Published in final edited form as:

Curr Protoc Cell Biol. 2009 June ; CHAPTER: Unit-26.5. doi:10.1002/0471143030.cb2605s43.

HIV-1 Interactions with Cells: From Viral Binding to Cell-Cell Transmission

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Abstract

Characterization of HIV-1 interactions with host cells is critical for the cell biology study of HIV-1. This unit describes a set of methods and protocols to perform quantitative assays of HIV-1 binding, internalization, infection, and cell-cell transmission. The basic protocols include: (1) Generating infectious single-cycle or replication-competent HIV-1 stocks, (2) HIV-1 binding and internalization assay, (3) HIV-1 infection of target cells and quantification of viral infection, and (4) HIV-1 cell-cell transmission assays. In addition to these basic protocols, alternative protocols and methods are also included. These functional assays provide useful tools to quantitatively study HIV-1 infection and viral transmission. The background information, critical parameters, anticipated results, and the time considerations of the protocols will be discussed in the commentary.

Keywords

Human immunodeficiency virus type 1 (HIV-1); binding; internalization; infection; viral transmission; interactions

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of acquired immune deficiency syndrome (AIDS), the leading infectious disease worldwide. Despite great effort and tremendous progress in HIV-1 and AIDS studies, eradication of HIV-1 infection and treatment of AIDS remain to be a long-term challenge (Fauci, 2007; Karlsson Hedestam et al., 2008). Understanding the multifaceted interactions between HIV-1 and host cells can potentially facilitate the development of therapeutic interventions against HIV-1 infection (Goff, 2007; Wu and KewalRamani, 2006). Quantitative analysis of HIV-1 interactions with host cells represents a critical aspect in the cell biology study of HIV-1 infection and dissemination. In this unit, we describe the use of a set of quantitative assays to measure HIV-1 binding, endocytosis, infection in target cells, and cell-cell viral transmission. These quantitative assays provide useful tools to study HIV-1 interactions with host cells.

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Internet Resources The *Virology Manual for HIV Laboratories* is freely available to download from the following websites:

http://www.niaid.nih.gov/DAIDS/vir_manual/

<http://aactg.s-3.com/labmanual.htm>

The website of the NIH AIDS Research and Reference Reagent Program

<https://www.aidsreagent.org/Index.cfm>

STRATEGIC PLANNING

Biosafety precautions

Infection of replication-competent HIV-1 can cause AIDS in humans. In general, performing experiments that involve using infectious HIV-1 should carefully follow the *Virology Manual for HIV Laboratories* (available to download from the websites listed in the Internet Resources). This manual was developed by the Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) and collaborating investigators. The manual addresses the biosafety issues of HIV laboratories in detail, and provides recommendations for specimen processing, storage and shipping, etc. Based on the manual, the following areas of the biosafety guidelines should be followed in order to effectively eliminate or minimize accidental exposure to HIV-1. (a) The use of universal precautions; (b) Establishing appropriate engineering controls; (c) Implementing appropriate work practice controls; (d) Using necessary personal protective equipment; (e) Implementing appropriate housekeeping procedures.

HIV research reagents

The NIH AIDS Research and Reference Reagent Program is a unique worldwide resource of state-of-the-art reagents for HIV and other pathogens. The program provides registered researchers with varieties of valuable reagents for studies of HIV and other retroviruses. Many of the expression plasmids, cell lines, and other reagents described in this unit can be obtained from the program. Please refer to the website listed in the Internet Resources for details.

BASIC PROTOCOL 1 GENERATING INFECTIOUS SINGLE-CYCLE OR REPLICATION-COMPETENT HIV-1 STOCKS

This method describes cellular transfection with HIV-1 proviral DNA as a common protocol to generate HIV-1 stocks. Replication-competent HIV-1 can be generated by transfection of human or animal cell lines with a full-length HIV-1 proviral construct (Adachi et al., 1986). Additionally, human CD4⁺ T cells or activated human peripheral blood mononuclear cells can be infected with replication-competent HIV-1 derived from transfection, and then expanded viral stocks can be obtained from the supernatants several days after the infection (Dong et al., 2007; Freed et al., 1995). Single-cycle reporter HIV is an infectious HIV vector that can establish an integrated provirus but cannot undergo additional cycles of replication, owing to an inactivated viral envelope gene. The viral genome encodes a reporter gene, which is expressed in infected permissive cells, enabling measurement of infectivity.

To generate single-cycle HIV-1, an *env*-deleted/inactivated HIV-1 proviral DNA and a construct expressing HIV-1 envelope glycoproteins (Env) can be cotransfected into cells (Aiken, 1997; Dong et al., 2007; Janas et al., 2008; Wang et al., 2007a; Wang et al., 2007b; Wu et al., 2002b). This method is also termed as HIV-1 pseudotyping. Based on different experimental purposes, various viral Env proteins from different tropic HIV-1 strains, simian immunodeficiency virus, or vesicular stomatitis virus G protein (VSV-G) can be incorporated in HIV-1 particles to generate single-cycle HIV-1 vectors (Aiken, 1997; Dong et al., 2007; Janas et al., 2008; Wu et al., 2002a). VSV-G-pseudotyped HIV-1 efficiently enters a broad range of cell types through a low-pH-dependent endocytic pathway (Aiken, 1997; Dong et al., 2007; Janas et al., 2008).

Materials

- Expression plasmids: R5-tropic Env of HIV-1_{JRFL} (pJRFL) or HIV-1_{ADA} (pADA), X4-tropic Env of HIV-1_{HXB2} (pHXB2), and VSV-G-expressing pVSV-G (kind gifts from Vineet KewalRamani, National Cancer Institute, Frederick, MD) (Dong et al.,

2007; Wang et al., 2007a; Wang et al., 2007b). The HIV-1 proviral DNA pHIV-Luc (pLaiΔenvLuc2) has an *env*-deleted and *nef*-inactivated viral genome with a luciferase reporter insertion, but contains all other viral genes (Yamashita and Emerman, 2004) (kind gifts from Michael Emerman, Fred Hutchinson Cancer Research Center). The green fluorescent protein-expressing HIV-1 vector (pHIV-GFP) was generated from a pNL4-3-based HIV-1 provirus that has deletions in the *env*, *vif*, *vpr*, *vpu*, and *nef* genes; the *GFP* gene was inserted in place of the *nef* open reading frame (Unutmaz et al., 1999). HIV-1 full length proviral construct pNL4-3 and pNLAD8 (Freed et al., 1995) (kind gifts from Eric Freed, National Cancer Institute, Frederick, MD).

- Human embryonic kidney cell line HEK293T cells
- Human CD4⁺ T cell line Hut/CCR5 cells
- Cell culture media (see Reagents and Solutions)
- Tissue culture plates (standard 10-cm diameter dishes or 6-well plates)
- 2.5 M CaCl₂, autoclaved or filter-sterilized (0.2 micron filter)
- 5 M NaCl, autoclaved
- 1 M BES [N,N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid from Cal Biochem], filter-sterilized (0.2-micron filter)
- 1 M Na₂HPO₄, filter-sterilized (0.2-micron filter)
- 5 ml-round-bottom, polystyrene tubes (Falcon catalog # 2058)
- Cryotubes (sterile)
- Tissue-culture-grade double distilled water (ddH₂O)
- Centrifuge (refrigerated)
- Additional equipment and reagents for cell culture

Preparation of transfection reagents

1. To prepare 0.25 M CaCl₂, dilute 2.5 M stock with tissue culture quality ddH₂O. To sterilize the solution, use a 0.2-micron sterile filter, and freeze 10 ml aliquots at -20°C.
2. To prepare 2X BBS (BES-Buffered Saline), please refer to Reagents and Solutions. The solution pH should be 6.96 with a ± 0.02 variance. Filter sterilize using a 0.2-micron filter, and freeze 10 ml aliquots at -20 °C.

Alternatively, commercially available transfection kits can be used.

Plate cells

The day before transfection, seed HEK293T cells in 10 cm-diameter plates at $2.5 \sim 3 \times 10^6$ cells in 10 ml growth media.

Alternatively, for transfections in 6-well plates, seed 3.0×10^5 cells/well in 2 ml of media. Cells should reach ~ 80% confluence 24 hr later. The authors recommend not to plate cells that have been overly confluent for more than 1 day.

Transfection

1. Replace with fresh culture medium 1~ 3 hr before transfection.

This step is optional if media of cultured cells appear to be fresh. Cold media should be pre-warmed to 37°C. Use extra caution when replacing media since cells can easily detach.

2. Quickly thaw 2X BBS and 0.25 M CaCl₂ aliquots in 37°C water bath and leave at room temperature after thawing.
3. Prepare appropriate amounts of DNA that will be used in transfection according to Table 1.

When using full-length HIV-1 proviral DNA (pNL4-3 or pNLADA, etc.) to generate replication-competent HIV-1, do not add Env-expressing vectors to the transfection mixture.

4. Aliquot appropriate proviral vector DNA and desired Env-expressing vector DNA into 5 ml-polystyrene tubes.

For 10 cm-diameter plates, combine 875 µl of 0.25 M CaCl₂ with prepared DNA and mix. For each sample well of the 6-well plate, combine 175 µl of 0.25 M CaCl₂ with prepared DNA and mix.

5. Add 2X BBS (pH 6.95) to the 0.25 M CaCl₂ and DNA mixture.

For 10 cm-diameter plates, add 875 µl of 2X BBS. For each well of the six-well plate, combine 175 µl of 2X BBS. Add 2X BBS drop-wise to the CaCl₂ and DNA mixture, and then gently vortex the transfection mixture for 2-5 seconds. The solution should turn cloudy from the formation of precipitation complexes.

6. After 13-15 min, gently and evenly add transfection mixture to HEK293T cells, drop by drop.

It is recommended by the authors to not exceed the 15 min incubation time period.

7. Place cells in an incubator designated for HIV-1 infections overnight.

DNA precipitation will form slowly and evenly overnight.

8. The next day, gently aspirate supernatants of HEK293T cells, and replace with prewarmed (37°C) culture media.

The authors recommend replacing media 16-20 hr post-transfection. Replacing the media will reduce the number of cells that may detach from the plate. Use appropriate biosafety precautions when replacing media, HIV-containing supernatant is now infectious. All tissue culture work from now on should be performed in a biosafety hood using appropriate precautions.

Harvest viral stocks

9. Harvest viral supernatants 2 days post transfection.

Collect supernatants in 15-ml conical tubes, and centrifuge at 1,200 rpm (270 × g) for 5 min at 4°C. Carefully transfer supernatants to a fresh tube, avoiding disruption of the cell pellet. Cellular debris can be further removed via filtering the supernatant through a 0.45-micron filter. Aliquot viral supernatant to cryotubes (0.5 ~ 1 ml/vial), and then quickly freeze in -80 °C freezer.

10. Generate replication-competent HIV-1 by infection of CD4⁺ T cells.

Alternatively, to expand replication-competent HIV-1 stocks in CD4⁺ T cells, Hut/CCR5 cells or activated peripheral blood lymphocytes (1 × 10⁶) can be infected with HEK293T-derived HIV-1_{NLAD8} (5 ng p24 equivalent), and supernatants will be

harvested 5 days post-infection. Please refer to step 9 for harvesting viral supernatants. HIV-1 Gag p24 protein concentrations of HIV-1_{NLAD8} stocks can be measured using an enzyme-linked immunosorbent assay (ELISA) according to the kit instruction.

Biosafety precautions

Follow the biosafety protocol and use 10% bleach to inactivate HIV-1 in the plates, tubes, plastic pipettes, etc.

BASIC PROTOCOL 2 HIV-1 BINDING AND INTERNALIZATION ASSAYS

HIV-1 can bind to cells through viral receptor CD4 and multiple cellular attachment factors (Geijtenbeek et al., 2002; Gummuluru et al., 2003; Turville et al., 2001; Turville et al., 2002). A C-type lectin, dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN), functions as an attachment factor of HIV-1 and facilitates HIV-1 binding, infection and viral transmission (Geijtenbeek et al., 2000; Wu and KewalRamani, 2006). DC-SIGN also mediates HIV-1 internalization in cells (Kwon et al., 2002; Wang et al., 2007a; Wu and KewalRamani, 2006). This protocol describes HIV-1 binding and internalization assays using Raji/DC-SIGN cells as an example. Raji/DC-SIGN cells are derived from a human Raji B cell line and stably express high-levels of exogenous human DC-SIGN (Wang et al., 2007a; Wu et al., 2004a; Wu et al., 2004b). CD4- and DC-SIGN-negative parental Raji cells are used as a background control.

HIV-1 incubated with Raji/DC-SIGN cells at 4°C primarily remains on the cell surfaces. After several washes, cell surface-bound virus can be measured by quantifying cell-associated HIV-1 Gag p24 (Wang et al., 2007a). In contrast, Raji/DC-SIGN cells incubated with HIV-1 at 37°C can bind virus on the cell surfaces and internalize HIV-1 intracellularly. When cells are incubated with HIV-1 at 4°C, washed and treated with a proteolysis reagent, such as trypsin, the majority of cell surface-bound HIV-1 can be stripped (Wang et al., 2007a; Wang et al., 2007b). When cells are incubated with HIV-1 at 37°C, washed, and then treated with trypsin, the cell-associated HIV-1 represents internalized virus as well as cell-surface-bound viruses that are protected from the proteolysis (Wang et al., 2007a; Wang et al., 2007b).

To examine the cell-HIV-1 interactions in the absence of potentially confounding effects of productive viral infection, aldrithiol-2 (AT-2)-inactivated HIV-1 can be used in the binding and internalization assays (Rossio et al., 1998; Wang et al., 2007a). Inactivation of HIV-1 with AT-2 modifies the essential zinc fingers in the viral nucleocapsid protein, but does not alter virion Env, thereby maintaining HIV-1 binding and fusion with target cells (Rossio et al., 1998). Thus, AT-2-inactivated HIV-1 is conformationally authentic and interacts with cells similarly to infectious HIV-1 (Frank et al., 2002; Rossio et al., 1998; Wang et al., 2007a; Wang et al., 2007b). Depending on different experimental purposes, p24-quantified infectious HIV-1 and different cell types can also be used to measure viral binding and internalization in similar assays.

Materials

- AT-2-inactivated R5-tropic HIV (Bal/Supt1-CCR5 cl30) (a gift from Jeffery Lifson, AIDS Vaccine Program, SAIC-Fredrick)
- Raji cells, Raji/DC-SIGN cells or another appropriate cell types
- Cell culture media (see reagents and solutions)
- Sterile PBS (without Ca²⁺ and Mg²⁺)
- 0.25% (w/v) trypsin without EDTA

- 1X cell lysis buffer (see below)
- 10% (v/v) Triton X-100
- HIV-1 p24 ELISA kit (anti-p24-coated plates from the AIDS Vaccine Program, SAIC, Frederick, MD) or p24 ELISA kit from PerkinElmer.
- RPMI-1640 medium with 10% (v/v) fetal bovine serum (FBS)
- Cell-culture grade ddH₂O
- 1.5-ml screw-cap tubes and eppendorf tubes
- Refrigerated microcentrifuge (e.g. rotor model F241.5P in Beckman Coulter microfuge 22R centrifuge)
- Additional equipment and reagents for cell culture

Prepare cells and virus for incubation

1. Aliquot Raji cells and Raji/DC-SIGN cells (3×10^5 per sample). Centrifuge cells at 3000 rpm ($\sim 900 \times g$) for 3 min at room temperature. Remove supernatant carefully.
2. Wash cells with PBS.
Centrifuge cells at 3000 rpm for 3 min at room temperature. Remove supernatant carefully.
3. Resuspend cells in culture media containing 20 ng of p24-equivalent AT-2-inactivated HIV-1.
The authors recommend that the final volume be raised to 200 μ l using RPMI media with 10% FBS. For cells being incubated at 4°C, the media should be chilled. Also, warm media can be used for the samples that will be incubated at 37°C.
4. Incubate at desired temperature (4°C or 37°C) for 2 hr.
The screw-cap tubes should be slightly loosened during virus incubation. Incubation of samples at 4°C can be performed on ice in a 4°C refrigerator.

Wash, trypsinize and lyse cells

5. After virus incubation, wash the cells, as in step 2, with 1ml of PBS 3-5 times to remove unbound cell-free HIV-1.
6. Treat desired samples with trypsin.
Use 0.25% trypsin, and incubate cells at room temperature for 4 min. The trypsin concentration and incubation time should be pre-tested and adjusted when using different cell types. Neutralize trypsin with cell culture medium containing 10% FBS. For samples not being treated with trypsin, incubate in cell medium only.
7. Centrifuge cells at 3000 rpm for 3 min at 4°C. Remove supernatant carefully, avoiding disruption of the pellet.
8. Resuspend the pellet in 1 ml of cold PBS to wash 3- 5 times.
Centrifuge cells at 3000 rpm, and remove supernatant carefully.
9. Resuspend cell pellet with 200 μ l of 1X lysis buffer. Add 20 μ l of 10% Triton X-100 (1/10 vol.) to each sample to release p24 according to the kit instruction.
10. Incubate samples at 37°C for 1 hr to inactivate virus, if infectious HIV-1 stocks are used.

11. Analyze samples by p24 ELISA assay according to kit instructions.

Alternatively, samples can be stored at -80°C , and analyzed in the future.

BASIC PROTOCOL 3 HIV-1 INFECTION OF TARGET CELLS AND QUANTIFICATION OF VIRAL INFECTION

Stable cell lines that express a reporter gene upon HIV-1 infection can be used to titrate HIV-1 infectivity. This protocol describes a flow cytometry-based HIV-1 titration assay using GHOST cells, which are human osteosarcoma cells expressing high levels of CD4 and CCR5. These cells contain a *GFP* gene under the control of the long terminal repeat (LTR) promoter of HIV-2, which is expressed during HIV-1 infection via Tat transactivation, acting as a sensitive indicator of HIV-1 infection (Cecilia et al., 1998). GHOST cells expressing the CXCR4 and CCR5 coreceptor are referred as GHOST/X4/R5 cells (Cecilia et al., 1998; Wu et al., 2002b). Approximately, 40% of GHOST/X4/R5 cells are positive for CD4; 84% of the cells are positive for CXCR4; 66% of the cells are positive for CCR5. The parental GHOST cells are 3.8% positive for CXCR4 and 0.8% positive for CCR5 (Cecilia et al., 1998).

Materials

- HIV indicator cell line GHOST/X4/R5 cells (kind gift from Vineet KewalRamani, National Cancer Institute, Frederick, MD)
- Infectious HIV-1 stocks
- Cell culture media (see Reagents and Solutions)
- Cell culture-grade neomycin, hygromycin B, and puromycin
- 4% (w/v) Paraformaldehyde
- Sterile PBS (without Ca^{2+} and Mg^{2+})
- Sterile 1mM EDTA
- 0.05% (w/v) trypsin with 0.2 g/L of EDTA
- PBS/2% (v/v) FBS
- 12-well tissue culture plate
- Flow cytometer
- Additional equipment and reagents for cell culture

Plate cells

1. The day before infection, plate 2×10^4 cells per well in a 12-well plate.
Cells can be plated in nonselective culture media for single-round infection experiments. To maintain high levels expression of receptor and coreceptors, GHOST/X4/R5 cells should be cultured in selective media (see Reagents and Solutions).

Virus infection

2. Add appropriate volumes of HIV-1 to GHOST/X4/R5 cells for incubation (Day 1).
Use appropriate biosafety precautions when handling virus stocks, and perform experiment in a designated biosafety cabinet. When titering virus pseudotyped with JRFL or HXB2, use 50, 100 and 200 μL of viral stocks for infections. When titering virus pseudotyped with VSV-G, use 12.5, 25 and 50 μL of viral stocks for infection.

It is recommended to not use more than 50 μ l of HIV-VSV-G for titration due to the cytotoxic effects of VSV-G. For replication-competent virus, 25 to 100 μ l of viral stocks can normally be used in infections. Infections can be performed in a final volume of 300 μ l per well of a 12-well plate. Alternatively, 10 μ g/ml of polybrene can be added in the infection to enhance the infection efficiency. It is important that a mock control (no virus input) is always included in the experiment.

3. After incubating the virus for 2 hr at 37°C, raise the volume to 1 ml with media.

Alternatively, supernatant can be replaced with 1 ml of media, or infections can be performed in a 0.5 ml final volume overnight (not recommended for VSV-G-pseudotyped HIV-1 due to cytotoxicity).

4. Replace media 24 hr post infection (Day 2).

Harvest infected cells

5. One or two days after replacing the media, harvest infected GHOST/X4/R5 cells (Day 3 or 4).

Gently wash cells twice with PBS. Add 300 μ l of PBS containing 1mM EDTA to each well, and place on a shaking platform for 15 min at room temperature. Alternatively, cells can be trypsinized (0.05% trypsin with EDTA), and neutralized with 10% FBS-containing DMEM. Before proceeding to step 5, wash cells with PBS. To calculate the virus titer, it is important to count and calculate the average total cell number/sample when harvesting cells. It is suggested that the mock control be counted.

6. Resuspend the samples in 300 μ l of PBS. Add 300 μ l of 4% paraformaldehyde to each sample for a final concentration of 2% paraformaldehyde.

The authors recommend thoroughly pipetting to mix cells in paraformaldehyde solution to prevent cell clumping.

7. Incubate cells on ice for at least 1 hr.

The authors recommend not exceeding 6 hr of incubation time.

8. After incubation, add 1 ml of PBS to wash cells and centrifuge cells at 7000 rpm ($\sim 4000 \times g$) for 15 seconds.

9. Resuspend cells in 200 μ l of PBS/2% FBS.

Detect GFP expression in infected cells

10. Analyze by flow cytometry for GFP expression.

There should be approximately over 10-fold shift in the mean GFP-fluorescence of infected cells over the mock control. Please see Figure 1 for examples.

11. Calculate virus titer based on the following equation:

Infectious Units (IU)/ml = (cell number) $\times$ (% of GFP-positive cells) $\times$ (dilution factor). For example, using 200 μ l of HIV-JRFL sample, the calculation would be as follows: $(1.39 \times 10^6) \times 8.56\% \times 5 = 5.95 \times 10^5$ IU/ml. Please refer to Table 2 for calculated virus titers as examples.

Biosafety precautions

Follow the biosafety protocol and use 10% bleach to inactivate HIV-1 in the plates, tubes, plastic pipettes, etc.

ALTERNATIVE PROTOCOL 1 VIRAL INFECTION AND QUANTIFICATION USING REPORTER HIV-1

Instead of using HIV-1 indicator cell lines in viral infection assays, a reporter gene such as *luciferase* or *GFP* engineered into HIV-1 genome can be expressed in infected cells and be used as a marker for viral infection (Unutmaz et al., 1999; Wu et al., 2002b). These virally expressed reporters in the infected cells can be measured to quantify HIV-1 infection. Additionally, when using wild-type, replication-competent HIV-1 in infection assays, HIV-1 p24 in the supernatant of infected cells can be measured to quantify viral production (Dong et al., 2007; Wang et al., 2007a). We describe these assays as an alternative protocol of HIV-1 infection assays, in which, Hut/CCR5 cells, a human CD4⁺ T cell line that expresses high levels of CD4, CXCR4 and CCR5, are used as an example.

Materials

- HIV-1 stocks with known viral infectivity
- Hut/CCR5 cells
- Cell culture media (see Reagents and Solutions)
- Cell culture-grade neomycin and puromycin
- 1.5-ml screw-cap tubes and eppendorf tubes
- Additional equipment and reagents for cell culture

Viral infection

Now that the virus stocks have been quantified, cells can be infected at a specific multiplicity of infection (MOI).

1. To calculate the volume of virus used at a specific MOI, use the following equation:

$$\text{MOI} = [(\text{virus stock IU/ml}) \times (\text{volume of virus used})] / (\text{number of cells in infection}).$$
 An example calculation is that 1×10^5 Hut/CCR5 cells are infected at a 0.5 MOI by a virus stock with a titer of 1.67×10^6 IU/ml. Therefore, to determine the amount of virus needed to infect 1×10^5 cells at a 0.5 MOI is as follows: $[(1.67 \times 10^6 \text{ IU/ml}) \times (\text{volume of virus used})] / (1 \times 10^5 \text{ cells}) = 0.5 \text{ MOI}$, where the volume equals 30 μl per sample.

2. Before adding virus, wash cells once with PBS.

Centrifuge aliquoted cells (1×10^5) at 3000 rpm ($\sim 900 \times g$) for 3 minutes. Remove supernatant. Resuspend cell pellet in 1 ml of PBS. Centrifuge at 3000 rpm for 3 min, and carefully remove supernatant. If using adherent cells, please see the basic protocol 3 using GHOST/X4/R5 cells for further details. Hut/CCR5 cells can be cultured in nonselective culture media for single-round infection experiments. To maintain high levels expression of receptor and coreceptors, Hut/CCR5 cells should be cultured in selective media (see Reagents and Solutions).

3. Add desired amount of virus to cells and mix well by pipetting.

Depending on the volume of virus used, the authors recommend a final incubation volume between 200 μl and 500 μl for optimal infection conditions. Prewarmed cell culture medium can be added so that the final volume for the virus incubation is within the recommended incubation volume. Additionally, incubations should be performed in 1.5 ml screw-cap tubes. Always include a mock control (no virus) with all virus infection experiments.

4. Incubate cells at 37 °C for 1.5 ~ 2 hr.

The authors recommend loosening the caps of samples during the incubation. Alternatively, viral incubation can be performed using 24-well culture plates.

5. After the virus incubation, wash the samples three times with PBS.

Please refer to step 2 of this protocol for specific details.

6. Resuspend cells in culture medium and place cells in culture.

7. Harvest cells 2 ~ 4 days later.

SUPPORT PROTOCOL 1 DETECTION OF LUCIFERASE ACTIVITY IN INFECTED CELLS

If using a luciferase reporter HIV-1, viral infection can be detected by measuring luciferase activity in infected cell lysates.

Materials

- 1.5 ml screw-cap tubes and eppendorf tubes
- 5X passive lysis buffer (Promega)
- Luciferase assay kit (Promega)
- 96-well black plate (Thermo)
- Chemluminescence microplate reader (Wallac 1420 VICTOR2 Multilabel Plate Reader)
- Microcentrifuge (e.g. rotor model F241.5P in Beckman Coulter microfuge 22R centrifuge)

Procedures

1. Transfer each sample of cells to a 1.5 ml-screw-cap tube, and centrifuge at 3000 rpm ($\sim 900 \times g$) for 3 min.
2. Remove supernatant, and resuspend pellet in 1 ml of PBS to wash cells.
3. Centrifuge at 3000 rpm for 3 min. Remove supernatant.
4. Use 100 μ l of 1X passive lysis buffer (Promega) to resuspend pellet.

The 1X passive lysis buffer should rapidly inactivate HIV-1. As an extra precaution, lysates can be transferred to a fresh tube to ensure that sample does not contain any infectious virus.

5. Shake samples for 5 min at room temperature. Incubation time can be increased to 10 minutes if using more than 5×10^4 cells per sample.
6. Centrifuge samples at 7000 rpm ($\sim 4000 \times g$) for 2 min.
7. If storing samples at -80 °C to run luciferase assay in the future, transfer supernatant to a fresh eppendorf tube. Otherwise, samples can be directly transferred to a 96-well black plate for detection of luciferase activity.

Samples can be aliquoted in 40 μ l volumes in duplicate. Use caution to not disrupt the pellet when aliquoting.

8. Perform the Luciferase assay according to the product instruction. Luciferase substrate should be dispensed in 100 μ l volumes.
9. Detect luciferase activity of cell lysates according to kit instructions.

SUPPORT PROTOCOL 2 GFP DETECTION IN INFECTED CELLS

If using a GFP reporter HIV-1, viral infection can be detected by measuring GFP expression in infected cells.

Materials

- 4% (w/v) Paraformaldehyde
- Sterile PBS (without Ca^{2+} and Mg^{2+})
- PBS/2% (v/v) FBS
- Flow cytometer
- 1.5 ml screw-cap tubes and eppendorf tubes
- Microcentrifuge (e.g. rotor model F241.5P in Beckman Coulter microfuge 22R centrifuge)

Procedures

1. Wash infected cells with 1 ml of PBS.
2. Resuspend the samples in 300 μ l of PBS. Add 300 μ l of 4% paraformaldehyde to each sample for a final concentration of 2% paraformaldehyde.
The authors recommend thoroughly pipetting to mix cells in paraformaldehyde solution to prevent cell clumping.
3. Incubate cells on ice for at least 1 hr.
The authors recommend not exceeding 6 hr of incubation time.
4. After incubation, add 1 ml of PBS to wash cells and centrifuge cells at 7000 rpm ($\sim 4000 \times g$) for 15 seconds.
5. Resuspend cells in 200 μ l of PBS/2% FBS.
6. Analyze for GFP expression by flow cytometry.
Including a positive control and a mock infection control. There should be approximately a 10~ 20-fold shift in the mean GFP-fluorescence of efficiently infected cells over the mock control.

SUPPORT PROTOCOL 3 HIV-1 p24 DETECTION IN SUPERNATANTS OF INFECTED CELLS

If using a replication-competent HIV-1 that does not encode a reporter gene, viral infection and production can be quantified by measuring HIV-1 p24 in cell supernatants.

Materials

- HIV-1 p24 ELISA kit (anti-p24-coated plates from the AIDS Vaccine Program, SAIC, Frederick, MD) or p24 ELISA kit from PerkinElmer.
- Cell lysis buffer

- 10% (w/v) Triton X-100

Detection of HIV-1 p24

1. Centrifuge cells at 3000 rpm ($\sim 900 \times g$) at 4°C for 5 min. Collect the supernatant carefully, avoiding disruption of the pellet.
2. Transfer the supernatant to a fresh screw-cap tube.
3. Add 10% Triton X-100, 1/10 vol. to each sample according to the kit.
4. Incubate samples at 37°C for 1 hr.
5. Analyze samples by p24 ELISA assay according to kit protocol.

Alternatively, samples can be stored at -80°C, and analyzed in the future.

BASIC PROTOCOL 4 HIV-1 CELL-CELL TRANSMISSION ASSAYS

Cell-cell transmission can enhance HIV-1 infection 100 ~ 1,000-fold compared with cell-free viral infection (Dimitrov et al., 1993). Elucidating the mechanisms of cell-cell transmission mediated HIV-1 can facilitate our understanding of viral pathogenesis and potentially aid in HIV-1 interventions (Piguet and Steinman, 2007; Wu and KewalRamani, 2006). HIV-1-pulsed dendritic cells efficiently transmit HIV-1 to cocultured CD4⁺ T cells (Wu and KewalRamani, 2006), which has been used as an *in vitro* model to study the mechanisms underlying cell-cell transmission (Cameron et al., 1992; Dong et al., 2007; Geijtenbeek et al., 2000; Pope et al., 1994; Wang et al., 2007a; Wang et al., 2007b; Wu et al., 2002a; Wu et al., 2002b). Exogenous DC-SIGN expressed on certain types of cells can mediate efficient HIV-1 *trans*-infection of cocultured CD4⁺ T cells (Wang et al., 2007a; Wang et al., 2007b; Wu et al., 2004a; Wu et al., 2004b). As an example, this basic protocol describes an HIV-1 transmission assay using Raji/DC-SIGN cells as donor cells and Hut/CCR5 T cells as targets. CD4⁻ and DC-SIGN-negative parental Raji cells are used as a background control. The viral transmission assay is referred to as the HIV-1 capture assay, which is performed when virus donor cells are preincubated with HIV-1 and washed to remove cell free virus, after which CD4⁺ T cells were added in coculture as infection targets.

Materials

- Single-cycle luciferase reporter HIV-1 stocks
- Cell culture media (see reagents and solutions)
- Sterile PBS (without Ca²⁺ and Mg²⁺)
- Tissue-culture-grade ddH₂O
- Luciferase activity detection kits (Promega)
- Refrigerated microcentrifuge (e.g. rotor model F241.5P in Beckman Coulter microfuge 22R centrifuge)
- 1.5 ml screw-cap tubes and eppendorf tubes
- 24-well tissue culture plates
- Wallace 1420 VICTOR2 Multilabel Plate Reader or equivalent instrument
- Additional equipment and reagents for cell culture

Incubate donor cells with HIV-1

1. Aliquot Raji/DC-SIGN cells (donor cells) 2.5×10^5 cells per sample into 1.5 ml screw-cap tubes. Use Raji cells as a background control.
2. Centrifuge cells at 3000 rpm ($\sim 900 \times g$) for 3 min at room temperature. Remove supernatant carefully, avoiding disruption of the pellet.
3. Resuspend the pellet in 1 ml of PBS to wash cells.
Centrifuge cells at 3000 rpm for 3 min at room temperature. Remove supernatant carefully. If using inhibition agents, such as anti-DC-SIGN (10 $\mu\text{g/ml}$) or mannan (20 $\mu\text{g/ml}$), preincubate cells with the inhibitors for 30 min at 37°C (Wu et al., 2002b).
4. Resuspend cells in 0.2~0.4 MOI ($\sim 1 \times 10^5$ infectious units) of HIV-1.
Please see Basic Protocol 3 for direct infection and how to calculate MOI. Based on viral input, the incubation volume should be 200 ~ 400 μl with culture media.
5. Incubate the cells in 1.5 ml screw-cap tubes for 2 hr at 37°C.
The authors recommend that you loosen the caps during incubation.

Wash donor cells and prepare target cells

6. Centrifuge for 2 min at 3000 rpm at 4°C. Remove supernatant carefully.
7. Wash each sample with 1 ml of PBS and centrifuge for 2 min at 3000 rpm. Remove supernatant carefully.
8. Prepare Hut/CCR5 target cells (5×10^5 cells/ml).
To enhance viral transmission efficiency, add polybrene (8 ~ 10 $\mu\text{g/ml}$) to target cells. When using adherent cells as target cells, plate cells one day before the coculture assay.

Coculture donor cells with target cells

9. Resuspend HIV-1-pulsed Raji/DC-SIGN cell pellets (from step 7) with 200 μl of the prepared Hut/CCR5 cells (1×10^5 cells) from step 8.
As a background control, include HIV-pulsed donor cells without coculture of target cells. When using adherent cells as target cells, remove media in 24-well plate, resuspend Raji/DC-SIGN cells with media (200 $\mu\text{l/sample}$), and then transfer them into each well of the adherent target cells.
10. Incubate the cells in screw-cap tubes for 1~2 hr at 37°C.
The authors recommend that you loosen the caps during incubation. Alternatively, coculture cells in a 24-well plate.
11. Transfer cells from each tube to a new 24-well plate. The final volume of medium should be 1 ml per sample per well.
Use RPMI-1640 media with 10% FCS.
12. Incubate for 2 ~ 3 days in an incubator designated for HIV-1-infected cells.
13. Refer to previous support protocols on quantifying viral infection of target cells when harvesting cells.

Biosafety precautions

Follow the biosafety protocol and use 10% bleach to inactivate HIV-1 in the plates, tubes, plastic pipettes, etc.

ALTERNATIVE PROTOCOL 2 HIV-1 ENHANCEMENT ASSAY

As an alternative method of HIV-1 cell-cell transmission assay, a viral enhancement assay can be used. This assay is performed using a smaller HIV-1 inoculum during the incubation with donor cells, and target cells are added directly to the coculture without removing the virus present in the culture medium (Wu et al., 2004b).

Materials

See Materials section of Basic Protocol 4.

Incubate donor cells with HIV-1

1. Aliquot Raji/DC-SIGN cells (donor cells) 2.5×10^5 cells per sample into the screw-cap tubes. Use Raji cells as a background control.
2. Centrifuge cells at 3000 rpm for 3 min at room temperature. Remove supernatant carefully, avoiding disruption of the pellet.
3. Resuspend the pellet in 1 ml of PBS to wash cells.
Centrifuge cells at 3000 rpm for 3 min at room temperature. Remove supernatant carefully. If using inhibition agents, such as anti-DC-SIGN (10 $\mu\text{g/ml}$) or mannan (20 $\mu\text{g/ml}$), preincubate cells with the inhibitors for 30 min at 37°C (Wu et al., 2002b).
4. Resuspend cells with 100 μl of culture media
Use nonselective media (see Reagents and Solutions)
5. Add 0.02~ 0.04 MOI ($\sim 1 \times 10^4$ infectious units) of HIV-1 to donor cells.
See Basic Protocol 3 for direct infection and calculation of MOI. Adjust the incubation volume to 200 μl with media.
6. Incubate the cells in screw-cap tubes for 2 hr at 37°C.
The authors recommend that you loosen the caps during incubation.

Prepare target cells and coculture with donor cells

7. Prepare Hut/CCR5 target cells (5×10^5 cells/ml).
To enhance viral transmission efficiency, add polybrene (8 ~ 10 $\mu\text{g/ml}$) to target cells. When using adherent cells as target cells, plate cells one day before the coculture assay.
8. Coculture HIV-1-incubated Raji/DC-SIGN cells (from step 6) with 200 μl of the prepared Hut/CCR5 cells (1×10^5 cells) from step 7.
As a background control, include HIV-incubated donor cells without coculture of target cells. When using adherent cells as target cells, remove media in 24-well plate, resuspend Raji/DC-SIGN cells with media (200 $\mu\text{l/sample}$), and then transfer them into each well of the adherent target cells.
9. Incubate the cells in screw-cap tubes for 1~ 2 hr at 37°C.

The authors recommend that you loosen the caps during incubation. Alternatively, coculture cells in a 24-well plate.

10. Transfer cells from each tube to a new 24-well plate. The final volume of medium should be 1 ml per sample per well.

Use non-selective culture media (see Reagents and Solutions).

11. Incubate for 2 ~ 3 days in an incubator designated for HIV-infected cells.
12. Refer to previous support protocols on quantifying viral infection of target cells when harvesting cells.

Biosafety precautions

Follow the biosafety protocol and use 10% bleach to inactivate HIV-1 in the plates, tubes, plastic pipettes, etc.

REAGENTS AND SOLUTIONS

2X BBS (BES-Buffered Saline), pH 6.96 ± 0.02

5.6 ml of 5M NaCl

5.0 ml of 1M BES

150 µl of 1M Na₂HPO₄

Tissue-culture quality sterile ddH₂O (raised to a final volume of 100 ml)

Solution should be filter sterilized through a 0.2-micron filter and be in 10 ml aliquots, stored at -20°C.

Cell lysis buffer (1X)

1% NP40 (Sigma: IGEPAL CA-630)

100 mM NaCl, dilute from 5 M stock (50X)

10 mM Tris-HCl, pH 7.5, dilute from 1 M stock (100 X)

1 mM EDTA, dilute from 0.5 M stock (500X)

Alternatively, a 10X lysis buffer stock solution can be prepared and stored at 4°C, so that 1X fresh lysis buffer can be conveniently diluted with ddH₂O for the assay.

Dulbecco-Phosphate-buffered saline (PBS, 1X)

137.9 mM NaCl

2.7 mM KCl

1.5 mM KH₂PO₄

8.1 mM Na₂HPO₄

Adjust pH to 7.3 using NaOH or HCl, if necessary

Autoclave solution

Store up to 6 months at room temperature

Paraformaldehyde fixative (pH 7.2)

Prepare a fresh 4% (w/v) solution of paraformaldehyde, analytical grade, in PBS.

Adjust the pH of the solution with HCl so that the pH is 7.2.

Solution can be stored in dark for up to 3 months at 4°C. Use caution when handling; formaldehyde is a carcinogen. The solution should be prepared in a fume hood.

PBS/2% (v/v) FBS

Add 2% FBS to 1X PBS just before use.

Cell Culture Media

It is optional to add penicillin (100 U/ml) and streptomycin (100 µg/ml) in the following media. All media should be stored at 4°C.

GHOST/X4/R5 cell medium

Dulbecco's Modified Eagle's Medium (DMEM)

10% (v/v) FBS.

Neomycin (500 µg/ml)

Hygromycin B (50 µg/ml)

Puromycin (1 µg/ml)

HEK293T cell medium

DMEM

10% (v/v) FBS

Hut/CCR5 cell medium

RPMI-1640

10% (v/v) FBS

Neomycin (500 µg/ml)

Puromycin (1 µg/ml)

Raji/DC-SIGN cell medium

RPMI-1640

10% (v/v) FBS

COMMENTARY

Background Information

In this unit, we describe a set of basic protocols to perform quantitative assays of HIV-1 binding, internalization, infection, and cell-cell transmission. Although not covered, many other assays and protocols have been described in the literature, which will be briefly introduced here. Based on various experimental purposes and requirements, numerous different methods can be used to study HIV-1 interactions with host cells. For instance, a flow cytometry-based HIV-1 binding assay using GFP-tagged virus has been used in CD4⁺ primary T cells and cell lines (Schaeffer et al., 2004). A sensitive and specific enzyme-based assay has been reported to detect HIV-1 virion fusion in primary T lymphocytes (Cavrois et al., 2002). LTR promoter-based HIV-1 indicator cell lines have been widely used in measuring HIV-1 infectivity or viral infection (Cecilia et al., 1998; Derdeyn et al., 2000). However, there could be a background

transcriptional activity of the LTR promoter that is independent of HIV-1 infection and Tat expression. Moreover, cellular activation states can also influence the activity of LTR promoter. To overcome these potential caveats, a Rev-dependent HIV-1 indicator T cell line using GFP as a reporter has been recently generated (Wu et al., 2007). This CD4⁺ T cell line appears to be sensitive to both CCR5- and CXCR4-tropic HIV-1 and shows no measurable background of GFP expression (Wu et al., 2007).

Although various reporter-expressing HIV-1 vectors provide convenient tools to measure viral infection (Connor et al., 1995; Unutmaz et al., 1999), the reverse transcriptase assay (Adachi et al., 1986; Freed et al., 1995) remains to be a reliable and sensitive method to measure HIV-1 replication (Hatzioannou et al., 2006). Moreover, quantitative real-time PCR to measure HIV-1 reverse transcription, integration, and gene transcription is an important tool in dissecting molecular mechanisms of viral replication (Butler et al., 2001; Dong et al., 2007; Wu and Marsh, 2001). For HIV-1 cell-cell transmission assay, the basic protocol described in this unit may need to be adjusted according to the cell types used in the experiments. In addition to DCs (Wu and KewalRamani, 2006), macrophages can mediate HIV-1 transmission to CD4⁺ T cells (Sharova et al., 2005). Furthermore, efficient cell-cell transmission of HIV-1 also occurs between CD4⁺ T cells (Jolly et al., 2007a; Jolly et al., 2007b; Sowinski et al., 2008).

Critical Parameters and Troubleshooting

Generating virus stocks—When generating virus stocks, it is imperative that all transfection reagents are at the appropriate pH. When mixing the transfection reagents, do not incubate the transfection mixture for longer than 20 min. To maximize the efficiency of transfection, use an earlier passage of HEK293T cells; the seeded cells should not be overly confluent before the transfection. Additionally, replace media when specified to ensure high-titer and yield of the virus stock. Lastly, the DNA being used in transfection reactions should be of high quality. If experiencing difficulty with generating virus of appropriate titer, check the pH of the transfection solutions, the passage number of HEK293T cells, and the quality of the DNA preparations. For the DNA concentrations, it is recommended using a stock around 1 mg/ml, because too dilute of a stock may potentially alter the pH of the transfection reaction.

HIV-1 binding and internalization assay—When performing the binding assay, keep consistent temperatures of sample incubation at 4°C or 37 °C. The samples that are incubated at 4°C and treated with trypsin represent background levels of the assay. The concentration of trypsin should be titrated for different cell types since some cells might be more sensitive to proteolysis. After trypsin treatment, use caution when removing the supernatant after washes since the sample can be very fragile. Lastly, include a parental cell line as a control if looking at specific attachment factors transduced in a cell line (e.g. comparing HIV binding in parental Raji cells and Raji/DC-SIGN cells). For p24 ELISA detection, see troubleshooting in the kit instruction.

HIV-1 infection and cell-cell transmission—For direct infection of target cells, cell viability and the quality of the virus stock will affect the quality of the assay. If the stock is too dilute, larger than desired incubation volumes will have to be used, and the infectivity of the virus may not be as high when compared to a more concentrated virus stock at the same MOI. Low-titer viral stocks can be concentrated by ultracentrifugation or ultrafiltration. Furthermore, if performing infections with different HIV-1 pseudotypes in parallel, it is important to maintain the same incubation volumes if directly comparing the virus infections. Always include a mock infection control for background levels.

These critical parameters should also apply for the HIV cell-cell transmission assays. Another important control in viral transmission assays is to use donor cells with HIV-1-incubation, but

without the coculture of target cells. Donor cells that are negative for HIV-1 receptor or appropriate coreceptors should not be directly infected by HIV-1, and therefore should have similar values as compared to the mock control. Notably, when using HIV-1-permissive donor cells, such as immature dendritic cells, HIV-1-pulsed dendritic cells can become infected 3-5 days post-infection if high viral inputs are used (Dong et al., 2007).

Anticipated Results

Generating HIV-1 stocks—For HIV pseudotyped with HIV-1 Env, such as HXB2 or JRFL, the titer of the virus generated from transfection should range between $2 \sim 5 \times 10^5$ infectious units (IU)/ml, whereas HIV pseudotyped with VSV-G has a titer ranging from $1 \sim 10 \times 10^6$ IU/ml. A 10 ~ 20-fold shift in GFP expression should be expected when titrating the virus stocks by flow cytometry assay. Please see Figure 1 and Table 2 for viral titration examples.

HIV-1 binding and internalization assay—HIV-1 binding and internalization can be enhanced around two-fold in Raji/DC-SIGN cells, relative to parental Raji cells (Wu et al., 2004b). When using 20 ng of p24-equivalent HIV-1 with 3×10^5 of Raji/DC-SIGN cells, the p24 detection in cell lysates should be approximately 500 pg/ml (Wang et al., 2007a). Notably, cell-associated p24 values will vary based on the cell types, virus input, incubation temperature and time, and the proteolysis treatment after viral incubation (Dong et al., 2007; Wang et al., 2007a; Wang et al., 2007b; Wu et al., 2004b).

HIV-1 infection assays—The level of infection detected in target cells will be dependent upon the number of cells used, the virus input, as well as the type of virus and cells that are used in the infection. Using luciferase reporter HIV-1 in viral infection assay (Alternative Protocol 1 and Support Protocol 1), the background levels were approximately 100 counts per second for the mock control. As an example, 2×10^5 Hut/CCR5 cells infected with HIV-1-VSV-G at an MOI of 0.1, and harvested 3 days post infection, had luciferase activity approximately 330-fold higher relative to that of the mock control. In contrast, Hut/CCR5 infected with HIV-1-JRFL had luciferase activity 39-fold higher compared to the mock control (Janas et al., 2008; Janas and Wu, unpublished data).

HIV-1 cell-cell transmission assays—If using HIV-1 capture assay (Basic Protocol 4), when compared to the negative control (HIV-pulsed Raji/DC-SIGN cells without target cells), Raji/DC-SIGN cell-mediated HIV-1-JRFL transmission to Hut/CCR5 target cells can be ≥ 350 -fold higher (Wang et al., 2007a; Wu et al., 2004a). If using HIV-1 enhancement assay (Alternative Protocol 2), expect to see around 10-fold enhancement of viral infection in the cocultures. Notably, the relative efficiency of HIV-1 cell-cell transmission may vary depending on the cell types and viruses (Wang et al., 2007b).

Time Considerations

Generating virus stocks—For virus stock generation, plan for 4 days. Day 1 will consist of plating cells. The transfection will be performed on the second day, and will take approximately 25~30 min depending on how much virus stock is being generated. On day 3, the media is replaced. The virus is harvested on day 4, and will take approximately 1 hr.

HIV-1 binding and internalization assay—The binding assay should take approximately 3~4 hr to perform, depending on the sample number (not including virus inactivation). The p24 ELISA detection normally takes approximately 6 hr.

HIV-1 infection of target cells—For virus titrations, the experiment will span 4 days. On day 1, the cells will be plated. On the second day, the cells are infected. On the third day, cell media is replaced. On the fourth day, the cells can be harvested for GFP analysis by flow

cytometry. Sample preparation for flow cytometry analysis can take from 1.5 to 6 hr, depending on how long the investigator chooses to fix the cells in paraformaldehyde. When performing other direct infections of target cells, anticipate approximately 4 hr on the day of infection (and 5 hr for transmission assay), and an additional 2–3 days until the cells are harvested for analysis. Harvesting the cells takes approximately 1 hr for the luciferase assay, and may be shorter if collecting cell supernatants for p24 analysis.

Acknowledgments

We thank Michael Emerman, Eric Freed, Jeffery Lifson, Vineet KewalRamani, and the AIDS Research and Reference Reagent Program, NIAID, NIH, for the kind gift of reagents. The authors thank Constance Kwas and other members of their laboratory for critical reading the manuscripts and helpful discussions. The work published by the authors' laboratory was supported by grants from the National Institutes of Health (AI068493) and the Campbell Foundation to L.W.

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Fig 1. Infectivity titration of HIV-1 pseudotyped with different envelope proteins
HIV-1 indicator cells (GHOST/X4/R5) were infected with various single-cycle HIV-1 pseudotypes with different viral inputs. GFP-positive cells were measured 3 days post infection by flow cytometry (FACSCalibur, BD Bioscience) and analyzed with CellQuest Pro program (BD Bioscience). GFP-positive cell populations were determined based on the settings of mock control, which used medium instead of virus in the infection assay. A total of 10,000 cells per sample were acquired and analyzed. The amounts of viral input are labeled on the top of each dot plot. The percentages of GFP-positive cells are shown in the bottom right corner of each dot plot.

Table 1Amount of HIV proviral DNA and Env-expressing vectors used for transfection ^a

Cell culture plates (cell number)	HIV proviral DNA (pHIV-Luc, pHIV-GFP, or pNL4-3 etc.)	Envelope (Env)-expressing vectors ^b (One of the expressing vector)	
		HIV Env-expressing vectors (μg) (pJRFL or pHXB2 etc.)	pVSV-G (μg)
10-cm plate (2.5×10^6)	10	10	5
6-well plate (3.0×10^5 /well)	2	2	1

^a All DNA used in transfection should be prepared with Qiagen DNA purification kits or equivalent products to ensure the purity of DNA and minimal endotoxin contamination.

^b When using full-length HIV-1 proviral DNA (pNL4-3 or pNLADA etc.) to generate replication-competent HIV-1, no need to add Env-expressing vectors.

Table 2
Summary results of single-cycle HIV-1 titration assay^a

Viral input (μl/sample) ^c	GFP-positive cells (%) [Viral titer infectious unit per ml (IU/ml)] ^b				Average titer ^d (IU/ml)
	12.5	25	50	100	200
HIV-1 types					
HIV-JRFL (R5-tropic)	N.D. ^e	N.D.	1.16 [3.22×10 ⁵]	3.34 [4.64×10 ⁵]	8.56 [5.95×10 ⁵]
HIV-HXB2 (X4-tropic)	N.D.	N.D.	2.46 [6.84×10 ⁵]	6.06 [8.42×10 ⁵]	11.60 [8.06×10 ⁵]
HIV-VSV-G	61.52 [6.84×10 ⁷]	81.54 [4.53×10 ⁷]	94.22 [2.62×10 ⁷]	N.D.	N.D.
					4.60×10 ⁵
					7.77×10 ⁵
					4.66×10 ⁷

^a GHOST/X4/R5 cells and single-cycle HIV-1 pseudotypes were used in the titration assay (see basic protocol 3).

^b Values in brackets denote individual virus titer calculated as the following:
Virus titer [infectious unit (IU)/ml] = Cell number × % of GFP-positive cells × dilution factor. The dilution factor = 1,000 μl/viral input (μl).

^c Average cell number, 1.39 × 10⁶/sample.

^d Average titer of the results derived from the three different viral inputs.

^e N.D. Not done.