CO₂ for 1 h.
5. Prepare 1 ml of 1.34x10⁶ cell/ml PHA activated PBMC in IL-2 medium.
6. Add 75 µl of prepared PBMC in all test tubes and incubate at 37°C, 5% CO₂ for overnight.
7. Wash 3 times with 10 ml of RPMI-1640.
8. Centrifuge and remove supernatant.
9. Add 400 µl of IL-2 medium in all test tubes and place 200 µl of suspension into 2 plates of 96-well plate. One is incubated at 37°C, 5% CO₂ for 2 days. The other plate is incubated for 8 days and change medium at day 4.
10. The supernatants from the first plate (2 days) are collected to RNA extraction by using QIAamp viral RNA mini kit (QIAGEN). Then 5 µl of RNA are used to determine viral load by real time RT-PCR. The real time RT-PCR is performed by using LightCycler (Roche) and using HIV 1 RG RT PCR (Artus) as reagent.
11. For the other plate (8 days), 100 µl of supernatants are collected and performed by using p24 ELISA test kit (Organon Teknika).
12. The values of viral load and p24 are collected and analyzed. The neutralizing index is expressed as a ratio of the TCID₅₀ obtained in the presence of HIV-1 seronegative over that obtained in the presence of test serum. The neutralizing index ≤ 3 is considered negative, while neutralizing index 3-9, 10-100 or >100 are considered as weak, medium and strong neutralizing activity, respectively.

2.5 Viral load detection by real time RT-PCR

140 µl of supernatant from neutralization test are extracted by QIAamp viral RNA mini kit (QIAGEN) and RNA is eluted by 60 µl of elution buffer. This RNA is collected and kept at -80 °C until use. 8 µl of RNA are used to determine viral load. 12 µl of master mix in HIV 1 RG RT PCR kit (Artus) and 8 µl of RNA are added in LightCycler capillary tube. Then RT-PCR is performed and using Taqman probe which specific for HIV-1 for detect viral load at channel F1/F2.

2.6 Reagents

1. RPMI 1640 medium

Dissolve 1 pack of RPMI 1640 powder in 950 ml of deionized distilled water by stirring. Add 2 g NaCO₃ and adjust pH to 7.2 with 1N HCl. Add deionized distilled water to 1,000 ml. Sterilize by filtering through 0.45 µm millipore filter. Store at 4 °C until use.

2. IL-2 medium

Mix 90 ml of RPMI 1640 medium, 10 ml of Fetal bovine serum, 100 µl of IL-2 (10,000 U/ml), 1 ml of L-glutamine, 200 µl of penicillin (50,000U/ml) and 200 µl of Streptomycin (50,000 U/ml).

3. PHA medium

Mix 90 ml of RPMI 1640 medium, 10 ml of Fetal bovine serum, 20 µl of PHA (2.5 mg/ml), 1 ml of L-glutamine, 200 µl of penicillin (50,000U/ml) and 200 µl of Streptomycin (50,000 U/ml).