Virus Neutralization Laboratory SOP
PLAQUE REDUCTION ASSAY

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I. Purpose

Research on HIV vaccines as well as studies on HIV pathogenesis in human and SIV in the macaque model, require the availability of simple and standardized assays for quantification of neutralizing antibodies to primary virus isolates. We describe here plaque reduction assays that have high specificity, sensitivity and reproducibility, and are simple to perform.

II. Scope

We have recently developed and standardized assays using human cell lines engineered to express CD4 and co-receptors for HIV and SIV entry. One cell line originated from a glioma (U87) and the other from an osteosarcoma (HOS). Both cell lines and their derivatives form monolayer cultures, a prerequisite for counting plaques. HIV-infected U87.CD4-CCR5 or –CXCR4 cells form syncytia, that is, plaques that can be stained with hematoxylin and enumerated by light microscopy. In addition to CD4 and co-receptors (most often used CCR5 and CXCR6 by SIV), GHOST(3) cells have been engineered to express the green fluorescent protein following virus infection. Infected cells show green fluorescence and can be enumerated by fluorescence microscopy. Neutralization is determined by the ability of a serum to reduce the number of plaque forming units (PFU) relative to controls exposed to medium or negative serum. Both assays are run in microtiter format and neutralization is evaluated after 3 days. Intra-assay variation has been used for estimation of the cut-off for neutralization.

III. Equipment

A. Biological safety cabinet class II (placed in a Biological Level-3 containment)
B. Humidified 37°C incubator with 5% CO_2.
C. Electric Pipettor (Drummond PipetteAid, VWR)
D. Adjustable pipettes (Finnpipettes)
E. Microscope (inverted light and fluorescence microscope)
F. Hemacytometer

IV. Materials

A. Disposable plastic pipettes (Sarstedt)
B. 1-1000 pipette tips (Finntips)
C. 15 and 50 ml conical centrifuge tubes (Techno Plastic Products, TPP)
D. Tube racks
E. 48-well or 96-well (for U87.CD4 or GHOST(3) cells, respectively) flat-bottom microtiter plates with lid.
F. Gloves, protective sleeves etc. according to safety regulations for the BL-3 containment.
G. Autoclavable boxes, autoclave bags and tape.

V. Reagents

A. U87.CD4-CCR5 or -CXCR4-expressing cells
B. GHOST(3)-CCR5 or -CXCR4-expressing cells. GHOST(3) cells also carry the HIV-2 LTR driven green fluorescent protein (GFP) marker which becomes activated upon infection with HIV-1 (HIV-2 or SIV). The cell lines are available from the repositories in the USA and UK.
C. Dulbecco's Modified Eagle's Medium (DMEM) and DMEM (High Glucose) (Gibco) supplemented with 7.5% or 10% (for GHOST(3) or U87.CD4 cells, respectively) fetal calf serum (FCS, FDA approved, Boule Nordic AB) and antibiotics (final concentration: penicillin 50U/ml and streptomycin 50µg/ml). (Invitrogen)
D. EDTA 5mM pH8. (Merck)
E. Polybrene 2µg/ml final concentration. (Sigma)
F. Phosphate-buffered saline (PBS).
G. Hematoxylin (Merck)
H. Normal human serum or plasma (negative control)
I. Serum or plasma from selected HIV-1 positive individuals (positive control)
J. Test reagents: Virus stocks of HIV or SIV isolates produced in peripheral blood mononuclear cells (PBMC).
K. Sera, plasma or the IgG derivative, and/or monoclonal antibodies.
L. Trypan blue 0.4% (Merck)
M. 70% ethanol

VI. Procedures

The methods described herein outline two plaque reduction assays for measurement of neutralizing antibodies to HIV. Both assays utilize human cell lines engineered to stably express CD4 and coreceptors for HIV entry. Plaques are composed of virus infected cells and are scored as syncytia by light (U87 series) or fluorescence microscope (GHOST(3) series).

Virus has to be titrated prior to performing the assay, since neutralization is dependent on the dose of input virus (Shi et al. 2002). In the present series of experiments we used 40-50 plaque forming units (PFU) per well (recommended range 10-100 PFU/well). (See SOPs Nordqvist & Fenyö 2005).

The neutralization assay is similar in the two indicator cell lines and therefore it will be described once. It has to be remembered, however, that there are differences in the handling of U87.CD4 and GHOST(3) cells. These differences are highlighted in Table 1.

Table 1. Overview of the methodology of the two assays.

Cells	U87.CD4 & coreceptors	GHOST(3) & coreceptors
Format	48-well plate*	96-well plate
Number of cells/well	$2 \times 10^4 - 4 \times 10^4$	4.8×10^3
Volume/well of serum-virus mixture added	200 µl	150 µl
Readout	Plaques: *Syncytial cells* Counted by light microscopy after staining with hematoxylin	Plaques: *Green cells* Counted by UV microscopy (fluorescence can be quantified by flow cytometry)

* The wells of 48-well plates provide a larger area than 96-well plates and allow convenient reading of plaques

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A. Day -1: **Seed cells in microtiter plates** according Table 1. Cultures should have reached half confluence when infected.

B. Day 0: **Set up the assay**

1. Mix serum or plasma (45 µl) or antibody and virus stock (usually 90 µl) in a sterile capped tube or 24-well (alternatively, 48-well) microtiter plate and dilute with D-MEM to a total volume of 900 µl. This results in a final dilution factor of 1/20 for the serum and 1/10 for the virus. Use a separate tube for each antibody concentration tested.
2. Incubate the serum-virus mixture at 37°C for 1 hr.
3. Prepare further dilutions of the serum-virus mixture in two or three 5-fold dilution steps in the following way:
   - Add 800 µl of medium to six (or nine) wells.
   - Add 200µl of the serum/antibody-virus mixture to the first triplicate wells (1/5 dilution)
   - Change tip, mix and transfer 200 µl to the adjacent well containing 800µl of medium (a 1/25 dilution), and so on. Ensure thorough and consistent mixing of each well and replace tips between dilutions.
4. Distribute aliquots (see Table 1 for volume) of serum/antibody-virus mixtures, "stock" and dilutions, into triplicate wells containing the cells. Remove medium from one row at the time and add serum-virus mixtures (virus or medium) immediately. Care must be taken that the cell monolayers do not dry out during this procedure.
5. .Control wells in triplicate:
   i. Positive virus controls consist of wells with cells and virus but no serum and/or HIV/SIV negative serum;
   ii. Cell controls consist of wells with cells only;
   iii. Positive serum control consists of wells with cells + virus + serum with known neutralizing activity.
6. Incubate overnight at 37°C with 5% CO_2 in a humidified incubator.

C. Day 1:

1. **Wash** plates by rinsing once with DMEM (without FCS) (500 µl/well for 48-well plates and 200 µl/well for 96-well plates).
2. Add 1000 µl or 200 µl, respectively, of fresh DMEM and incubate plates further.

D. Day 3: **Readout**

U87-CD4 cells:

1. Wash plates by rinsing two times with PBS (500 µl/well).
2. Fix with methanol:acetone (1:1) for 3-5 minutes.
3. Stain with hematoxylin for 2-3 minutes, wash with tap water and air dry. Hematoxylin stains cell nuclei, which turn dark blue after contact with tap water.
4. Count syncytial cells under a light microscope. The number of plaques (distinct grouping of syncytial cells is counted in wells infected with the lowest virus dilution that allows identification of individual plaques, that is, in a dilution that produces 20-30 plaques per well. Virus titers are calculated as plaque-forming units (PFU) per milliliter =

$$\frac{\text{average number of plaques in triplicate wells X virus dilution}}{\text{volume in the well*}}$$

*Refers to the volume of the virus added, either 200 µl or 150 µl (cp. B4 above).

GHOST(3) cells:
Count fluorescent cells in a fluorescence microscope. Fluorescence can be observed 48 hours after infection, peaks at 72 hours and decays thereafter. The number of plaques (distinct grouping of syncytial cells or single cells that show green fluorescence) is counted in wells infected with the lowest virus dilution that allows identification of individual plaques, that is, in a dilution that produces 20-30 plaques per well. For calculation of plaque-forming units per milliliter (PFU/ml) see above.

E. Calculate the neutralizing capacity of the serum by the formula:

(PFU with serum/PFU without serum) x 100

The neutralizing capacity of a serum is thus expressed as the degree of reduction in plaque-forming units in the presence of serum relative to wells with no serum.

F. Calculation of cutoff value for neutralization

A major concern was the reproducibility of the virus titer and its implication for the cutoff value of neutralization. Because both neutralization assays are based on plaque reduction in the presence of serum, the intra-assay variation is important (Shi et al. 2002). To establish the accuracy of the intra-assay variation of virus titer determinations, three assays were performed on the same day. Fifteen serum-virus combinations were tested on U87.CD4-CCR5 cells and four serum-virus combinations on GHOST(3)-CCR5 cells. We calculated the percent difference for each individual determination relative to the mean of the three repeat determinations. The range of differences gave a SD±9.1% for U87.CD4-CCR5 cells and SD±9.66% and SD±9.89% (for negative and positive sera, respectively) for GHOST(3)-CCR5 cells (Nordqvist & Fenyö 2005). On the basis of these data we chose a cutoff for neutralization (i.e. plaque reduction) of 30%, which represents 3.3 or 3.1 standard deviations in assays performed in the two different indicator cell systems. By using this cutoff the risk of falsely calling a serum neutralizing should be less than 1%.

G. Comments

1. U87.CD4 and GHOST(3) cell lines have been engineered to stably express CD4, co-receptors for HIV and, in the case of GHOST(3) cells also the green fluorescent protein (GFP) (Deng et al 1996, 1997). The cell lines are available from the repositories in the USA and UK (http://www.aidsreagent.org, http://www.nibsc.ac.uk/catalog/aids-reagent). Stability of the different markers is, however, highly variable. It is therefore mandatory that upon receipt of the indicator cell lines, each laboratory freezes a large stock of cells (at least 10 tubes each). Continuous passage of any of the U87.CD4 cell series should not exceed two months. After this time the cultures have to be discarded and a new tube from the stock thawed out. GHOST(3) cells grow faster than U87.CD4 cells and may be even more prone to lose markers. It is therefore advisable to thaw out the cells shortly before use for experiments (2-3 days before). The cell lines may then be used for a maximum of six weeks.

It is important to include control viruses with defined co-receptor usage in each experiment. Using the same virus stock, co-receptor usage patterns (also time to syncytium induction in U87.CD4 cells and the proportion of cells showing fluorescence in GHOST(3) cells) should be highly repeatable between experiments. Another way of testing receptor expression is by flow cytometry using monoclonal antibodies to CD4 or the chemokine receptors (anti-CCR3, -CCR5, -CXCR4 and –CXCR6 are commercially available) (Vödrös et al 2001).

2. Should the proportion of receptor positive cells decrease with time the cell lines may be re-selected by culturing in selective medium for two weeks. Expression of the different markers can be selected according to the following scheme:

- CD4 - 300 µg/ml of G418 for U87.CD4 and 500 µg/ml for GHOST(3)
- CCR1, CCR2, CCR3, CCR5, CXCR4, BOB, Bonzo - 1 µg/ml puromycin
- GFP - 100µg/ml Hygromycin for GHOST(3) (reduce to 50µg/ml if cells appear too sensitive)

Following selection passage cells at least once in DMEM 10% (or DMEM 7,5% for GHOST(3) cells), then freeze stocks and use for experiments.

3. GHOST(3) cells show a background expression of CXCR4. Virus isolates able to use this receptor show a background expression at various levels more or less across the entire panel of GHOST(3) cells. Use of a specific receptor need sometimes be verified by using the CXCR4 antagonist, AMD3100 (Vödrös & Fenyö 2005).

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