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Edwin Yunhao Gong *Editor*

Antiviral Methods and Protocols

Second Edition

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Second Edition

Edited by

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Cover illustration: The blue background is the crystal violet staining of the whole cell monolayer. The white dots are virus particles (one white dot is derived from one virion). In the last well, only one virion is present at that drug concentration (the test compound at that concentration totally inhibits virus replication), and one can count the number of white dots to calculate the drug effect. This image represents duplicate wells for each drug test concentration.

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Preface

Viruses are the leading cause of disease and death worldwide, and antiviral drugs are one of the most effective ways, in addition to vaccines, to control and manage viral infections. Although antiviral drugs have been successfully developed for some viral diseases, there remains a clear unmet medical need to develop novel antiviral agents for the control and management of many viruses that currently have no or limited treatment options as well as a need to overcome the limitations associated with the existing antiviral drugs, such as adverse effects and emergence of drug-resistant mutations.

The first edition of *Antiviral Methods and Protocols* was published in the *Methods in Molecular Medicine* series (discontinued some years ago) in 2000. This new edition, published in the *Methods in Molecular Biology* series, reflects the major technical advances for the last 13 years in antiviral research and discovery. Although it is flagged as the second edition, all chapters are completely new, and none of the chapters is simply updated from the previous edition. This edition focuses on many important human viruses that cause major problems in public health, such as human immunodeficiency virus type 1 (HIV-1), hepatitis viruses (hepatitis B and C viruses), herpes viruses, and influenza virus. Many new chapters describing antiviral methods and protocols against respiratory viruses, such as human respiratory syncytial virus (4 chapters), and some important emerging viruses, such as dengue virus (7 chapters), West Nile virus (1 chapter), and chikungunya virus (2 chapters), are included. In addition, as the in vivo evaluation of antiviral efficacy and modes of action using animal models is very important to antiviral drug discovery and development, several chapters describing animal models for evaluation of antiviral agents in vivo are also presented.

Consistent with the approach of the *Methods in Molecular Biology* series, each chapter contains detailed cutting-edge research techniques that are currently used in the field of antiviral research. Many methods and protocols presented in this edition are fully validated, high-throughput antiviral assays that can be used for screening compound libraries in pharmaceutical companies and research institutions. Overall, this new edition of *Antiviral Methods and Protocols* will serve as a laboratory reference for pharmaceutical and academic biologists, medicinal chemists, and pharmacologists as well as for virologists in the field of antiviral research and drug discovery.

I would like to thank Professor John Walker, chief editor of the series, for his advice. I also wish to express my gratitude to the editors from Springer, especially Patrick Marton, David Casey, Anne Meagher and Paul Wehn, for making this new edition possible, and to Sundaramoorthy Balasubramanian, Project Manager at SPi Global, India, for his valuable contribution to edit this book, and to all the chapter authors for their valuable contributions to this book.

Beerse, Belgium ***Edwin Yunhao Gong***

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Part I

Human Immunodeficiency Virus Type 1 (HIV-1)

Chapter 1

A Fluorescence-Based High-Throughput Screening Assay to Identify HIV-1 Inhibitors

Peggy Geluykens, Koen Van Acker, Johan Vingerhoets, Christel Van den Eynde, Marnix Van Loock, and Géry Dams

Abstract

Highly active antiretroviral therapy (HAART) dramatically increases the long-term survival rates of human immunodeficiency virus type 1 (HIV-1) infected patients. Yet, poor adherence to therapy, adverse effects and the occurrence of resistant viruses can compromise the efficacy of HAART regiments. Therefore, there remains a clear unmet medical need for novel drugs and treatment options. In this chapter, we describe an HIV-1 antiviral high-throughput screening assay based on an HIV-1 permissive T lymphoblastoid MT4 cell line, stably transfected with a construct carrying an HIV-1 long terminal repeat promoter driving the expression of a reporter gene (enhanced green fluorescent protein). This assay runs in a 384-well format and enables the identification of HIV-1 inhibitors during a high-throughput screening campaign. In parallel, a cytotoxicity assay is performed to evaluate the compound-related in vitro toxicity.

Key words HIV-1, High-throughput screening, Fluorescence-based assay, HIV-1 inhibitors

1 Introduction

In 2009, approximately 33 million people were living with HIV-1 (WHO, AIDS epidemic update, 2010). More importantly, it was reported for the first time that the spread of HIV-1 was halted and even reversed. This success can be contributed to the implementation of HAART [1]. Nevertheless, there remains a significant unmet need as the long-term success of HAART can be compromised by poor adherence to therapy, adverse effects and HIV-1's propensity to develop resistance against antiretroviral drugs, which limit treatment options for patients.

Cell-based antiviral assays are widely used to underpin the discovery of antiviral compounds and offer tractable methodologies for large scale operations such as high-throughput screening and smaller, routine monitoring of medicinal chemistry efforts during hit to lead and lead optimization processes [2]. Cell-based antiviral assays offer

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an attractive way to identify HIV-1 inhibitors, as these "holistic" assays encompass, in one single test, the complete range of antiviral and cellular targets which are critical for viral replication. This enables targeting the virus in its natural cellular environment, whereas biochemical assays are mostly focused on one specific target and the active compounds in these assays are not necessarily able to enter cells due to their chemical properties. In the first generation cell-based assays, the antiviral activity of compounds was evaluated based on their inhibition of viral cytopathic effects (CPE) in cell culture. In these assays, an HIV-1-susceptible cell line is infected with the wild-type virus in the presence of a potential antiviral compound. When viral replication proceeds, the cells are killed by the virus, whereas antiviral active compounds protect the cells from virus-mediated cell death. In parallel, the cytotoxicity of the compounds is assessed on uninfected cells, which enables the determination of the compound's selectivity. However, observing virus-induced CPE requires several days (>5 days) of incubation, which makes the assay more prone to compound-related toxicity. Therefore, the tolerance towards toxic compounds is low, which compromises the ability to identify compounds with low selectivity indexes. Pauwels et al. [3] described such a CPE-based antiviral assay, which was based on the infection of human MT4 cells with laboratory-adapted HIV-1 strains. Due to the excellent replication rate of these HIV-1 strains in the MT4 cell line, HIV-1-mediated cell death can be detected with the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), at 5 days post-infection [4]. The first HIV-1 non-nucleoside reverse transcriptase (RT) inhibitor was discovered with such an MT4/MTT assay [5].

Later on, the second generation antiviral screening assays were developed, in which target cells are equipped with a reporter gene to assess the inhibition of viral replication by small molecules. Upon HIV-1 infection and replication, the reporter gene is expressed in an HIV-1 dependent manner. Antiviral compounds which inhibit HIV-1 replication also inhibit the reporter gene expression. Throughout the years, several reporter genes have been used to develop screening assays, including chloramphenicol acetyltransferase, luciferase, green fluorescent protein, alkaline phosphatase, and β-galactosidase [6]. As described above, the selectivity of the compounds is determined by running a cytotoxicity test in parallel using mock-infected reporter cells in which the reporter gene expression is controlled by a constitutive promoter.

Here, we describe in details a cell-based reporter assay using stably transfected MT4 cells that carry an enhanced green fluorescent protein (EGFP) reporter gene under the control of the HIV-1 long terminal repeat (LTR) promoter [7]. These cells express basal levels of EGFP that can be easily detected by fluorescence microscopy or flow cytometry. During HIV-1 replication, the viral Tat protein is expressed and binds to the LTR, up-regulating the EGFP

expression levels at least tenfold. Consequently, the infected cells can be discriminated from noninfected cells by their fluorescence intensity. Active antiviral compounds inhibit the HIV-1 replication cycle and as such will reduce the Tat-induced EGFP signal. EGFP expression levels can be quantified by automated fluorescence microscopy or flow cytometry. The EGFP-based assay has several advantages over the CPE assays. First, EGFP is an intrinsically fluorescent protein which does not require any substrate, making the assay homogeneous and noninvasive. Due to this mix-and-read principle, the readout is completely harmless to the cells and the same plate can be measured repeatedly, which introduces flexibility and reduces costs considerably. Second, the EGFP signal is generated before the MT4 cells die, allowing detection of viral infection in living cells which results in a shorter incubation time as the assay does not rely on life–death discrimination. Third, the microscopic readout allows for the convenient retrieval of images and raw data afterwards. One disadvantage of the EGFP-based assay, however, is that the intrinsically fluorescent compounds or serum components can interfere with the fluorescent readout of the assay.

2 Materials

2.1 Reagents

1. HIV-1 IIIB virus: kindly provided by Dr Guido van der Groen (Institute of Tropical Medicine, Antwerp, Belgium).
2. MT4 cells (human T lymphotropic virus type 1-transformed human T lymphoblastoid cell line): kindly provided by Dr Naoki Yamamoto (National Institute of Infectious Diseases, AIDS Research Center, Tokyo, Japan).
3. MT4-LTR-EGFP cells were obtained by transfecting MT4 cells with a selectable construct encompassing the HIV-1 LTR promoter driving the expression of EGFP and subsequent selection of stably transfected cells [7] (*see* **Note 1**).
4. MT4-CMV-EGFP cells were obtained by stably transforming MT4 cells with a CMV–EGFP construct [7].
5. RPMI/10 % FBS: RPMI-1640 with Ultra Glutamine and HEPES without phenol red (Lonza-BioWhittaker®), supplemented with 10 % fetal bovine serum (Sigma) and 0.02 mg/mL gentamicin (Gibco).
6. Geneticin® (G418; Gibco).

2.2 Consumables

1. Corning® 384 Well Flat Clear Bottom Black Polystyrene TC-Treated Microplates (Corning).
2. Corning® 96 Well Clear V-Bottom TC-Treated Microplates (Corning).
3. Tissue culture flasks.

2.3 Equipment

1. Z2™ Coulter counter®, Analyzer (Beckman Coulter).
2. Humidified 37 °C incubator (5 % CO_2).
3. Allegra® X-15R Centrifuge (Beckman Coulter).
4. Multidrop combi reagent dispenser with dispensing cassettes (Thermo Scientific).
5. Automated fluorescence microscope equipped with a 488 nm (blue) laser or a fluorescence spectrophotometer.

3 Methods

3.1 Preparation of Virus Stocks

1. Thaw a tube of IIIB virus in the incubator at 37 °C, e.g., a virus stock with a titer of 2.6×10^6 tissue culture infectious dose per mL ($TCID_{50}$/mL).
2. Pellet 3×10^7 MT4-LTR-EGFP cells by centrifuging for 5 min at $500 \times g$ in a 50 mL sterile tube.
3. Discard the supernatant and infect the cells with IIIB at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell. In this example, the virus titer is 2.6×10^5 $TCID_{50}$/mL, thus add 1.15 mL of virus stock on the pellet.
4. Mix the cell pellet and virus dilution by briefly shaking the tube and incubate for 60 min at 37 °C. Meanwhile, preheat a 175 cm^2 cell culture flask containing 200 mL RPMI/10 % FBS at 37 °C.
5. Resuspend the cells with virus in the 200 mL preheated RPMI/10 % FBS.
6. Incubate the culture flask in the incubator at 37 °C.
7. Microscopically assess CPE and GFP-signal on a daily basis until full CPE is observed, typically 5–7 days post-infection.
8. To harvest the virus, transfer the supernatant into a 500 mL sterile tube and centrifuge at $2{,}000 \times g$ and 4 °C for 10 min to pellet the cells.
9. Transfer the supernatant to a new 500 mL sterile tube.
10. Aliquot the viral supernatant into 1.5 mL or 4.5 mL cryovials (one extra 1.5 mL tube for titration, *see* Subheading 3.2) and store at −80 °C.

3.2 Titration of the Virus Stocks

1. Perform virus dilutions using a 96-well V-bottom plate in a following plate layout: column 1 is the medium control (MC), columns 2–11 are assigned to prepare a fivefold serial dilution of the virus, column 12 is the cell control (CC). To determine the virus titer ($TCID_{50}$/mL), the virus dilution series is repeated eight times (rows A to H). Therefore, dispense 100 μL of RPMI/10 % FBS into column 1 (MC) and columns 3–12 of a 96-well V-bottom plate using a multi-channel pipette or multidrop combi reagent dispenser.

2. Thaw virus at 37 °C. Add 125 μL undiluted virus to each well of column 2 with a multi-channel pipette.
3. Make a 1/5 serial dilution of the virus from columns 2 to 11 in the 96-well plate by pipetting 25 μL of column 2 into the 100 μL RPMI/10 % FBS of column 3. Mix thoroughly by pipetting up and down and transfer 25 μL to the next column. Repeat this through column 11 (*see* **Note 2**).
4. Prepare RPMI/10 % FBS containing 2 % DMSO, 4 mL for each 384-well plate.
5. Dispense 10 μL of RPMI/10 % FBS containing 2 % DMSO in an entire 384-well black plate using a multidrop combi reagent dispenser.
6. Transfer four times 15 μL from each well of the 96-well plates to each of the quadruplicate wells in the black 384-well plate (e.g., well A1 of the 96-well plate corresponds to wells A1, A2, B1, and B2 of the 384-well plate).
7. Prepare an MT4-LTR-EGFP cell suspension in RPMI/10 % FBS; 6 mL at 4×10^5 cells/mL for each 384-well black plate.
8. Dispense 15 μL of the MT4-LTR-EGFP cell suspension into columns 3–24 using a multidrop combi reagent dispenser (columns 3–22 contain the serial dilution of the virus stock, columns 23 and 24 are CC). In addition, dispense 15 μL of RPMI/10 % FBS into columns 1 and 2 (MC).
9. Incubate the 384-well plate for 3 days in a humidified incubator at 37 °C.
10. Afterwards, count the wells that show CPE or fluorescence for every virus dilution and calculate the titer ($TCID_{50}$/mL) according to the method of Reed and Muench [8].

3.3 HIV-1 Antiviral High-Throughput Screening Assay

3.3.1 Preparation of the Compound Test Plates

1. Prepare fourfold serial dilutions of test compounds in RPMI/10 % FBS at 4× final concentrations and add 10 μL/well of each test concentration to the corresponding wells of columns 3–22 of a 384-well black plate (the DMSO concentration in each well is 2 %, *see* **Note 3**). Typically, we test four concentrations for each compound and 80 compounds per plate. For each set of test compounds, prepare duplicate plates: one for testing the antiviral activity and one for assessing the cytotoxicity. In our laboratory, the stock solutions of test compounds are made in 100 % DMSO and diluted to test concentrations using medium.
2. In the activity plates, dispense 10 μL of RPMI/10 % FBS containing 2 % DMSO in columns 1, 2, 23, and 24. In this plate format, columns 1 and 2 are used as virus control (VC; absence of compounds); columns 3–22 contain the serial dilutions of the test compounds; and columns 23 and 24 serve as cell control (CC; absence of virus and compounds).

3. In the toxicity plates, dispense 10 μL of RPMI/10 % FBS containing 2 % DMSO in columns 1, 2, 23, and 24 columns. In this plate format, columns 1 and 2 are used as CC, columns 3–22 contain the serial dilutions of the test compounds, and columns 23 and 24 serve as MC.

3.3.2 Antiviral Testing

1. For activity testing, prepare 6 mL of MT4-LTR-EGFP cell suspension in RPMI/10 % FBS (4×10^5 cells/mL) for every 384-well test plate. In parallel, prepare 12 mL of MT4-CMV-EGFP cell suspension in RPMI/10 % FBS (2×10^5 cells/mL) for each toxicity 384-well plate.
2. Thaw the virus stock at 37 °C and prepare a virus dilution in RPMI/10 % FBS to generate an MOI of 0.0025 $TCID_{50}$/cell. Prepare 6 mL for each 384-well activity test plate; e.g., to obtain an MOI of 0.0025 using a virus stock with a titer of 3.33×10^5 $TCID_{50}$/mL, 3 μL virus stock per mL of virus dilution is required.
3. In each 384-well activity plate, dispense 15 μL of RPMI/10 % FBS into the wells of columns 23 and 24 using a multidrop combi reagent dispenser, and dispense 15 μL virus dilutions into the wells of columns 1–22. In addition, dispense 15 μL of the MT4-LTR-EGFP cell suspension (6,000 cells/well) into all wells of the plate.
4. In each 384-well toxicity plate, dispense 30 μL of RPMI/10 % FBS into the wells of columns 23 and 24 and dispense 30 μL of the MT4-CMV-EGFP cell suspension (6,000 cells/well) into the wells of column 1 until 22, using a multidrop combi reagent dispenser.
5. Place the plates in a humidified incubator at 37 °C and incubate for 3 days.
6. Three days post-infection, measure the EGFP fluorescence using an automatic fluorescence microscope at 488 nm (blue laser).

3.3.3 Data Analysis

1. Calculate the dose response curves and determine the half maximal effective concentration (EC_{50}), which represents the concentration of a compound at which the virus replication is inhibited by 50 %, as measured by a 50 % reduction of the EGFP fluorescent intensity compared with the VC. The EC_{50} is calculated using linear interpolation of a dose response curve. For each compound concentration the percentage inhibition is determined. The percent inhibition (I) for every test concentration is calculated using the following formula: $I = 100 \times \left((S_{VC} - S_T)/(S_{VC} - S_{CC})\right)$; S_T, S_{CC}, and S_{VC} are the amount of EGFP signal in the test compounds, CC, and VC, respectively. In parallel, determine also the half maximal cytotoxic concentration (CC_{50}), defined as the concentration required to reduce the EGFP fluorescent intensity by 50 %

compared to that of the untreated control wells. Both EC_{50} and CC_{50} for each compound can be calculated using computer software such as GraphPad Prism.

2. Determine the selectivity index (SI), defined as the ratio of the CC_{50} to the EC_{50}. We use an SI≥4 as the definition of "hits".

4 Notes

1. The MT4-LTR-EGFP and the MT4-CMV-EGFP cell lines are maintained in RPMI/10 % FBS supplemented with 0.5 mg/mL geneticin for selective pressure. The cell lines are incubated in a humidified incubator at 37 °C. The cells are passaged every 3 or 4 days. For a 3- or 4-day cell culture, 7.5×10^4 cells/mL or 3.75×10^4 cells/mL are seeded in a T175 cell culture flask, respectively. The cells are cultured for maximum 30 passages. Prior to an experiment, all cell lines are cultured in medium without the geneticin selection agent.
2. To avoid carryover of virus, transfer the virus suspension from the highest concentration to the first dilution with minimal touching of the medium present in the well. Discard the tip and use a new tip to mix medium and virus before transferring the suspension to the next dilution. Use this procedure for every dilution step.
3. The final DMSO concentration in each well cannot exceed 0.5 % because of the toxicity to the cells. The volume of the compounds in a plate is 10 μL/well and is fourfold diluted with the cell suspension (and virus suspension) in a total volume of 40 μL. Therefore, the DMSO concentration in the compounds' dilutions is 2 % and should be the same in all wells. The cell and virus control wells should contain the same DMSO concentration as the compound wells.

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Chapter 2

A Homogeneous Time-Resolved Fluorescence Assay to Identify Inhibitors of HIV-1 Fusion

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Abstract

The human immunodeficiency virus type 1 (HIV-1) initiates infection through sequential interactions with CD4 and chemokine coreceptors unmasking the gp41 subunit of the viral envelope protein. Consequently, the N-terminal heptad repeats of gp41 form a trimeric coiled-coil groove in which the C-terminal heptad repeats collapse, generating a stable six-helix bundle. This brings the viral and cell membrane in close proximity enabling fusion and the release of viral genome in the cytosol of the host cell.

In this chapter, we describe a homogeneous time-resolved fluorescence assay to identify inhibitors of HIV-1 fusion, based on the ability of soluble peptides, derived from the N- and C-terminal domains of gp41, to form a stable six-helix bundle in vitro. Labeling of the peptides with allophycocyanin and the lanthanide europium results in a Föster resonance energy transfer (FRET) signal upon formation of the six-helix bundle. Compounds interfering with the six-helix bundle formation inhibit the HIV-1 fusion process and suppress the FRET signal.

Key words HIV-1, Gp41, Fusion inhibitor, Screening, FRET, HTRF

1 Introduction

HIV-1 capsid is enveloped with a lipid bilayer spiked with trimeric gp120–gp41 glycoprotein complexes [1, 2]. These spikes play a pivotal role in the HIV-1 entry process and are anchored in the viral membrane via the gp41 transmembrane protein to which the exterior gp120 subunits are non-covalently attached. The HIV-1 entry process is initiated upon binding of gp120 to the cellular CD4 receptor inducing conformational changes which in turn enables binding of gp120 to chemokine co-receptors. This latter interaction facilitates insertion of the N-terminal fusion domain of gp41 into the cell membrane. During this process, the N-terminal heptad repeat domains (HRN) form a trimeric coiled-coil structure. Next, the gp41 C-terminal heptad repeat domains (HRC) collapse into these hydrophobic grooves resulting in the formation

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of a stable six-helix bundle, a trimer of heterodimers. Finally, this brings viral and cell membranes into close proximity promoting fusion and delivery of the viral capsid to the cytoplasm. Hence, interfering with six-helix bundle formation prevents the virus from entering the cell, precluding HIV-1 infection.

HRC-derived peptides are potent inhibitors of six-helix bundle formation with reported antiviral activities at nanomolar concentrations, e.g., T-20 [3], T-1249 [4], and C-34 [5, 6]. More importantly, the six-helix bundle is a validated target for HIV-1 antiviral drug development, as T-20 (enfuvirtide) was approved for clinical use [7]. Yet, a search for a small molecule fusion inhibitor is still justified as the T-20 peptide can be administered only by injection, is prone to proteolytic cleavage, and resistance does occur [8].

HRC-derived peptides not only are potent inhibitors but also enable the in vitro formation of six-helix bundles, when mixed together with the HRN-derived peptides. Therefore, combining synthetic peptides overlapping gp41 residues 546–581 (N36) and 628–655 (C34) offers an attractive approach to develop biochemical assays which enable identification of peptidomimetic or small molecule inhibitors of six-helix bundle formation [9–12].

Here, we describe a homogeneous time-resolved fluorescence (HTRF) assay to measure the inhibition of six-helix bundle formation, based on N36–C34 interactions (Fig. 1). To address aggregation issues of N36 in the absence of C34, the N-terminal part of N36 is fused to a soluble alpha-helical peptide derived from GCN4, denoted IQ [10, 13]. The resulting fusion peptide, IQN36, is labeled with the light emitting fluorophore allophycocyanin (APC) and the C34 peptide is labeled with the UV-excitable fluorophore europium (Eu). Formation of the six-helix bundle brings both fluorophores in close proximity enabling FRET between the UV-excitated Eu donor and APC acceptor, resulting in emission of red light. Since this technique allows detection of changes in the nanometer range, compounds that interfere with the binding of C34 to IQN36 will quantitatively suppress the UV-induced signal.

2 Materials

2.1 Peptides and Reagents

1. Biotin-labeled IQN36 (Table 1): IQN36 is synthesized using standard protocols (Abgent, San Diego) and biotinylated at the N-terminus of the IQ moiety (PerkinElmer). The stock solution is prepared in DMSO at a concentration of 2 mM and stored at 4 °C.
2. Europium labeled C34 (C34-Eu) (Table 1): C34 is synthesized using standard protocols (Abgent, San Diego) and Eu-labeled at its C-terminus (PerkinElmer). Spin down briefly the tube before reconstituting C34-Eu in HEPES buffer to a concentration of 100 μM. Mix by vortexing and store aliquots at −20 °C.

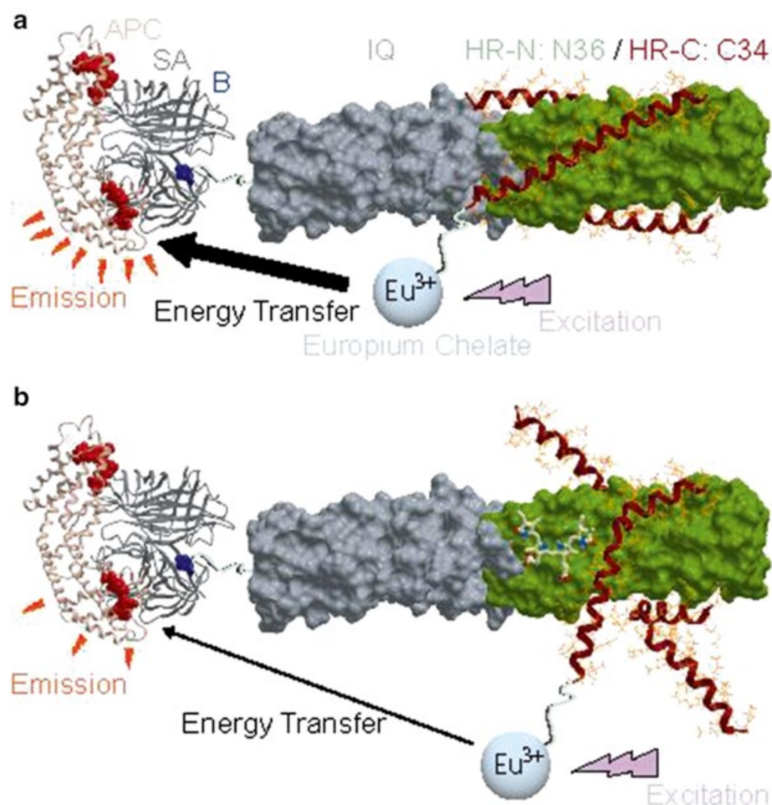

Fig. 1 (**a**) Structure of the Eu/allophycocyanin (APC)-based assay. C34 and N36 are derived from the gp41 heptad-repeats, respectively, the C- and N-terminal. A chelate of the UV-excitable fluorescent donor Eu^{3+} is bound to C34 via a short linker. N36 is fused with an IQ moiety for solubility reasons and labeled with the light-emitting acceptor APC via a biotin-streptavidin linker. IQN36-APC moieties trimerize to give rise to a coiled-coil structure. When C34 binds the hydrophobic grooves of the IQN36 trimer, both fluorophores come in close proximity, resulting in FRET and the emission of APC-fluorescence. *SA* streptavidin, *B* biotin. (**b**) Mechanism of FRET inhibition. Compounds that interfere with the binding of C34 to IQN36 will quantitatively suppress the UV-induced signal [14]. Reproduced from Dams et al. (2007) with permission from SAGE Publications

Table 1
Amino acid sequence of IQN36 and C34

Peptide	Amino acid sequence
IQN36	RMKQIEDKIEEIESKQKKIENEIARIKKLISGIVQQQNNLLRAIEAQQHLLQ LTVWGIKQLQARIL
C34	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL

3. Streptavidin conjugated to SureLight-Allophycocyanin (streptavidin-APC, PerkinElmer): reconstitute to 1 mL with deionized H_2O and allow the vial to sit on ice for 20–30 min. Mix gently by vortexing or tapping the tube before use. The stock solution is stored at 4 °C and the concentration is batch dependent.
4. 100 mM HEPES buffer (pH 7.2): Weigh 23.83 g of HEPES and add PBS to a final volume of 1 L. Adjust the pH to 7.2 and sterilize the solution through a 0.22 μm filtration; store at 4 °C.

2.2 Consumables

1. White 384-well non-tissue culture treated plates (Costar, Corning).
2. Costar® 50 mL reagent reservoir (Costar, Corning).
3. Corning® 28 mm diameter syringe filter, 0.2 μm (Corning).

2.3 Equipment

1. Multidrop combi liquid dispenser with dispensing cassettes (Thermo Scientific).
2. Viewlux™ (PerkinElmer) or other fluorescent readers.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Preparation of 384-Well Test Plates

Perform fourfold serial dilutions to prepare 4× final concentrations of test compounds in PBS and add 10 μL of each test concentration to each of the quadruple wells of a 384-well plate (final concentration of DMSO is 0.5 %, *see* **Note 1**). Typically, we test eight compounds per plate with nine concentrations of each compound. In this plate format, columns 1–16 contain the serial dilutions of the test compounds, columns 17–20 are used as positive control (no compounds) and columns 21–24 as negative control (no APC-labeled IQN36). In our laboratory, stock solutions of test compounds are made in DMSO and dispensed in 384-well plates using a 96-channel liquid handler.

3.2 Preparation of Working Solutions

All working solutions need to be freshly prepared and are diluted in 100 mM HEPES buffer (pH 7.2).

1. Prepare a 40 nM working solution of streptavidin-APC (6 mL per 384-well plate).
2. Prepare a 400 nM working solution of IQN36-biotin (5 mL per 384-well plate).
3. Prepare a 500 nM working solution of C34-Eu (5 mL per 384-well plate).

3.3 HTRF Assay

1. For each 384-well plate, prepare 1 mL of negative control mix by adding equal volumes of streptavidin-APC working solution and 100 mM HEPES buffer. Incubate the mix for 30 min at room temperature.
2. For each 384-well plate, prepare 10 mL of test mix by adding equal volumes of streptavidin-APC and IQN36-biotin working solutions. Incubate the mix for 30 min at room temperature.
3. Dispense 20 μL/well of the negative control mix to columns 21–24 (negative control wells) using a multidrop combi liquid dispenser (final concentration of streptavidin-APC is 10 nM).
4. Dispense 20 μL/well of the test mix to columns 1–20 (test wells and positive control wells; the final concentration of streptavidin-APC is 10 nM and the final concentration of IQN36-biotin is 100 nM) (*see* **Note 2**).
5. Incubate the plates at room temperature for 2 h (*see* **Note 3**).
6. Add 10 μL working solution of Eu-labeled C34 peptide to all wells (final concentration of C34-Eu is 125 nM) (*see* **Note 2**).
7. Incubate the plates at room temperature for 30 min.
8. Measure the FRET signal using a Viewlux™ reader with the following instrument settings: a standard filter set for the LANCE® assay (PerkinElmer) (a DUG11 (330/75) excitation filter, a 400LP dichroic mirror, a 618/8 emission filter for the fluorescence of the Eu^{3+} donor, and a 671/8 emission filter for the fluorescence of the APC acceptor); a chopper wheel system provides a 50 μs delay after the excitation flash, to suppress the fast-decaying signals due to background fluorescence and direct excitation of the acceptor, and is followed by a 354 μs signal integration period [14].
9. Calculate the 50 % inhibitory concentration (IC_{50}) of the test compounds (*see* **Note 4**).

4 Notes

1. Although DMSO concentrations up to 5 % do not have a negative influence on the results, a final DMSO concentration of 0.5 % is typically applied.
2. Optimal concentrations of labeled peptides are selected from a three-dimensional analysis encompassing a concentration range of (a) streptavidin-APC, (b) IQN36-biotin, and (c) C34-Eu to generate robust dose–response curves, a high signal-to-background ratio, a good sensitivity, an acceptable Z', and an economical use of C34-Eu [14]. The Z'-factor is a simple statistical parameter to evaluate the quality of high-throughput screening assays [15].

(a) The optimal concentration of streptavidin-APC is 10 nM as this yields the best signal-to-background ratio. Higher concentrations do not improve the signal-to-noise ratio, whereas concentrations below 1 nM compromise the acceptor signal.

(b) To optimize the IQN36-biotin concentrations, the trimerization level as well as the solubility of the peptide is considered. Under the experimental conditions of this assay, 100 nM yields the best results.

(c) The cost of the C34-Eu peptide is a point to consider when screening large numbers of compounds to generate millions of data points. Concentrations of 250 nM or more yield high signal-to-background ratios and a Z′ factor of 0.9 [14]. However, a C34-Eu concentration of 125 nM still results in a Z' factor of 0.8, which is still acceptable for a biochemical high-throughput screening assay. In addition, 50 % reduction of C34-Eu usage positively influences the cost/benefit ratio of the assay.

3. Prior to the addition of C34-Eu, test compounds are preincubated with IQN36-APC for 2–4 h, allowing the inhibitor to bind the IQN36 coiled-coil without having to compete with C34-Eu after the stable six-helix bundle is formed.

4. The IC_{50} is defined as the concentration of compound achieving 50 % inhibition of the FRET signal in compound-treated wells compared to the positive control. First, the percentage inhibition is determined for every compound concentration. The percent inhibition (I) is calculated using the following formula: $I = 100 \times (S_{PC} - S_T)/(S_{PC} - S_{NC})$; S_T, $S_{NC,}$ and S_{PC} are the amount of fluorescent signal in the test compounds, NC (negative control) and PC (positive control), respectively. The IC_{50} is calculated using linear interpolation of a dose response curve. For each compound concentration, the median inhibition of the quadruple replicates is calculated. If the medium inhibition values of all tested concentration is higher or lower than 50 %, the lowest concentration is reported with an "<" sensor, or the highest concentration is reported with a ">" sensor, respectively. If the median inhibition value of a tested concentration is exactly 50 % this value is reported. In all other cases, the IC_{50} value is calculated following the formula:

$$IC_{50} = \exp\left(\ln(C_h) - \ln(C_h / C_l) \times (I_h - 0.5)/(I_h - I_l)\right)$$

C_h and C_l are the compounds concentration of which the inhibition values (I_h and I_l) are below and above 50 % inhibition, respectively. The formula uses a linear interpolation in the log-concentration domain. Calculations can be performed using computer software such as GraphPad Prism. All dose–response curves of active compounds are reviewed by the operator.

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Liesbet Smeulders and Lieve Bunkens contributed equally to the work.

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Chapter 3

Identification of HIV-1 Reverse Transcriptase Inhibitors Using a Scintillation Proximity Assay

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Abstract

The human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) converts the viral single-stranded RNA into double-stranded DNA. The inhibition of reverse transcription in the viral life cycle has proven its efficacy as a clinically relevant antiviral target, but the appearance of resistance mutations remains a major cause of treatment failure and stresses the continuous need for new antiviral compounds. In this chapter, we describe an HIV-1 RT scintillation proximity assay (SPA) to identify inhibitors of the RT. The assay uses an RNA/DNA (poly(rA)/oligo(dT)) template/primer bound to SPA beads, which contain scintillant. Reverse transcriptase extends the primer by incorporating [^{3}H]dTTP and dTTP, which results in light production by the scintillant in the bead. Compounds that inhibit reverse transcriptase will prevent the incorporation of tritiated dTTP resulting in a reduction of emitted light compared to the untreated controls.

Key words HIV-1, Reverse Transcriptase, SPA technology, Antiviral inhibitors

1 Introduction

Upon entry into the host cell, the HIV-1 virus is uncoated and the viral RT converts the single-stranded RNA genome into double-stranded DNA (dsDNA). Within the context of the pre-integration complex, the dsDNA is translocated to the nucleus and integrates in the genome of the host cell [1]. The different steps of reverse transcription are well characterized and have been reviewed elsewhere [2]. The RT incorporates deoxyribonucleotide triphosphates (dNTPs) at the 3′ end of the primer, using the viral RNA as a template. The inhibition of this step in the viral life cycle played a pivotal role in the establishment of highly active antiretroviral therapy as RT inhibitors are included in the current standard of care [3]. Both nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs) have proven their clinical efficacy. NRTIs are phosphorylated in the host cell and the NRTI-triphosphate competes with the natural dNTPs for binding in the

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active site. These compounds are chain terminators because they lack the 3′-OH group [4]. In contrast, NNRTIs are another chemical class of small molecules that bind to a pocket close to the active site of RT [5, 6]. The appearance of resistance mutations to these compounds stresses the continuous need for new compounds with another mechanism of action [7]. Recently, a novel type of RT inhibitor, called Indopy-1, has been described [8]. This class of compounds also targets the RT by binding to the active site of the enzyme; but unlike NRTIs, they do not require to be phosphorylated to block the DNA synthesis, although they share the same binding site [8].

SPA is a homogeneous technology based on the fact that in an aqueous environment weak β-emitters, like [^{3}H], need to be in close proximity of scintillant molecules before they can transfer their energy to the scintillant [9, 10]. This energy transfer results in the emission of light, which can be measured using a photomultiplier. The chemical incorporation of the scintillant into beads eliminated the requirement of separating bound from unbound radiolabeled molecules, resulting in a homogeneous (mix-and-read) assay [11]. The combination of these beads with the [^{3}H] has proven ideal because tritium has a very short path length of 1.5 μm, which requires that the tritium-labeled molecules interact with the coated beads in order to be in the vicinity of the scintillant to emit lights. Therefore, the energy of unbound [^{3}H] is dissipated in the solution, generating only low levels of background signal [10].

In this chapter, we describe an RT assay using SPA (Fig. 1) that makes use of a 5′-biotinylated 16 mer oligo(T) DNA primer annealed to a 300-base poly(rA) template. This RNA/DNA (poly(rA)/oligo(dT)) template/primer is bound to the SPA beads via a streptavidin/biotin linkage. In the presence of RT, [^{3}H]dTTP and dTTP are incorporated through primer extension. In the presence of RT inhibitors the incorporation of [^{3}H]dTTP and dTTP is prevented, resulting in a reduced signal compared with untreated controls.

2 Materials

2.1 Reagents

1. RPMI/10 % FBS: RPMI-1640 supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % L-glutamine and 0.02 mg/mL gentamicin (Gibco).
2. HIV-1 reverse transcriptase (GE Healthcare, 200 units/vial).
3. Quan-T-RT™ [^{3}H]-Reverse Transcriptase kit (GE Healthcare) (*see* **Note 1**).
4. Efavirenz, a marketed NNRTI [12, 13] as a reference compound.