Production and Use of HIV-1 Luciferase Reporter Viruses

Presented in this unit are protocols for the preparation and use of HIV-1-based luciferase reporter viruses. These viruses are competent for only a single cycle of infection and thus serve as useful tools for accurate quantitation of the early steps in the virus replication cycle, starting from entry into the cell, up to transcription of the integrated provirus. Measurement of reporter gene activity is much easier and more quantitative than classical supernatant p24 ELISA. In addition, because the reporter viruses can be pseudotyped (prepared with heterologous envelope glycoproteins), findings can be definitively attributed to envelope glycoprotein function. The first procedure describes a method for the production of reporter virus stocks (see Basic Protocol 1). This is followed by the basic method for infecting cells with the HIV-1 luciferase reporter virus and measuring the resulting signal (see Basic Protocol 2). Two versions of the protocol are presented, one for infection of adherent cells and the other for nonadherent cells. The third protocol describes how to evaluate test compounds that inhibit entry of the virus into the cell (see Basic Protocol 3). The protocol can be adapted with slight modifications for evaluating neutralizing antibodies that target the viral glycoprotein or molecules that act postentry. For the first two protocols, amounts of virus, numbers of cells, and other parameters can be varied and should be tested for particular applications. Signal intensities may vary for different viruses and different cell type combinations. For higher throughput applications, the assay can be scaled for 384-well format.

CAUTION: The mutation in *env* greatly reduces the biohazard of working with reporter virus as compared to live virus; however, single-cycle viruses are not considered harmless because the stocks contain low levels of replication-competent virus that result from recombination during transfection or reversion. The viruses must, therefore, be used under conditions similar to those used for live HIV-1. Use containment procedures as for live HIV—ie., Biosafety level 3 (BSL3). Transfections can be prepared with BSL2 procedures and then moved into the BSL3 area. All materials are then to be handled with gloves and lab coat, and protective safety glasses worn. Aspirate discarded liquids into bleach and autoclave plasticware before disposal.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: pNL-Luc reporter virus plasmids can be obtained from the NIH Aids Research and Reference Reagent Program. Similiar vectors are also available from several other laboratories. pNL-Luc4 reporter virus is now available in which the luciferase gene has been replaced with the codon-optimized luciferase gene. This plasmid yields viruses that result in higher luciferase activity upon infection of primary cells. Envelope glycoprotein vectors are available from many laboratories and from the NIH AIDS Research and Reference Reagent Program, HEK293 and -293T cells (see Critical Parameters) are available from the American Type Culture Collection, and cell lines expressing individual chemokine receptors are available through the NIH AIDS Research and Reference Reagent Program (see Internet Resources).

NOTE: The Gag p24 ELISA is not described in this unit, but can be performed with home-made reagents or with a commercially available kit (e.g., Abbott, Coulter, DuPont Alliance/PerkinElmer).

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PRODUCTION OF HIV-1 LUCIFERASE REPORTER VIRUS

Reporter virus is generated by cotransfecting HEK293 cells with reporter virus plasmid DNA and an equal amount of envelope glycoprotein expression vector. The transfection protocol is based on the calcium phosphate method (Graham and van der Eb, 1973), which results in >80% of cells expressing a cotransfected enhanced green fluorescence protein (EGFP) expression plasmid. Reporter viruses can also be produced by lipofection which yields virus stocks of similar titer and is somewhat easier, but considerably more expensive to perform due to the cost of the lipid. While the protocol presented is for transfection of a single 10-cm plate, it may be scaled up by using multiple dishes of HEK293 cells or by doubling the DNA and HBS cocktail and using 15-cm culture dishes. A single transfected dish will yield 10 ml virus stock. For virus at 100 ng p24/ml, this will be sufficient for 1000 infections in a 96-well format. To serve as a negative control, a transfection should be performed in the absence of glycoprotein expression vector. In this case, virus will be produced, but will be noninfectious.

Materials

Confluent plate of HEK293 (ATCC# CRL-1573) or HEK293T (ATCC# CRL-11268) cells
PBS (Life Technologies)
Trypsin-Versene (Biowhittaker)
DMEM-10 (Biowhittaker, Life Technologies; also see *UNIT 7.2*)
Plasmid mixture: equal mixture of pNL-Luc reporter (available from authors) and envelope expression plasmids (NIH AIDS Research and References Reagent Program; see Internet Resources)
2 M $CaCl_2$ (see recipe)
2× HBS (see recipe)
Gag p24 ELISA kit (e.g., Dupont)

10-cm plates
2054 tubes, sterile (Becton-Dickinson)
0.45-µm syringe filter

Prepare reporter virus by transfection of HEK293 cells

1. Trypsinize a confluent plate of HEK293 or HEK293T cells by aspirating the culture medium, adding 5 ml PBS, aspirating the PBS, and then adding 1 to 2 ml trypsin versene. Incubate 2 to 5 min at room temperature or 37°C until the cells detach from the surface.

   See Critical Parameters for information on choice of cell type.

2. Resuspend the cells in 5 ml DMEM-10. Count cells and transfer 2.0×10^6 cells into a 10-cm plate in 10 ml medium. Incubate overnight.

   The cells will be ~25% confluent the next day.

3. The following day, add 20 µg plasmid mixture (10 µg reporter virus and 10 µg envelope glycoprotein plasmid) in a total volume of 450 µl, into a 1.5-ml microcentrifuge tube. Add 62 µl of 2 M $CaCl_2$ and vortex.

   These steps are performed under sterile conditions in a laminar flow hood using sterile pipets and pipet tips. Although the plasmid DNA is not sterile, it should be kept as clean as possible. For pseudotyping with the VSV-G (see Background Information), 6.0 µg VSV-G expression plasmid is sufficient.

4. Add 500 µl 2× HBS into a sterile 2054 tube. Add the $CaCl_2$/DNA solution dropwise from a Pasteur pipet at a rate of about 2 drops/sec without mixing. Immediately after completing the addition, flick several times to mix. Incubate 20 min on ice.

   A fine precipitate should form that is visible only at high power magnification. The precipitate will not be easily visible until several hours after transfection.

5. Add the precipitate drop-wise to the 10-cm plate of cells (step 2). Incubate overnight and change the medium the next day.

6. After 48 hours post-transfection, harvest the supernatant (which contains the virions) and centrifuge 5 min at 300 × *g* (1200 rpm in most table-top centrifuges), 4°C. Filter the supernatant through a 0.45-µm syringe filter and freeze in 0.5- to 1.0-ml aliquots at −80°C. Quantitate the virus using a Gag p24 ELISA kit.

   Virus can be stored at –80°C for several years without affecting activity. A successful prep will yield 50 to 300 ng p24/ml.

INFECT CELLS WITH HIV-1 LUCIFERASE REPORTER VIRUS

BASIC PROTOCOL 2

The NL-Luc-R^-E^- reporter virus (Chen et al., 1994; Connor et al., 1995) used in this protocol and produced as described (see Basic Protocol 1) contains the firefly luciferase gene (*Photinus pyralis*), which is detected by flash or glow reagents. The glow reagents are preferable because they produce light at a nearly constant level for several hours, allowing for flexibility in the timing of the experiment. The NL-R-Luc-R^-E^- reporter virus contains the Renilla (*Renilla reniformis*) luciferase gene (Mariani et al., 2000). The two luciferase genes can be detected individually in the same culture using Stop and Glow reagents (Promega), making it possible to use both viruses together and yet quantify them separately. This strategy has been used in some applications for normalization purposes (Mariani et al., 2000).

See Critical Parameters for a description of important controls.

Materials

Cells (NIH AIDS Research and Reference Reagent Program; see Internet Resources)
Reporter virus, frozen (see Basic Protocol 1)
DMEM-10 (*UNIT 7.2*)
Luc-Lite luciferase assay reagent (Packard)

96-well culture dishes
Microtiter plate luminometer
Hemacytometer
96-well black microtiter plates
Transparent microplate adhesion sealing film (Packard)

Infect adherent cells

1a. Plate cells the day before infection in 96-well culture dishes at 2×10^3 cells/well. Incubate overnight.

   Alternatively, 24-well plates can be used with 8×10^3 cells/well.

2a. The next day, thaw a frozen aliquot of reporter virus in a 37°C water bath (do not vortex). Transfer the thawed sample tube to an ice bucket. Adjust the virus to 20 ng p24/ml in DMEM-10.

While vortexing reduces the infectivity of the virus, keeping it cold during preparation will preserve its infectivity.

3a. Remove the medium from the plate of cells and add 50 µl fresh medium. Add 50 µl diluted virus to each well. Assay three replicates per infection.

4a. Measure luciferase activity 3 days post-infection by adding 100 µl Luc Lite assay reagent, incubating the plate 30 min at room temperature, and then reading in a microplate luminometer.

Infect suspension (nonadherent) cells

1b. The day of infection, pellet 1.0×10^7 cells by centrifuging 5 min at $300 \times g$ (1200 rpm in most tabletop centrifuges), room temperature

2b. Remove supernatant, resuspend the cells in 2.0 ml DMEM-10, and determine the number of cells per milliliter using a hemacytometer. Adjust cell density to 5.0×10^5/ml with medium and distribute 50 µl into the wells (2.5×10^4 cells/well).

Nearly any type of infectable transformed T cell line is suitable, including Jurkat, CEM, CEMx174, SupT1, Hut78, and PM-1. For primary activated lymphocytes, increase the number of cells/well 5-fold.

3b. Thaw a frozen aliquot of reporter virus in a 37°C water bath (do not vortex). Transfer the thawed sample tube to an ice bucket. Adjust the virus to 20 ng p24/ml medium. Add 50 µl diluted virus to each well. Incubate 3 days.

While vortexing reduces the infectivity of the virus, keeping it cold during preparation will preserve its infectivity.

4b. Add 100 µl Luc Lite luciferase assay reagent to each well. Transfer 100 µl of each lysate to the corresponding wells of a black 96-well microtiter plate. Cover the plate with transparent microplate adhesion sealing film and read the luciferase activity in a microplate luminometer.

For primary cells, assay 5 days postinfection.

BASIC PROTOCOL 3

EVALUATION OF CELL ENTRY INHIBITORS WITH HIV-1 LUCIFERASE REPORTER VIRUS

This protocol is for the evaluation of small molecules targeted to CCR5 (e.g., TAK-779, Baba et al., 1999; SCH-C, Striziki et al., 2001), but can easily be adapted to testing CXCR4 inhibitors (Schols et al., 1997), testing peptide inhibitors of gp41 (Munoz-Barroso et al., 1998), or titering neutralizing antibodies. A nonadherent transformed T cell line is used as the target in this example, although activated primary lymphocytes can be substituted. Adherent cells can be used with the modifications described above (see Basic Protocol 2).

Several different reporter viruses can be used in a single assay, each pseudotyped by a different envelope glycoprotein. VSV-G (Naldini et al., 1996; Reiser et al., 1996; Sharma et al., 1996) or A-MuLV Env pseudotypes (Page et al., 1990) should always be included to measure nonspecific and post-entry effects of inhibitors (see Commentary). See Critical Parameters for a discussion of important controls.

Materials

Cells (NIH AIDS Research and Reference Reagent Program; see Internet Resources)
Medium (e.g., RPMI-10; InVitrogen, Biowhittaker, Life Technologies; also see *UNIT 7.2*)
Viral entry inhibitor in DMSO
DMSO
Reporter virus, frozen (see Basic Protocol 1)
Luc-Lite luciferase assay reagent (Packard)

Hemacytometer
96-well culture dishes
Multichannel micropipettor (optional)
96-well black microtiter plates
Transparent microplate adhesion sealing film
Microtiter plate luminometer

1. Pellet 1×10^7 cells by centrifuging 5 min at $300 \times g$, room temperature. Remove supernatant, resuspend the cells in 2 ml medium, and determine the number of cells per milliliter with a hemacytometer. Adjust the cell density to 6.25×10^5/ml and distribute 40 µl into each well of a 96-well culture dish (2.5×10^4 cells/well).

   If activated primary T cells are used, then the cell number per well should be increased 5-fold.

2. In medium, make a 5-fold serial dilution of the viral entry inhibitor in DMSO from 10^{-1} to 10^{-9} as follows. Add 40 µl medium into nine sterile microcentrifuge tubes. Add 10 µl inhibitor into the first tube, vortex, and serially transfer 10 µl into each tube. Because the DMSO will cause a small increase in infection, generate a parallel serial dilution of DMSO and use it as a control for its effects on virus entry.

   If the inhibitor stock is 1 mM in DMSO, this dilution scheme will test a range of inhibitor concentration from 20 µM to 0.05 nM, which is sufficient to cover the range of known antiviral compounds.

   The assay can also be used to titer neutralizing antibodies. Prepare a 5-fold dilution series of antibody or inactivated serum in DMEM-10. Add 100 µl of each dilution to a 96-well culture dish. Add 20 µl diluted reporter virus to each well and incubate 1 hr at 37°C. Transfer 60 µl of each well to the cells prepared in step 1. Incubate 6 hr or overnight. Continue at step 5.

3. Transfer 10 µl of each dilution to the cells in triplicate (step 1). Leave three wells with no test compound. To these add 10 µl medium. Incubate for 30 min at 37°C.

4. Thaw an aliquot of reporter virus in a 37°C water bath (do not vortex). Transfer the thawed sample tube to an ice bucket and adjust the virus to 20 ng p24/ml medium. Add 50 µl diluted virus to each well. Incubate 6 hr or overnight.

   While vortexing reduces the infectivity of the virus by partially detaching the HIV envelope from the virion, keeping it cold during preparation preserves its infectivity.

5. The next day, centrifuge the plate 5 min at $300 \times g$, room temperature. Using a 200-µl micropipettor or multichannel micropipettor, remove as much of the medium as possible without disturbing the cells. Add 100 µl medium to each well.

6. Harvest the cultures 3 days postinfection by first adding 100 µl Luc Lite luciferase assay reagent and then transferring 100 µl of each lysate to the corresponding wells of a 96-well black microtiter plate.

7. Cover the plate with transparent microplate adhesion sealing film and read the luciferase activity in a microplate luminometer

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

$CaCl_2$, 2 M

Add 29.40 g $CaCl_2 \cdot 2H_2O$ to water and adjust volume to 100 ml. Pass through a 0.45-µm filter to sterilze. Store in aliquots up to 2 to 3 years at –20°C; after thawing, store up to 6 months at 4°C.

HBS, 2×

1.0 g HEPES
1.6 g NaCl
0.074 g KCl
0.025 g Na_2HPO_4 (for 7 H_2O use 0.047 g)
Adjust pH to 7.05 to 7.15
Adjust volume to 100 ml with H_2O
Pass through a 0.45-µm filter to sterilize
Store in aliquots up to 2 to 3 years at –20°C; after thawing, store up to 6 months at 4°C

Accurate pH and the correct amount of phosphate are critical for achieving high transfection efficiency.

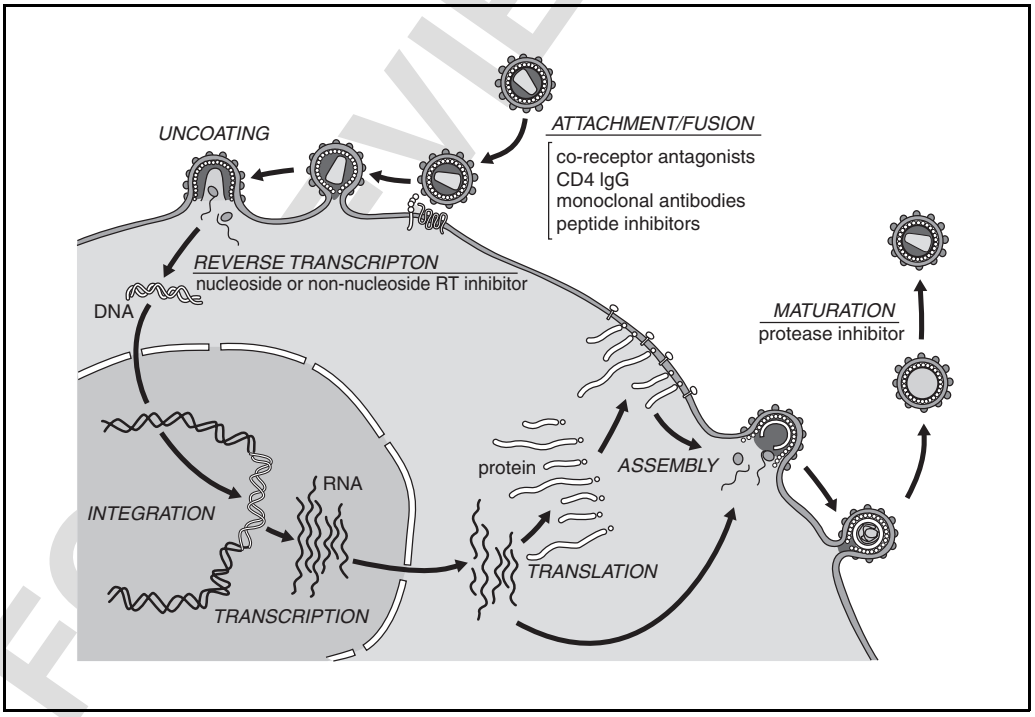

Figure 12.5.1 HIV-1 life cycle. Inhibitors are listed that act at various stages of viral replication. Entry inhibitors interfere with receptor interaction or membrane fusion. Inhibitors of reverse transcription are active postentry, but before integration. Protease inhibitors target the viral protease and inhibit maturation after virus release by the infected cell.

COMMENTARY

Background Information

Reporter viruses are an important research tool for the analysis of the cellular and viral components in HIV-1 replication. They allow for rapid and quantitative measurement of virus infection and can be used to great advantage for the screening and evaluation of antivirals.

Most reporter viruses consist of infectious HIV-1 proviral DNA that has been engineered to contain a reporter gene either in *nef* (a gene whose function is dispensible for single-round infection in vitro) or *env*. Expression of the reporter requires only the early steps of the virus replication cycle (entry into the cell, reverse transcription, integration, and provirus transcription). Subsequent steps in virus replication (virus assembly and budding) are not measured and do not affect reporter gene activity (see Fig. 12.5.1).

Reporter viruses have been constructed that contain genes for firefly luciferase (*Photinus pyralis*), Renilla luciferase (*Renilla reniformis*; Mariani et al., 2000), enhanced green fluorescent protein (EGFP; Page et al., 1997), alkaline phosphatase (He and Landau, 1995), and chloramphenicol acetyltransferase (CAT; Parolin et al., 1996). Viruses containing drug resistance markers such as *gpt*^{r}, *neo*^{r}, or *hygro*^{r} have also been useful (Page et al., 1990). The protocols presented in this unit use pNL-LucR$^-$E$^-$, a virus that contains the firefly luciferase gene in Nef, and has frameshifts engineered in *env* and *vpr* (viral protein R). Inactivation of Vpr eliminates potential negative effects caused by the apoptotic effects of Vpr.

Inactivation of *env* restricts the virus to a single round of replication. Because it is replication-defective, the virus must be produced by transfection and harvested as a culture supernatant. The virus is quantitated and frozen in aliquots for use as needed. Because they are *env*$^-$, an envelope glycoprotein expression vector plasmid DNA is included in the transfection and the viruses are released from the transfected

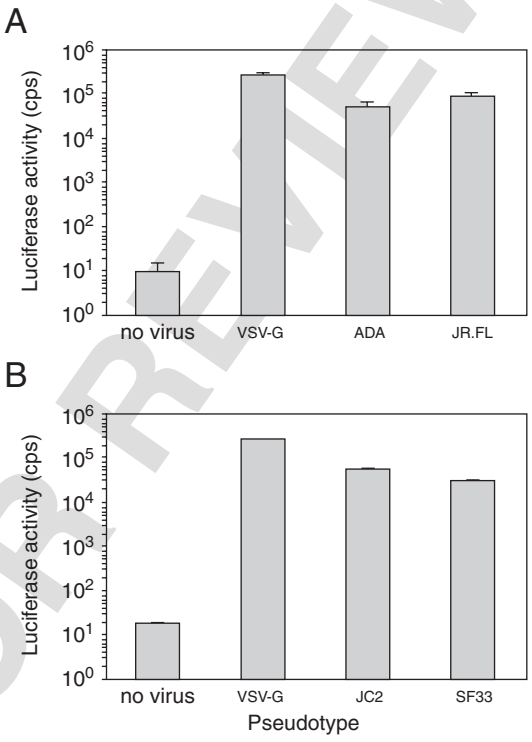

Figure 12.5.2 Infection of (**A**) HOS.CD4.CCR5 and (**B**) HOS.CD4.CXCR4 with NL-LucR$^-$E$^-$. Cells were infected with 1 ng p24, pseudotyped by CCR5-tropic (ADA, JR.FL) or CXCR4-tropic (JC2, SF33) glycoproteins, or with VSV-G. Luciferase activity was measured 3 days postinfection and plotted as counts per second (cps). Infection of 4×10^3 cells per 96-well dish with 1 ng HIV-1 luciferase reporter virus resulted in 5×10^4 to 2×10^5 cps. Cells not infected showed a background activity of 10 to 20 cps.

cells as pseudotypes bearing the transfected glycoprotein. A panel of pseudotypes can be generated, each with a tropism and entry mechanism dictated by the incorporated glycoprotein. Because retroviruses are fairly promiscuous with respect to glycoprotein incorporation, a wide range of infectious pseudotypes can be generated (Landau et al., 1991). Figure 12.5.2 shows the infection of HOS.CD4.CCR5 and HOS.CD4.CXCR4 cells with NL-Luc virus pseudotyped either with CCR5-tropic HIV-1 envelope (strains ADA and JR.FL), CXCR4-tropic HIV-1 envelope (strains JC2 and SF33), or VSV-G glycoprotein.

Because of the promiscuous nature of HIV-1 envelope glycoprotein incorporation, reporter viruses can readily be produced as pseudotypes in which the glycoprotein is derived from heterologous viruses (Landau et al., 1991). HIV-1 glycoproteins vary with respect to coreceptor usage (either CCR5 or CXCR4), CD4 dependence, and clade. Nevertheless, any HIV-1 glycoprotein is expected to efficiently pseudotype the NL-Luc-R$^-$E$^-$ core to form infectious virus. A-MuLV env and VSV-G pseudotypes enter cells independent of CD4 and coreceptor. Thus, they provide a means for definitively determining whether an inhibitor targets viral entry into cells or whether postentry or nonspecific cellular toxicities are at work (see Fig. 12.5.1). For example, an inhibitor that reduces infection of JR.FL-pseudotyped reporter virus, but does not inhibit VSV-G pseudotypes, acts at entry; however, whether the inhibitor targets the virus or the cell is not clear and distinguishing these two possibilities may not be straightforward. One means to test this is to add the inhibitor to cells, remove it following a brief incubation, and then add the reporter virus. If infection is blocked, the inhibitor most likely acts on the cell (either on CD4, CCR5, or CXCR4). If no effect is observed, no conclusion can be drawn. The converse, adding the inhibitor to the virus, is less practical, because removing the inhibitor by centrifugation reduces infectivity and takes too long.

Pharmacologic inhibitors of HIV-1 replication can be characterized by virus growth kinetics in culture using replication-competent virus. Inhibitors are added to activated primary CD4$^+$ lymphocytes or to an infectable transformed T cell line and virus replication is quantitated by measuring supernatant over a 2-week period by p24 ELISA or a reverse transcriptase assay. This method measures the effect of the inhibitor through multiple rounds of virus replication. However, generating growth curves is labor-intensive, requiring frequent sampling and passaging of the infected cultures. Growth curves are not readily suited for high throughput formats, are expensive, and do not provide information about which step in the virus replication cycle is being affected. Input virus may be carried over and mistaken for virus production at early points in the growth curve. In addition, there is the biohazard associated with working with live virus.

Single-cycle reporter viruses offer a useful alternative for characterizing HIV-1 inhibitors, particularly those that target early steps in virus replication. The measurement is sensitive (<0.001 pg luciferase enzyme can be detected), rapid, accurate, and relatively inexpensive. Because the viruses are competent only for a single round of replication, conclusions can be drawn concerning virus entry. Input virus is not measured since reporter gene activity requires infection. The assay is amenable to high throughput formats. This strategy also provides a means to evaluate the specific step in virus replication at which an inhibitor acts, which is not as easy as it might seem.

Although single-cycle reporter viruses are most suited for the evaluation of entry inhibitors, they can also be used to evaluate reverse transcriptase, integrase, or Tat inhibitors. Inhibitors, such as the protease inhibitors, that act late in the virus life cycle can also be evaluated, but this requires modification of the protocol. For these situations, the inhibitors are added to the HEK293 cells shortly following transfection with reporter virus DNA. The viruses are then harvested and used to infect a susceptible target cell.

Reporter viruses are useful for characterizing HIV-1 coreceptor usage by primary isolates. The primary virus *env* gene is amplified and cloned into an expression vector such as pcDNA-I (Invitrogen). Pseudotyped virus is prepared by cotransfection with pNL-LucR$^-$E$^-$ and the virus is used to infect a panel of cultured cell lines that stably express CD4 and individual chemokine receptors. HOS.CD4 and U87.CD4 cells expressing different CC-chemokine receptors or CXCR4 (Deng et al., 1996) are available through the National Institutes of Health AIDS Research and Reference Reagent Program (see Internet Resources). HOS.CD4 cells grow rapidly, but express trace amounts of endogenous CXCR4. The endogenous CXCR4 may allow low level entry of virus even in the presence of an effective CCR5 antagonist. U87.CD4 cells are more difficult to culture, but do not express detectable CXCR4.

Critical Parameters

Choice of cell line

Generation of high titered virus requires efficient cell transfection. If the p24 concentration of the virus-containing supernatant is <50 ng/ml, the transfection needs to be modified (see Troubleshooting). HEK293 and HEK293T cell lines differ by virtue of the expression of SV40 T antigen. HEK293T cells may express higher levels of the envelope glycoprotein if the vector has an SV40 replication origin; however, the effect on virus titer seems to be minimal.

Culturing the HEK293 cells requires care. They cannot be allowed to become overly confluent. Trypsinization must be sufficient to easily detach the cells from the dish to form a single cell suspension with little aspiration. For transfection, take care to spread the cells evenly on the dish and gently swirl to disperse them evenly on the plate.

Luciferase activity is higher in some transformed T cell lines than others and therefore comparisons of luciferase activity between cell types may be misleading. This may not be due to real differences in infectability, but rather to differences in the stability of luciferase. Differences in CD4 and coreceptor expression levels between cell lines can also affect the assay. CD4 and CXCR4 are expressed at a high level on most transformed T cell lines, but CCR5 is generally low to undetectable. Expression of transduced or transfected CCR5 is usually higher than that of the endogenous protein. Levels of all three molecules should be tested periodically by flow cytometry (Mariani et al., 2000).

Luminometers

Microtiter plate luminometers have significant amounts of cross-talk between wells (regardless of manufacturer's claims to the contrary). The cross-talk results in spill-over of signal from neighboring wells and can be quite noticeable when a low activity well is adjacent to a high activity well. Cross-talk is influenced by the type of assay plate used. The authors find that only the solid black plates are acceptable. A cross-talk measurement should be performed by reading a plate containing alternating blank and strongly positive columns. The number of counts per second (cps) present in the blank wells over background is a measure of cross-talk. To minimize cross-talk, it is advisable to avoid having wells containing an extremely large signal, or at least to least a blank column to separate wells containing high levels of luciferase activity. VSV-G pseudotypes have titers 10- to 100-fold higher than those bearing an HIV-1 glycoprotein. It is advisable to use smaller amounts of these (2- to 10-fold less) to reduce cross-talk on the assay plate.

Controls

Several controls are informative. A triplicate set of wells to which no virus is added is used to indicate assay background and variability. A second set is used for infection with virus lack-

Table 12.5.1 Selected HIV-1 Entry and Fusion Inhibitors[a]

Inhibitor	Target	EC_{50}[b]	IC_{50}[c]	Manufacturer[d]	Reference
TAK-779	CCR5	12–37 nM	1 nM	Takeda Chemical Industry	Baba et al., 1999; Shiraishi, et al., 2000
Sch-C	CCR5	0.4–9 nM	1 nM	Schering-Plough	Strizki et al., 2001
Merck CCR5 antagonists	CCR5	Unknown	1–35 nM	Merck	Dorn et al., 2001; Willoughby et al., 2001; Hale et al., 2001
AMD3100	CXCR4	0.01–0.1 nM	0.01–0.1 nM	AnorMED	Donzella et al., 1998
T-20	Transmembrane subunit of HIV-1 envelope protein	1.7 ng/ml	NA	Trimeris	Wild et al., 1993; Kilby et al., 1998

[a]Abbreviations: NA, not applicable.
[b]50% antiviral effective concentration. Can vary depending on the virus and the cell system used.
[c]50% concentration to inhibit binding of receptor ligand.
[d]Most compounds are not commercially available.

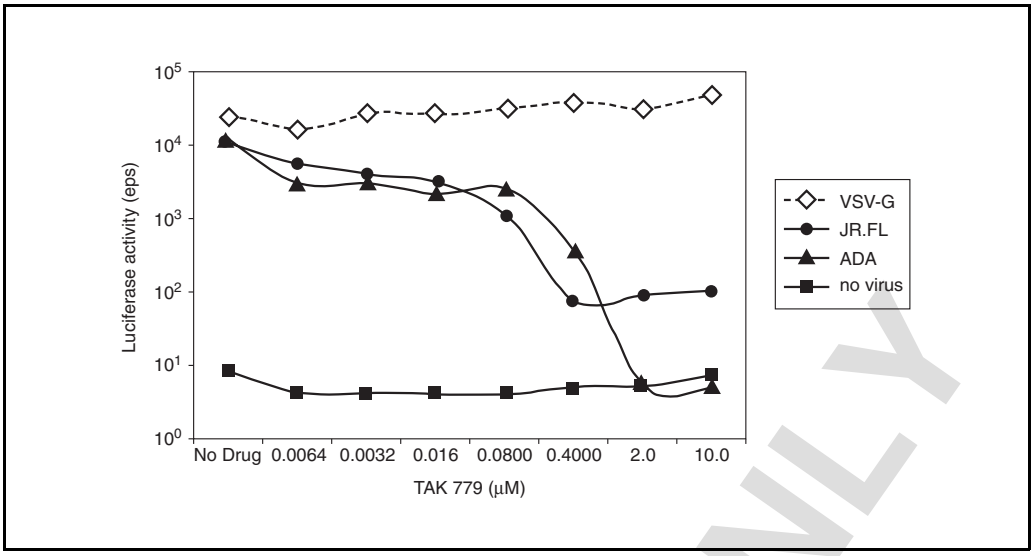

Figure 12.5.3 TAK 779 inhibition curve with NL-LucR$^-$E$^-$ pseudotyped by CCR5-tropic glycoproteins JR.FL and ADA. HOS.CD4.CCR5 cells were infected as described (see Basic Protocol 1). A noninfected control (closed squares) was also included. JR.FL (closed circles) and ADA (closed triangles), but not the VSV-G (open diamonds) pseudotype, were inhibited by the CCR5 antagonist TAK-779 (Baba et al., 1999).

ing an envelope glycoprotein to control for receptor/coreceptor-independent virus entry. For compound screening, a known inhibitor is added to one set of wells to demonstrate complete blocking. Useful inhibitors include MIP-1α, MIP-1β, TAK-779 for R5 viruses or AMD3100 for X4 viruses (see Table 12.5.1). Alternatively, an anti-CD4 monoclonal antibody such as Leu 3a can be used.

Troubleshooting

The most frequent problem encountered with the assay is low-activity reporter virus. Low luciferase activity is most likely due to insufficient transfection efficiency during preparation of the reporter virus. If the reporter virus p24 concentration is below 50 ng/ml, it is likely that the transfection was inefficient. Transfection efficiency can be monitored by spiking the transfection with 1 to 2 μg of an EGFP expression vector. Analysis by flow cytometry 48 hr post-transfection should show a broad curve of fluorescence for which >80% of the cells are positive. To improve the transfection efficiency, try thawing fresh HEK293 cells and changing transfection reagents. Plasmid DNAs must be pure, intact, and accurately quantitated. The quality and purity of the DNA should be checked on an ethidium bromide–stained agarose gel. Some HIV-1 glycoproteins are not potent or well expressed in the transfected cells. If this is the case, other vectors should be tested. Frozen aliquots of reporter virus of known activity should be kept on hand for standards and troubleshooting. If an experiment results in low luciferase activity, compare the standard alongside the current batch of reporter virus.

Many test compounds will be toxic at the highest concentrations tested. For example, at 10 μM, ~1% of random compounds tested were toxic (unpub. observ.). The VSV-G pseudotype allows for identification of such compounds, although this will also rule out compounds that specifically block a post-entry step.

Anticipated Results

In the absence of inhibitor, R5- and X4-specific viruses, should yield luciferase activity on the order of 1×10^5 cps for the conditions described in the protocol using adherent or nonadherent cells. Representative data are shown in Figure 12.5.2. Luciferase activity using primary cells will be 10- to 100-fold lower, since these cells are much more difficult to infect and are less metabolically active. VSV-G and A-MuLV pseudotypes will yield 10-fold higher luciferase activity. Uninfected wells will have a background of 50 to 200 cps and wells infected with env$^-$ virus will be <1000 cps. All assays points should be determined in triplicate and the data presented as the average of the triplicates. Standard deviation is calculated and should be <10%. Effective entry inhibitors will reduce the luciferase activity to close to background without decreasing the VSV-G pseudotype, at least until the inhibitor concentration

becomes toxic. A typical inhibition curve is shown in Figure 12.5.3.

Time Considerations

Production of reporter virus requires half a day followed by a 3-day incubation. An additional day is required for the p24 measurement. Setting up an infection with ten 96-well plates takes a day for a single worker. The plates are incubated 3 days and reading the assay and processing the data requires another day.

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Internet Resources

http://www.atcc.org

Online catalog from the American Type Culture Collection.

http://www.aidsreagent.org

Online catalog from the National Institutes of Health AIDS Research and Reference Reagent Program.

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