Chapter 27

Protocol for a Mammalian Cell-Based Assay for Monitoring the HIV-1 Protease Activity

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Abstract

Proteases are essential at different stages of the viral life cycle and for the establishment of a successful infection. Monitoring the catalytic activity of proteases in an easy and straightforward manner can thus drastically facilitate the discovery of novel antivirals, as well as help elucidate the activity and mechanism of action of the viral protease under study. In our laboratory, we have developed an assay in T-cells with a robust read-out to monitor the proteolytic activity of HIV-1 Protease (PR). The assay utilizes the prototypic transcription factor Gal4, which consists of the N-terminal DNA-binding domain and the C-terminal trans-activation domain. The assay is based upon (1) introduction of PR in between the two Gal4 domains to obtain a PR/Gal4 fusion protein and (2) utilization of the enhanced Green Fluorescent Protein as reporter of PR activity.

In order to overcome the possible cellular cytotoxicity of PR, the fusion protein in our assay is under the control of a tetracycline-inducible promoter. This ensures that it will be expressed only when needed, upon the addition of tetracycline or doxycycline. When active, PR has autocatalytic activity and cleaves itself from the Gal4 domains, resulting in the inability to induce eGFP expression. However, if PR activity is blocked or it is inactive, the two domains remain intact, resulting in eGFP expression. The assay can therefore be utilized to analyze the inhibitory effects of factors, peptides or compounds, designed on a rational- or nonrational-based approach, in the natural milieu of infection, where eGFP serves as a biosensor for PR activity.

Key words: HIV-1 protease, T-cell, Cell-based assay, Gal4 transcription factor, DNA-binding domain, Trans-activation domain, Green fluorescent protein (GFP), Drug screening, Drug discovery, Protease inhibitor, Autocatalytic activity

1. Introduction

Proteases are crucial at different stages of the viral life cycle and thus absolutely required in the establishment of a successful infection. HIV-1 encodes for an 11 kDa aspartic protease (PR) as part of a precursor poly-protein (1, 2). PR must cleave itself from the poly-protein before it can cleave the gag-pol precursor and nef

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(except for Envelope (3, 4), which is cleaved by host enzymes (5, 6). Hindering the proteolytic activity of HIV-1 PR, results in immature virions and aberrant infection. Hence, it is imperative to develop innovative assays to monitor HIV-1 PR activity, as well as to screen for new inhibitory compounds in a high-throughput manner. This assay can thus facilitate the development of novel antiretroviral drugs.

1.1. The assay

In our laboratory, we have developed a novel and straightforward assay to monitor HIV-1 PR activity (illustrated in Fig. 1) (7). While PR is active at the membrane or within the virions within the viral life cycle, in our assay PR is active in the cytoplasm and is totally membrane independent. To maximize the relevance of hits that will be found using this assay, it has been designed in T-cells, a cell line readily infected by HIV-1, in order to mimic the natural milieu of HIV infection. The assay utilizes Gal4 (8), which consists of an N-terminal DNA-binding domain (DBD) and a C-terminal trans-activation domain (TAD). The DBD recognizes and binds to the Gal4 responsive upstream activation sequences (UAS), which allows the TAD to activate transcription of the downstream reporter gene, the enhanced Green Fluorescent Protein (eGFP). The DBD and TAD must act in conjunction, and neither domain can act independently as a transcription factor (9).

The assay can be used to monitor the catalytic activity of other proteases: viral as well as host (10). The constructs described in

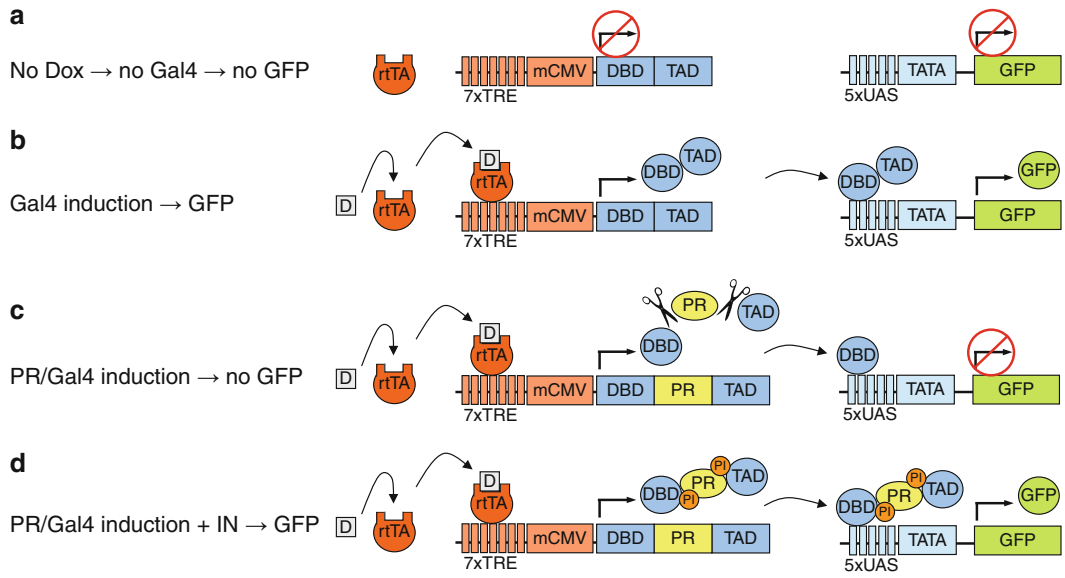

Fig. 1. Outline of the assay. *D* Doxycycline, *rtTA* Reverse tetracycline trans-activator, *DBD* DNA-binding domain, *TAD* Trans-activation domain, *TRE* Tet-responsive element, *UAS* Upstream activation sequence, *PR* Protease, *PI* Protease inhibitor, *GFP* Green fluorescent protein, *mCMV* Minimal Cytomegalovirus promoter, (adapted from Hilton and Wolkowicz (7)).

this paper can be used for this purpose as well, since the HIV-1 PR, including the cleavage sites, can be easily exchanged with any other protease of interest, and as such can serve as a platform to monitor proteases, provided their recognition/cleavage sites are included.

In our assay, wild-type PR, along with the PR cleavage sites, is introduced in between the DBD and TAD of Gal4 (PR/Gal4) (7, 11). When PR is active, it cleaves itself from the fusion protein, effectively separating the domains, resulting in no eGFP expression. However, if PR activity is inhibited or it is inactive, the domains remain intact and eGFP expression is induced. Thus, in our assay, eGFP acts as a biosensor of HIV-1 PR activity. A schematic of the assay is outlined in Fig. 1.

1.2. Tetracycline Inducible System

In order to circumvent the possible cytotoxicity of HIV-1 PR in mammalian cells (12), we adapted the tetracycline (tet) inducible system in order to express the PR fusion protein in an inducible manner. The tet-inducible (off/on) system consists of two elements: (1) Tet-responsive element (TRE), which has seven copies (7×) of the tet operator sequences (TetO) and a minimal CMV promoter, and (2) reverse tet-controlled trans-activator (rtTA). When doxycycline (dox) is added to the system (dox is a stable form of tet), it binds to rtTA, resulting in a conformational change, which allows the complex to bind to TetO, resulting in the transcription of downstream genes. In short, PR expression is tightly regulated by the tet system, and induced only upon the addition of dox.

1.3. Expression and Clonal Selection

All the assay elements are expressed via retro/lentiviral vectors to enable stable expression in mammalian cells. Two to three days after transduction with retro/lentiviral particles, T-cells are induced with dox. Cells expressing high levels of the reporter eGFP gene, with minimal background (highest dynamic range) are selected. A clonal population is generated to rule out genotypic differences and ensure that the population is homogenous.

2. Materials

2.1. Plasmids

The plasmids used to construct the clones for the assay are depicted in Fig. 2 (Plasmids are available upon request).

1. Transient expression: pFR-GFP (reporter construct), pcDNA 3.1 Gal4, pcDNA 3.1 PRm/Gal4, and pcDNA 3.1 PR/Gal4 (Zeocin), for transient expression studies (Fig. 2a) (see Note 1).
2. Stable expression: pH-5× UAS-GFP (reporter construct), pH-7× TRE-PR/Gal4, for stable but inducible expression of the PR/Gal4 fusion protein. pH-TRE is a self-inactivating lentiviral vector (with most of the 3′LTR U3 region removed) for

safety purposes as well as to ensure that the LTR promoter does not interfere with the UAS promoter of the reporter construct (Fig. 2b upper panel) (see Notes 2 and 3).

3. rtTA plasmid: pBMN-rtTA-i-mCherry is a Murine Leukemia Virus (MLV)-based plasmid for the stable expression of rtTA. The plasmid also expresses mCherry under internal ribosome entry site (IRES), enabling sorting for rtTA positive cells based on mCherry expression (Fig. 2b upper panel) (see Note 4).
4. Plasmids for viral production: pCMVΔ8.2 (packaging plasmid, expresses all the HIV-1 proteins, except Envelope), pCI-VSVg (expresses the Vesicular Stomatitis Envelope glycoprotein), pRSV-Vpr (expresses HIV-1 Viral Protein R, shown to enhance viral titers) (Fig. 2b lower panel).

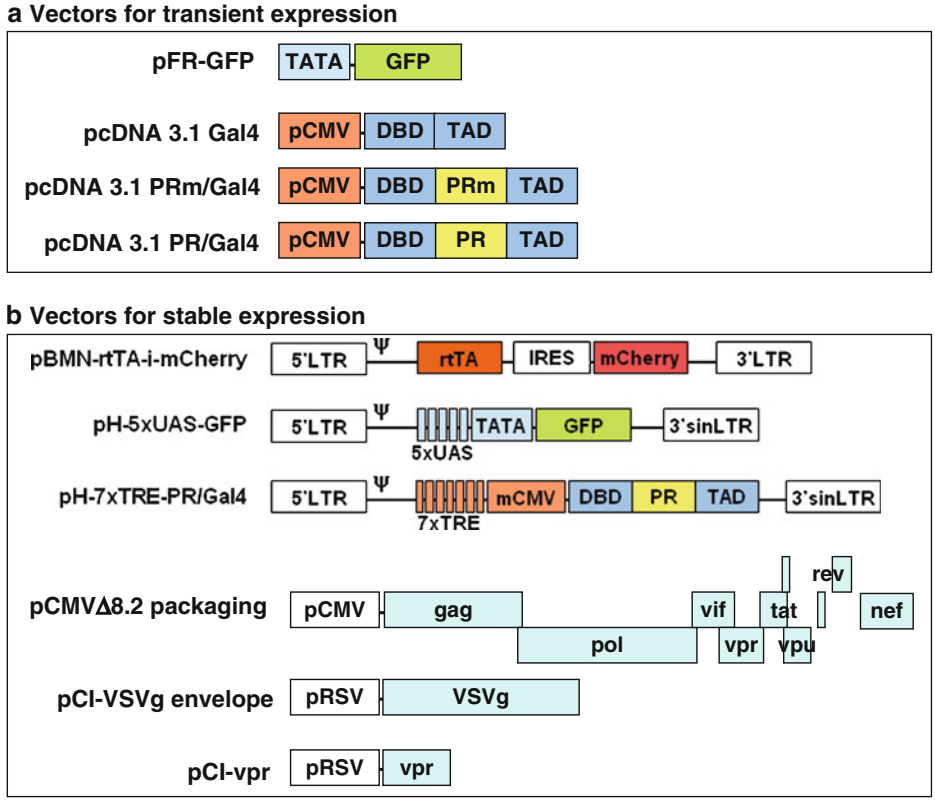

Fig. 2. Schematic representation of the plasmids. (**a**) Vectors for transient expression include the reporter pFR-GFP and Gal4, PRm/Gal4 and PR/Gal4 expression plasmids. (**b**) Vectors for stable expression include, in the *upper panel* transfer vectors: retroviral vector expressing rtTA (pBMN-rtTA-i-mCherry), lentiviral reporter vector (pH-5× UAS-GFP), and lentiviral vector expressing inducible PR/Gal4 fusion protein (pH-7× TRE-PR/Gal4) (see Note 4); and in the *lower panel* vectors for the production of HIV-based lentiviral particles: pCMVΔ8.2 packaging, pCI-VSVg envelope, and pCI-vpr regulatory plasmids. *LTR* Long terminal repeat, *sinLTR* Self-inactivating-LTR, *Ψ* Packaging signal, *rtTA* Reverse tetracycline trans-activator, *IRES* Internal ribosome entry site, *DBD* DNA-binding domain, *TAD* Trans-activation domain, *TRE* Tet-responsive element, *UAS* Upstream activation sequence, *mCMV* Minimal Cytomegalovirus promoter, *PR* Protease, *GFP* Green fluorescent protein, *VSVg* Vesicular Stomatitis Virus glycoprotein, *pRSV* Rous Sarcoma Virus promoter.

2.2. Validation of the Assay with Transient Expression

1. Cells:
   (a) HEK293T (ATCC CRL-11268) are adherent cells used for transient expression studies.
2. Plasmids:
   (a) pcDNA Gal4, PR/Gal4, PRm/Gal4, and pFR-GFP (Fig. 2a).
3. Media:
   (a) HEK293T are grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin–streptomycin and 2mM L-Glutamine and 10 % (v/v) fetal calf serum (FCS).
   (b) Cells are resuspended in 1× Phosphate Buffer Saline (PBS) for flow cytometric analysis (see Note 5).
4. Reagents:
   (a) Polyethylenimine linear 25 kD (PEI) is dissolved in nano pure water to obtain a stock concentration of 2 mg/mL. Aliquots are stored at –80 °C.
   (b) Stocks of FDA approved PR Inhibitors (PI), i.e., Darunavir are dissolved in DMSO and stored at –20 °C at a concentration of 10 mM. Working stocks (at various concentrations as needed) are stored at 4 °C. The reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.
5. Flow cytometry:
   (a) Flow cytometer with a 488 nm laser for the detection of GFP.

2.3. Production of Stable and Inducible Clones for Screening

1. Cells:
   (a) HEK293T (ATCC CRL-11268) and Phoenix GP cells are adherent cells used for the production of lentiviral and retroviral particles, respectively.
   (b) SupT1 (ATCC CRL-1942), a T-cell line readily infected by HIV-1 (see Note 6).
2. Plasmids:
   (a) pCI-VSVg, pRSV-Vpr and pCMVΔ8.2 for the production of retroviral particles and pH-7× TRE-PR/Gal4, pBMN-rtTA-i-mCherry and pH-5× UAS-GFP for the assay elements (Fig. 2b) (see Note 7).
3. Media:
   (a) HEK293T and Phoenix GP cells are grown in DMEM, supplemented with penicillin–streptomycin and 2mM L-Glutamine and 10 % (v/v) FCS.
   (b) SupT1 cells are grown in RPMI, supplemented with penicillin–streptomycin and 2mM L-Glutamine and 10 % (v/v) FCS at 37 °C and 5 % CO_2.

(c) Cells are sorted in 1× PBS with 1 % (v/v) FCS. (see Note 5)

4. Reagents:
   (a) Polyethylenimine linear 25 kD (PEI) is dissolved in nano pure water to obtain a stock concentration of 2 mg/mL. Aliquots are stored at −80 °C.
   (b) Polybrene (Hexadimethrene Bromide) is dissolved in nano pure water at 5 μg/ml and stored at −20 °C.
   (c) Stocks of FDA approved PR Inhibitors (PI), i.e., Darunavir are dissolved in DMSO and stored at −20 °C at a concentration of 10 mM. Working stocks (at various concentrations as needed) are stored at 4 °C. The reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.
   (d) Stock of doxycycline is dissolved in nano pure water at a concentration of 1mg/mL and stored at −20 °C.
5. Flow cytometry:
   (a) Fluorescence Activated Cell Sorter (FACS) with Automatic Cell Deposition Unit, (ACDU) (see Note 8).

2.4. Verification of Assay Responsiveness to FDA Approved Inhibitors

1. Cells:
   (a) Selected SupT1 clones from Subheading 3.3.
2. Media:
   (a) RPMI, supplemented with penicillin–streptomycin and 2 mM L-Glutamine and 10 % (v/v) FCS at 37 °C and 5 % CO_2.
   (b) Cells are resuspended in 1x PBS for flow cytometric analysis. (see Note 5)
3. Reagents:
   (a) Stocks of FDA approved PR Inhibitors (PI), i.e., Darunavir are dissolved in DMSO and stored at −20 °C at a concentration of 10 mM.
   (b) Stock of doxycycline is dissolved in nano pure water at a concentration of 1 mg/mL and stored at −20 °C.
4. Flow cytometry:
   (a) Flow cytometer with a 488 nm laser for the detection of GFP.

3. Methods

3.1. Plasmids

Prepare stock solutions of all necessary plasmids listed in the materials section to ensure pure plasmid samples (Maxiprep).

3.2. Validation of the Assay with Transient Expression

In order to validate the expected behavior of the proteins in the assay, transfect cells in the presence and absence of PI (see Note 9).

1. Grow HEK293T cells in a 6-well plate to about 50–60 % confluency (around 300,000 cells per well, 24 h prior to transfection) (see Note 10).
2. Incubate the cells in a humidified incubator in atmosphere enriched with 5 % CO_2 at 37 °C.
3. For transfections in the presence of PI, add PI (Darunavir) to the cells to a final concentration of 1 μM, at least 10 min *prior* to transfection (see Note 11).
4. In a 0.6 mL Eppendorf tube, add 125 μL DMEM only (without FCS, PenStrep or L-Glutamine), 15 μL of polyethylenimine linear 25 kD and 3 μg of plasmids (pcDNA Gal4, PR/Gal4 and PRm/Gal4, along with pFR- GFP) drop-wise and mix thoroughly.
5. Leave this mixture at room temperature for 20 min.
6. Add this mixture to all cells drop-wise.
7. Put the cells back in the humidified incubator with 5 % CO_2 at 37 °C.
8. 24 h post-transfection, replace the media of transfected cells.
9. 48 h post-transfection analyze the cells by flow cytometry and/or fluorescence microscopy.

In the presence of the reporter, PR/Gal4 and 1 μM Darunavir there should be maximal activation of eGFP. On the other hand, reporter alone or reporter with PR/Gal4 in the absence of PI should have minimal eGFP expression.

3.3. Production of Stable and Inducible Clones for Screening

Development of a stable cell line enables preselection of an optimal clonal cell line, which exhibits the most robust and reliable readout for PR inhibition. Due to the potential cytotoxicity of PR in cells, its expression is induced after the cells have been grown and prepared for screening.

Generation of viral particles for transduction of the target SupT1 cells (see Note 12).

Production of Retroviral Particles (pBMN-rtTA-i-mCherry)

1. Plate Phoenix GP cells at about 50–60 % confluency in 10 mL of fresh medium in a 10 cm plate (Phoenix GP cells express gag-pol of MLV and require only a transfer vector and an envelope-expressing vector, for the production of MLV-like particles).
2. After 24 h, transfect (same procedure as in Subheading 3.2) with 3 μg of pBMN-rtTA-i-mCherry and 3 μg of pCI-VSVg.
3. Incubate the cells in a humidified 5 % CO_2 incubator.

4. Replace the media 24 h post-transfection.
5. Collect the viral supernatant 48 h post-transfection, (see Note 13) filter out cellular debris using 45 μm filters (Pall Corporation) and store the viral stocks at −80 °C in aliquots (see Note 14 and 15).

Production of Lentiviral Particles (pH Vectors)

1. Grow HEK293T cells at about 50–60 % confluency in a 10 cm plate for each transfer vector used (pH vectors).
2. Transfect as described in Subheading 3.2 with 3 μg of the transfer vector (pH-7× TRE-PR/Gal4), 3 μg pCI-VSVg, 1.5 μg of pRSV-Vpr (shown to enhance viral titers) (13), and 2 μg of the packaging vector, pCMVΔ8.2, (kindly provided by Didier Trono, EPFL, Switzerland) (14).
3. Replace the media 24 h post-transfection.
4. Incubate the cells at 37 °C in a humidified incubator with 5 % CO_2.
5. Collect the viral supernatant 48 h post-transfection, filter out cellular debris using 45 μm filters (Pall Corporation), aliquot the viral stocks and store them at −80 °C.

Establishment of rtTA Cell Line (See Note 16)

1. Thaw the frozen viral stock of rtTA-i-mCherry.
2. Seed cells at a density of about 250,000 cells/well in 3 mL of fresh medium for each well of a 6-well plate.
3. Add 12 μL of polybrene (stock at 5 μg/mL) to the cells.
4. Add 1 mL of the viral stock to the polybrene-treated cells, followed by fresh media up to 3 mL and mix well (see Note 17).
5. Centrifuge the plate at 1,500×*g* for 80 min. at 32 °C in a hanging bucket rotor (Spin infection).
6. Resuspend the cells in the plate at the end of the cycle.
7. Incubate the cells in a humidified incubator with 5 % CO_2.
8. Resuspend the cells in fresh media 24 h post-infection.
9. 72 h post infection, analyze the cells by flow cytometry and sort a minimum of 50,000 mCherry positive cells (see Notes 5 and 18).
10. Expand the sorted cells, and reanalyze to verify the purity of rtTA-mCherry population.
11. If necessary, sort again to further purify the population.

Co-infection of the rtTA Cell Line with the Reporter and the Inducible PR/Gal4 Viral Particles

1. Thaw the 5× UAS GFP reporter and, PR/Gal4 fusion viral stocks.

2. Count the rtTA mCherry expressing cells and seed about 250,000 cells/well of a 6-well plate in 3 mL of fresh medium.
3. Add 12 μL of polybrene to the cells.
4. Add 1 mL of the viral stocks of pH-5× UAS-GFP and 1 mL of the pH-7× TRE-PR/Gal4 to the cells (see Note 17).
5. Add fresh medium to bring the final volume to 3 mL.
6. Centrifuge the plate at 1,500 × *g* for 80 min at 32 °C in a hanging bucket rotor (Spin infection).
7. Resuspend the cells at the end of the cycle in fresh medium.
8. Incubate the cells in a humidified incubator with 5 % CO_2.
9. Resuspend the cells in fresh media 24 h post-infection.

Establishment of an Efficient Reporter Cell Line

1. Expand the transfected HEK293T cell lines generated above for about 1 week.
2. Place 300,000 cells/well in 3 mL of fresh medium in each well of a 6-well plate.
3. Add Darunavir and dox to a final concentration of 1 μM and 1 μg/mL, respectively (see Notes 11 and 19).
4. After about 48 h, verify the presence of eGFP, under a fluorescent microscope.
5. Analyze the cells and sort for the cells expressing high levels of eGFP in an FACS.
6. Allow the sorted eGFP population to expand until fluorescence diminishes.
7. Add dox again to the cells and sort out the negative population to eliminate background activation.
8. Repeat purification once more (see Note 20).

Generation of Clonal Populations

1. Activate the cells with Darunavir and dox, as above.
2. Sort cells into at least two 96 well plates at a single cell per well.
3. Visualize the wells under a microscope to confirm that the wells truly contain single cells. Ignore and omit wells containing no cells or multiple cells.
4. Allow the cells to expand (this may require a few weeks).
5. Activate individual expanded clones with 1 μg/mL dox alone, and with both 1 μg/mL dox and 1 μM Darunavir.

Analyze cells by FACS to identify the ideal clone to be used for screening purposes as well as to monitor the catalytic activity of PR. The optimal clonal cell line will be one which has the lowest

eGFP ratio between cells with dox only compared to cells with dox and PI (i.e., cells with dox only should have little to no eGFP expression, but when PI is also added, these cells should have close to 100 % eGFP expression and the highest mean fluorescence intensity of eGFP as well).

3.4. Verification of Assay Responsiveness to FDA Approved Inhibitors

Expand the selected clonal cell line and test other FDA approved PI to verify that the responsiveness of the clone is not limited to a specific inhibitor.

1. In a 96-well plate, seed 50,000 cells/well in 3 mL fresh medium from the selected clonal population.
2. Pre-incubate these cells with the specific PI at varying concentrations for at least 10 min.
3. Activate with 1 μg/mL dox.
4. Analyze responsiveness to the PI using flow cytometry as described above.

Titration of Doxycycline

In order to avoid over-activating the PR/Gal4 fusion protein, titrate the concentration of dox required to maximally induce eGFP expression in the presence of a PI.

1. Perform the titration experiment in a 96-well plate with 50,000 cells/well.
2. Incubate cells in each well with 1 μM Darunavir and add varying concentrations of dox.
3. Analyze the cells by flow cytometry and determine the saturating point of dox.

4. Notes

1. pFR-GFP and pcDNA 3.1 PR/Gal4 constructs harbor the same elements as pH-5× UAS-GFP and pH-7× TRE-PR/Gal4 vectors, respectively, and are described in Fig. 2 top panel; the only difference being that they are not retro/lentiviral in nature.
2. When amplifying retroviral/lentiviral plasmids, grow bacteria harboring plasmids at 30 °C or 32 °C rather than 37 °C, since at higher temperatures there is a greater chance of recombination.
3. For the production of stable cell lines with inducible elements, the vectors used in this assay are highly recommended. Nevertheless, other vectors displaying similar characteristics can also be used.

4. rtTA is also available in a plasmid carrying a zeocin antibiotic resistance marker pBMN-rtTA-i-Zeocin, and therefore can be selected for with zeocin, rather than fluorescence (mCherry)-based sorting.
5. RPMI contains residual compounds that are considered to interfere with the fluorescent properties of the samples, and therefore it is typically recommended to fully remove the media before analyzing the samples by flow cytometry. However, we have not noticed any interference with the signals, by analyzing samples directly in their growth medium. However, for sorting, cells MUST be resuspended in the proper sorting buffer.
6. The cell line for the assay chosen here is SupT1. Other relevant cell types for HIV-1 such as other T-cell lines, macrophages, and so on can be used as well. Naturally, if adherent cells are used instead, tissue culture practices should be adapted to adherent versus non-adherent cells.
7. pH-7× TRE-PR/Gal4 is described here. A control vector for Gal4 activity (pH-7× TRE-Gal4) and another one for a mutant catalytically inactive version of PR (pH-7× TRE-PRm/Gal4) were utilized in the original assay to corroborate the expected behavior of the assay. While obviously Gal4 will serve as a control for maximum eGFP induction irrespective of the presence of inhibitors, the PRm/Gal4 mutant fusion will serve as a control for insertion of PR within the Gal4 domains. As this PR is inactive, eGFP expression is expected even in the absence of inhibitor.
8. Clones were sorted here using a flow cytometer with an automatic cell deposition unit. However, without this technology, the cells can be counted, diluted to one cell/well and incubated. This method of clonal expansion is far less reliable and requires careful observation of plated wells to confirm that only a single cell was deposited in each well. However, if a cell-sorter is not available, it is still a viable option.
9. A transient experiment to validate the expected behavior of the constructs *prior* to the more elaborate and time-consuming task of obtaining the final cell lines is highly recommended, but not compulsory.
10. All the transfections were done with cells at a confluency of 50–60 %; however, lower or higher cell concentrations can be used. In this case, transfection efficiency could be lower, or cell death higher, and therefore should be tested.
11. Any of the FDA approved PIs can be used to activate the expression of eGFP in the validation and selection steps outlined here. However, Darunavir has been observed in this assay to have the lowest IC50 and minimal cell death at higher

concentrations and is therefore recommended as a PI control. Indinavir was used as control in the original experiments (7).

12. While replicative virus is not used in the experiments outlined here, because of the production of MLV, and HIV particles (infectious for one round, but non-replicative), a Biosafety Level-2 environment is required for the development of the assay.
13. It is recommended that the viral supernatant be collected at 48 h. However, virus can still be collected at later time points, up to about 72 h. Harvesting outside of the 48–72 h window, however will probably significantly reduce the viral titer.
14. Filtering the viral supernatant with 45 μm filters is not mandatory, but it improves the infection efficiency. The cellular debris from the supernatant can also be removed by centrifugation at 525 × *g* for 5 min.
15. Upon freeze thawing the viral stock, it is typically assumed that about 50 % of the infectivity is lost. However, we have not noticed such drastic loss in infectivity. The virus can be stored at 4 °C for about 3 days. However, there is about 50 % loss in infectivity with each day at 4 °C. In other words, freeze thawing cycles should be avoided.
16. Development of an rtTA cell line *prior* to co-infection can be bypassed if infection efficiencies are high enough to infect the cells with all three viral particles (carrying each of the assay elements) simultaneously.
17. When transducing cells, it is a good idea to infect at various Multiplicities of Infection (MOI) to ensure efficient infection. High expression of reporter from one infectious event could prevent the need for an MOI larger than one.
18. While 50,000 cells is recommended in our protocol, sorting more or less cells is acceptable; however, growth and recovery times will then vary.
19. For the tetracycline inducible system, we use dox to induce expression, since it is more stable at room temperature. However, tetracycline would be expected to be a comparable alternative.
20. The method used here for enrichment of a responsive cell line to select the most inducible clone with the clearest eGFP readout was two rounds of sorting, first in the presence of PI and dox, and then only with dox. Other selection methods can also be used, such as using resistant markers (resistance to blasticidin, zeocin, and so on) and then selecting with the proper antibiotics. However, these methods should still incorporate FACS to enrich for the most responsive population and to eliminate background.

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