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PBMC Co-culture and Plaque Reduction Assay Using HT4-6C Cells

I. PBMC Coculture Procedure

Materials

Culture Medium	RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5-10 U/ml IL-2
Cells	PBMCs (uninfected), PHA-stimulated (3 µg/ml) for 48-72 hours
Virus	HIV-infected PBMCs or culture supernatant
Inactivation Medium	5% Triton-X100 or special diluent buffer containing 0.5% Triton-X100 (used to inactivate virus samples for p24 assay)
Miscellaneous	15 ml centrifuge tubes, T-25 flasks

Procedure

Day 0 (Thursday)

1) To coculture HIV-infected PBMCs with uninfected PBMCs:

   a) Quickly thaw cells. Combine 5 x 10^6 uninfected cells with 5 x 10^6 infected cells in a 15 ml centrifuge tube. Centrifuge at low speed (500 rpm) for 10 minutes. Aspirate off freeze medium and resuspend the cells in 1 ml culture medium. Incubate at 37°C for 2-3 hours.

   For HIV-infected supernatant:

   b) Add 0.5-1.0 ml of supernatant to 5 x 10^6 uninfected PBMCs in a 15 ml centrifuge tube. Incubate at 37°C for 2-3 hours.

2) After incubation, add fresh culture medium to bring the volume of the culture to 5.0 ml, and transfer the culture to a T-25 flask.

3) Remove 300 µl of supernatant, and add 30 µl of inactivation medium to this aliquot for p24 testing. Incubate the T-25 culture flask at 37°C.

Day 4 (Monday)

4) Add 5 ml of culture medium with 10 U/m IL-2 to the T-25 flask to bring the volume to 10 ml. Incubate at 37°C.

Day 7 (Thursday)

5) Collect 5 ml of medium into a 15 ml centrifuge tube, and remove 300 µl from this aliquot for p24 testing. Add 30 µl of inactivation medium to the p24 aliquot. Store the 4.7 ml aliquot at -80°C.

6) Add 5 ml of culture medium containing 10 U/ml IL-2 and 5 x 10^6 uninfected PBMCs to the T-25 flask, bringing the total volume back to 10 ml and the cell count to 10 x 10^6. Incubate at 37°C.

Day 11 (Monday)

7) Collect 5 ml of medium into a 15 ml centrifuge tube, and remove 300 µl from this aliquot for p24 testing. Add 30 µl of inactivation medium to the p24 aliquot. Store the 4.7 ml aliquot at -80°C.

8) Add 5 ml of culture medium containing 10 U/ml IL-2 to the T-25 flask to bring the volume back to 10 ml. Incubate at 37°C.

Day 14 (Thursday)

9) Resuspend the culture, and collect 5 ml of the cell suspension into a 15 ml centrifuge tube. Remove 300 µl from this aliquot for p24 testing. Add 30 µl of inactivation medium to the p24 aliquot. Store the 4.7 ml aliquot at -80°C.

10) Add 5 ml of culture medium containing 10 U/ml IL-2 and 5 x 10^6 uninfected PBMCs to the T-25 flask, bringing the total volume back to 10 ml and the cell count back to 10 x 10^6. Incubate at 37°C.

Day 18 (Monday)

11) Repeat steps 7-8.

Day 21 (Thursday)

12) Repeat steps 9-10.

Harvest

13) Samples with p24 values >20,000 pg/ml can be harvested and stored at -80°C. To harvest cells:

    a) Clarify the supernatant by centrifugation (2000 rpm, 10 minutes), and store as 0.5-1.0 ml aliquots.

    b) Alternatively, collect the supernatant into a 15 ml tube, freeze, then clarify and aliquot

the supernatant at a later time.

Comments and Suggestions

1) Cultures with p24 values <100,000 pg/ml should be cultured a minimum of 28 days.

2) If a culture exhibits low p24 levels, start a new culture from the 4.7 ml supernatant aliquot with the highest p24 value. Use 2 ml of supernatant, add 10 x 10^6 uninfected PBMCs, and bring the culture volume to 10 ml with fresh culture medium.

3) The stored 4.7 ml aliquots from cultures that have p24 values >200,000 pg/ml can be aliquoted as described in step 13 for use at a later date. If desired, samples can be pooled for harvest, and re-titered.

II Plaque Assay

Materials

Cells	HeLa CD4-Clone 6C (HT4-6C) Cells
Detachment Medium	1X Trypsin-EDTA (Gibco #25200-015)
Geneticin	G418 Sulfate (Gibco #860-1811), 10 mg/ml stock solution
Plaque Assay Medium	DMEM supplemented with 4% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 8 µg/ml DEAE Dextran, 0.5 µg/ml Polybrene
Cell Maintenance Medium	DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine
Titered Virus	Virus must be titered to determine the dilution that gives 100-200 plaques in the "NO DRUG CONTROL" wells.
Crystal Violet Solution	Add 5 grams crystal violet to 995 ml water and let sit overnight. The next day, filter the solution (Reeve Angel 802)
Miscellaneous	24-well tissue culture plates (Falcon #3047); 100% MeOH (cell fixative); Test drug(s)

Procedure

1) Culture HT4-6C cells in cell maintenance medium. The cells should be split into 75 ml flasks each week, with 3 x 10^4 cells/ml and 100 µl geneticin in a total volume of 20 ml per flask.

2) To perform the plaque assay, trypsinize the cells using detachment medium at 37°C for 5-10 minutes. Centrifuge the cells at 1000 rpm for 10 minutes, and resuspend in 5-10 ml maintenance medium.

Day 0

3) Evenly plate 1 ml of cells (2.5-3 x 10^4 cells/ml) into a 24-well culture plate. Incubate the cells at 37°C.

Day 1

4) Aspirate off medium and quickly replace with 200 µl/well titered virus. A virus dilution that gives 100-200 plaques in the "NO DRUG CONTROL" wells should be used. Incubate 2 hours at 37°C.

5) Add 800 µl of test drug at 1.25X concentration. Incubate 3 days at 37°C.

Day 4

6) Aspirate off the medium. Fix the plates by submerging them in 100% MeOH for 10-15 minutes.

7) Remove the plates from the MeOH and rinse once with water.

8) Stain the wells with 0.25 ml of 5% crystal violet for five minutes, then rinse the plates with water and air dry. The plates can now be read.

Suggestions for Plaque Assay

1) The cell concentration is determined by how well the cells grow in the incubator. If the incubator door is opened frequently, growth will be slower.

2) If the cells are too densely plated, the plaques will be obscured. It is important to evenly distribute the cells in the wells.

3) After aspirating medium from the wells, add the virus immediately to prevent the cells from drying out. It may be necessary to aspirate the medium from only a few wells at a time.

4) Always use a NO VIRUS CONTROL to familiarize yourself with the appearance of a true plaque.

5) Count the large multinucleated and usually darker stained cells. If you are not certain about a plaque, don't count it.

6) The virus strain used in the assay is important. Screen lab stocks of virus for those that create the largest plaques. Virus isolates from patients may have smaller, more difficult to read plaques.