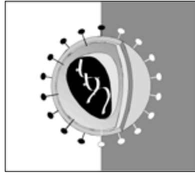

NIH AIDS Research & Reference Reagent Program
20301 Century Boulevard
Bldg. 6, Suite 200
Germantown, MD 20874
USA

Phone: 240 686-4740
Fax: 301-515-4015
www.aidsreagent.org

Focal Immunoassay (FIA) to Detect HIV

Dr. Bruce Chesebro
National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT 59840

The protocol described below utilizes several reagents available through the NIAID AIDS Research and Reference Reagent Program.

Reagents

HeLa-CD4 Cells	HeLa Clone 1022 (Repository Catalog #1109) and/or HeLa CD4 Clone 6C (Repository Catalog #459)
TNE	0.01 M Tris HCl pH 7.5, 0.15 M NaCl, 0.002 M EDTA
TNE-FBS (or PBS-FBS)	TNE (or PBS) containing 1% fetal bovine serum (FBS)
Primary Antibody	Monoclonal anti-HIV Gag (supernatant from Hybridoma H12-5C, Catalog #1513)
Secondary Antibody	HRP-conjugated goat anti-mouse or anti-human immunoglobulin (Cappel) in TNE-FBS or PBS-FBS. Dilution is usually 1:500, but this depends on the individual serum lot.
Peroxidase Substrate Stock	3-amino-9-ethyl-carbazole (AEC, Sigma) in dimethyl formamide (4 mg/ml). Store solution at -20°C in the dark. **NOTE:** AEC is a carcinogen. Use mask and gloves when weighing powder, and dispose of solutions properly.
Sodium Acetate/Acetic Acid Buffer	50 mM sodium acetate-acetic acid buffer, pH 5.0
Other Reagents	Various tissue culture reagents; DEAE-Dextran; Methanol; 95% ethanol; 30% H_2O_2

Preparation of Cells:

1. Maintain HeLa CD4 cells (Catalog # 1109) by serial passage every 4-6 days at 1:5-1:10 dilutions; maintain Catalog #459 at 1:20 dilution. For HIV infectivity assays, use cells from a **subconfluent** flask (<$3x10^6$ cells per 25 cm^2 flask). Seed target cells (Catalog #1109) into tissue culture wells at low density, i.e., 1 x10^5 cells/35 mm well in 4.0 ml medium, or 4.5 x 10^4/TC24 well, or 2 x 10^4/TC48. Use on half of this number of cells for #459.

2. Next day infect cells. For assaying infected cells from lymphoid suspension cultures, seed various dilutions of washed cell suspension directly into the medium. Next day remove cells by aspirating medium, and refeed dishes. For assaying cell-free virus, remove medium, add DEAE Dextran (8 µg/ml in serum-free medium), incubate 20 minutes at 37°C, remove fluid, add serum-free medium, and remove the medium when ready to infect cells. (**NOTE:** The cells must never be allowed to dry out during these procedures.) To infect, add fluid containing serial dilutions of virus in medium with 0.1% FBS to the wells (higher protein concentrations are inhibitory). Use 500 µl per 35 mm well, 200 µl per TC24 well, or 100 µl per TC48 well. Two hours later, add medium with 10% FCS. Do not substitute polybrene for DEAE-Dextran as it is inhibitory on these cells.

Focal Immunoassay Procedure:

Three days after infection (cells will be subconfluent), remove medium and perform focal immunoassay as follows:

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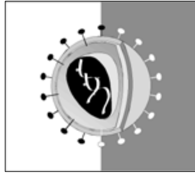
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1. Fix with methanol or 95% ethanol (1.5 ml per well) for approximately 5 minutes.

2. Pour off fixative and immediately rinse twice with TNE. **Do not allow the cells to dry** between the fixative and TNE steps. The dishes may be stored for several days at 4°C at this point.

3. Rinse once with TNE-FBS (or PBS-FBS). Add primary antibody, 100-200 µl per well. Incubate 45 minutes at room temperature, wash twice with TNE-FBS (or PBS-FBS).

4. Add appropriate secondary antibody (100-300 µl per well). Incubate 45 minutes at room temperature.

5. Rinse twice with TNE-FBS (or PBS-FBS).

6. Just prior to use, add 1 volume of peroxidase substrate stock to 19 volumes of sodium acetate-acetic acid buffer. Then add 1 µl of 30% H_2O_2 per 2 ml of solution, mix and add 1-2 ml to each well. Incubate 30 minutes at room temperature in the dark under a foil cover.

7. Rinse with water, allow to dry, and then count foci with a dissecting microscope at 10-40X total magnification (we use a Nikon SMZ 10 microscope). Use of light reflected off a dull surface, such as white paper, instead of a mirror will reduce the prominence of the highly refractile peroxidase-negative uninfected cells. Use of a standard inverted tissue culture microscope with 4X objective and 8-10X eyepieces gives very poor visualization of these foci on dry tissue culture plates, and is not recommended.

Screening AIDS Patient Sera for Use in FIA:

1. Add NP40 to serum to give a final concentration of 1%. Incubate 30 minutes at room temperature to inactivate HIV. The serum may be removed from AIDS lab at this time.

2. Dilute 1:10 with TNE and filter through a 22 µm filter to remove residual protein aggregates.

3. Dilute with TNE-FBS (or PBS-FBS) to final dilutions of 1:100, 1:300, 1:900, and test as primary antibody in FIA on MeOH fixed HIV-infected CD4-positive HeLa cells. For the secondary antibody, commercial HRP-conjugated anti-human antibody preparations are routinely used at 1:300-1:500 dilutions.

4. Select best sera for continued use and keep sterile at 4°C or frozen. Good sera should have no background on uninfected cells and show strong staining of HIV-infected cells at 1:300-1:900 dilutions. In our experience about 20% of AIDS patient sera are very good. The others either have a high background on uninfected cells or have a low titer, showing only weak staining of infected cells at a 1:100 dilution.

Detection of CD4 Antigen on HeLa-CD4 Cell Lines:

Detection of CD4 antigen on clone 6C or 1022 cells also can be done using indirect immunofluorescence on cells in suspension followed by fluorescence microscopy or flow cytometry. Cells are removed from plastic dishes by brief (7 minutes) trypsin treatment at 37°C. Trypsin is neutralized with 20% FBS. Cells are filtered through nylon mesh, then washed into PBS containing 1% FBS and 0.01 M sodium azide. Cells are kept on ice and in the presence of azide during subsequent immunological reactions. The cells are reacted with anti-CD4 monoclonal antibody (OKT4A, OKT4, or anti-Leu 3A) for 30 minutes, washed twice, reacted with FITC-conjugated anti-mouse immunoglobulin, washed 2-4 times, fixed with 1% formaldehyde in PBS,

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and then examined by fluorescence microscopy, with 10-40% of the cells appearing positive with varying levels of intensity.

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