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CEM-SS Microtiter Syncytial-Forming Assay For the Detection of Neutralizing Antibody or Antiviral Compounds Against HIV-1 and HIV-2

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Materials:

RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% PSN antibiotic mixture (100X), Gibco Labs, Life Technologies, Chagrin Falls, OH 44022

96-well flat bottomed plates, Costar, catalog #3569

Poly-L-Lysine Hydrobromide (PLL), Sigma, catalog #P-1399

DEAE-Dextran, Pharmacia

CEM-SS cells

CEM-SS cells have been biologically cloned for the following characteristics: 1) a monomorphic cell population capable of forming a loosely adherent monolayer in poly-L-lysine treated microtiter wells, 2) their ability to retain viability and become fusogenic following viral infection and thus form large, viral-induced syncytia. These cells are transformed and occasionally go through spontaneous periods of karyocytomegaly. This can last for 1-2 weeks in culture. During this period the cells may approach 3 to 4 cell diameters (40-50 u).

HIV-1 and HIV-2 Infectivity Assay

Neutralization procedure: (Allow five hours. Split cells 1:2 24 hours prior to experimentation.)

1. Run the following procedures simultaneously:

    a. PLL treat 96-well plate. Prepare PLL at 50 μg/ml in sterile distilled water. Use 50 μl/well to coat plate. Incubate 1 hour at room temperature.

    b. Inactivate serum sample (50 μl) at 56°C for 30 minutes.

    c. (This step is optional, depending upon assay requirements). DEAE-D treat logarithmically growing CEM-SS cells. Count cells; centrifuge at 1500 RPM for 5 minutes and resuspend cell pellet in DEAE-D at 25 μg/ml in RPMI 1640. Incubate 30 minutes at 37°C. Centrifuge at 1500 RPM for 5 minutes and resuspend cell pellet in medium at a concentration of 1×10^6 cells/ml. Make 10 ml per plate - no additional wash is required.

2. Prepare serum dilutions:

    Prepare a series of 50 μl dilution blanks with RPMI 1640 and make doubling dilutions by adding 50 μl of medium to the heat-inactivated serum sample. Allow extra 50 μl blanks for positive virus controls.

3. Neutralization:

    Add 50ul of test stock virus (100-200 syncytial forming units) to each of the doubling dilution serum samples and to the medium-only controls. Incubate at room temperature for 30 minutes.

4. Prepare 96 well plate:

Wash wells twice with 1x PBS and follow by pipetting 50 µl of CEM-SS cells into each well (50,000 cells/well if HIV-1 is to be tested; 75,000 cells/well if HIV-2 is to be used). Avoid introducing bubbles into the cell suspension - they will not pop during incubation and will cause severe disruption of cell distribution. Allow cells to attach at least 15 minutes at 37°C.

5. Infection: (Do not use an aspirator or multi-channel pipettor from this point on!):

Gently remove medium from the wells by tilting the plate and pipetting from the corner of each well to minimize accidental removal of cells from the monolayer. Remove medium from one row at a time to avoid dehydration of the cells. Immediately replace the medium with the 40-45 µl duplicate samples of the incubated virus-serum mixture. Wells should be pre-labeled for easy sample identification. Incubate the plate for 1 hour at 37°C.

6. Conclusion of infection:

Gently remove the virus-serum inoculum from the wells, taking care not to disturb the cell monolayer. Feed each well with 100 µl of fresh complete medium; the addition of Mycostatin (Nystatin) at 5 µl/ml final concentration (0.5%) is optional. Add the medium gently down the side of the well to avoid disrupting the monolayer.

7. Syncytia formation period:

Allow approximately 4-5 days for the formation of HIV-1 syncytia and 3 days for HIV-2. Feed each test well with an additional 100 µl of fresh medium on an intermediate day of the incubation. Count all syncytia in the wells containing virus stock controls. Take the mean number of syncytia derived from the other duplicate antibody/virus (Ab/v) treated wells and place in the formula:

mean number of syncytia in (Ab/v) wells = [V_n]

mean number of syncytia in virus control wells = [V_o]

Plot V_n/V_o (Y axis) vs. descending concentrations of serum (X axis) on log or semi-log paper

Calculation of the 90% inactivation point:

V_n = total number of HIV-1 induced syncytia produced under experimental conditions, i.e., serum, anti-viral peptides, etc.

V_o = total number of HIV-1 induced syncytia added to each microtiter well.

V_n/V_o = this ratio represents the virus surviving fraction at that serum dilution.

Serum Dilution	V_n	V_o (mean)	V_n/V_o
1:2	0,1	100	0.005
1:4	1,2	100	0.015
1:8	4,6	100	0.050
1:16	13,12	100	0.125

1:32	27,29	100	0.280

HIV-1 and HIV-2 Infectious Cell Center Assay (ICC):

1. Prepare and plate CEM-SS cells as previously stated, using 100,000 cells per well.

2. Transformed T-lymphoblastoid cells (i.e. H9, CEM, HUT-78, etc.) infected with HIV that are to be tested for evidence of virus production and/or fusogenic potential are added to the CEM-SS cells in incremental amounts starting with 100 and 1,000 cells. Primary peripheral blood lymphocytes or single cell suspensions of tissues can also be plated onto the CEM-SS cells, but because these preparations contain lower numbers of infected cells they should be used at higher concentrations per well (1,000, 10,000,...) than infected T cell lines. As many as 2 x 10^6 cells are evaluated from humans or chimpanzees.

3. Cells used in the ICC assay are evaluated 18-24 hours after plating out. Infected cells are visible as large, well-demarcated syncytial cells. The entire well is counted and expressed as # of virally infected cells/total # of cells plated.

Miniassay Modification for Samples in Short Supply:

Step 2 of assay procedure: Use 15 µl instead of 50 µl for medium blanks.

Step 3: Use 15 µl of virus instead of 50 µl; adjust concentration upward to compensate for reduced volume.

Step 5: Add 70 µl of medium to each neutralization well just prior to infecting CEM-SS cells.

Calculations:

15 µl undiluted serum +15 µl virus =30 µl	room temperature incubation	1:2 serum concentration noted in protocol refers to this incubation period only; it is not the final concentration
+70 µl medium =100 µl for infection		
1:2→1:1.67	It follows that:	1:4 1:13.3 1:8 → 1:26.7 1:16 → 1:53.3 1:32 → 1:106.7 1:64 → 1:213.3 1:128 →1:426.7

References:

Nara, P.L., Hatch, W.C., Dunlop, N.M., Robey, W.G., and Fischinger, P.J. Simple, rapid, quantitative micro-syncytium forming assay for the detection of neutralizing antibody against infectious HTLV-III/LAV. Abstract #31, p. 15. Third International Conference on Acquired Immunodeficiency Syndrome (AIDS), Washington, D.C., June 1-5, 1987.

Nara, P.L., Hatch, W.C., Dunlop, N.M., Robey, W.G., and Fischinger, P.J. Simple, rapid, quantitative micro-syncytium forming assay for the detection of neutralizing antibody against infectious HTLV-III/LAV. *AIDS Res Hum Retroviruses* **3**:283-302, 1987.

Nara, P.L. and Fischinger, P.J. Quantitative assay for HIV-1 and -2. *Nature* **332**:469-470, 1988.

Please refer all questions concerning the assay to Dr. Peter L. Nara, MS, DVM, PhD, and Ms. Nancy Dunlop, BS, MA at (301) 846-1335 (Frederick, MD, USA).

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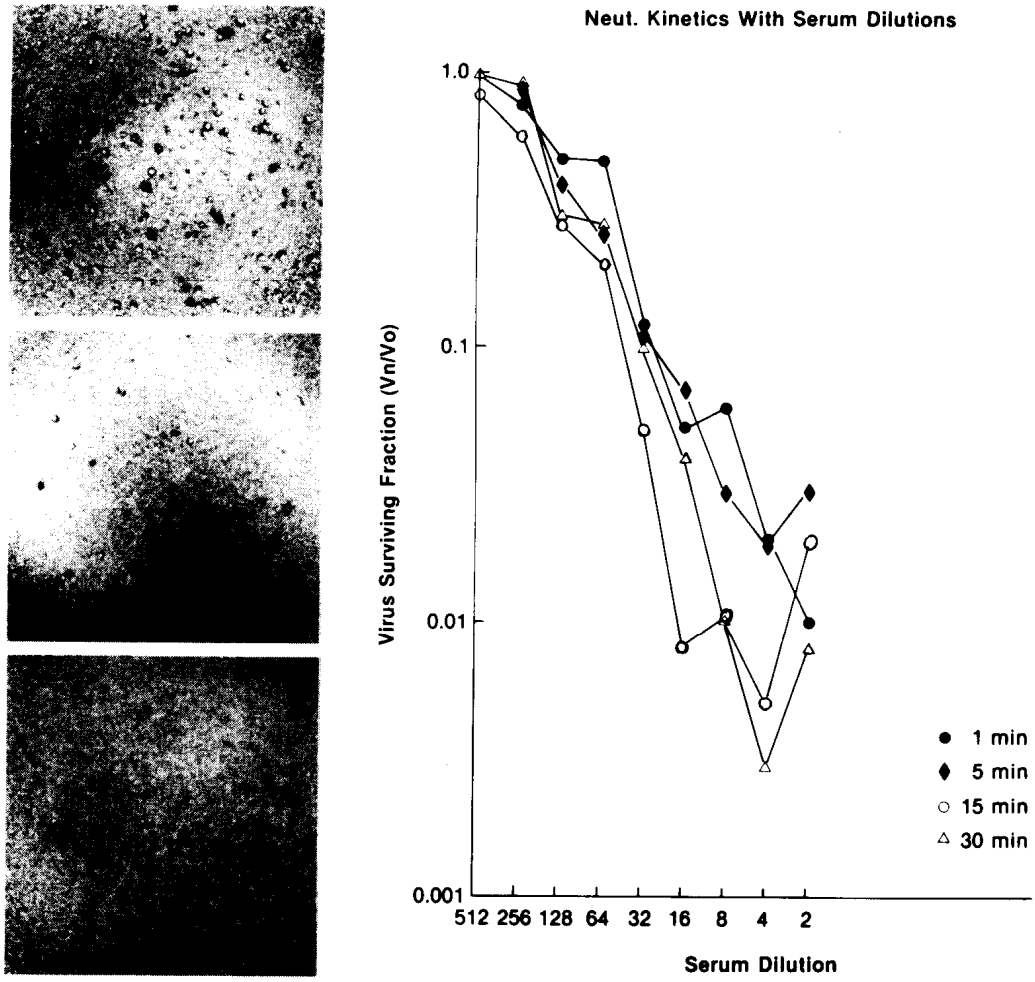

Figure 4–2 Neutralization kinetic analysis of an HIV-1-infected human [9]. Approximately 200 syncytial-forming units (SFU) of virus were mixed with various dilutions of antisera (1:4 to 1:512) for the indicated periods of time (i.e., 1 minute, 5 minutes, etc.) after which the serum/virus mixtures were added to preplated CEM-SS target cells for one hour. Virus/serum mixtures were removed, and media were added back to the microtiter wells. The 90% and 99% points of neutralization are represented at a V_n/V_o of 0.1 and 0.01 on the Y axis, respectively. Photomicrographs are representative of the HIV-1 induced syncytia present in the microtiter wells as V_n/V_o = 1.0 (top), V_n/V_o = 0.1 (middle) and V_n/V_o = 0.01 (bottom). Approximately 75% of the well is displayed (20x).