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 $1x10^6$ cells/ml. The PBMCs are maintained in tissue culture flask at 37 ^oC, 5% CO_2. 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 $TCID_{50}$ 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 37ºC, 5% CO_2. 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 $TCID_{50}$ is determined and stored the supernatant at -80 ºC for neutralization test.

2.3 Tissue culture infectious dose ($TCID_{50}$)

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 ºC, 5% CO_2 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 ºC, 5% CO_2. On day 4, the medium is changed and day 8, the culture medium is collected to measure p24 antigen. $TCID_{50}$ is calculated according to the

Spearman-Karber formula.

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

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 $TCID_{50}$ 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 56ºC.
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 $TCID_{50}$ with IL-2 medium for 1,400 μl. Then dilute virus HIV-1 by serial 2 fold dilution from 100 to 6.25 $TCID_{50}$.
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% CO_2 for 1 h.
5. Prepare 1 ml of $1.34x10^6$ 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% CO_2 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% CO_2 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 $TCID_{50}$ obtained in the presence of HIV-1 seronegative over that obtained in the presence of test serum. The neutralizing index $\leq$ 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 $NaCO_3$ 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 ^oC 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).