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Analysis of viral and cellular proteins in HIV-1 reverse transcription complexes by co-immunoprecipitation

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Abstract

Molecular details and temporal organization of the early (pre-integration) phase of HIV life cycle remain among the least investigated and most controversial problems in the biology of HIV. To perform reverse transcription and intracellular transport of the viral genetic material, HIV forms multimolecular complexes termed reverse transcription complexes (RTCs). Analysis of the kinetic of reverse transcription and nuclear import of RTCs, as well as assessment of the changes in their protein content in the course of reverse transcription and nuclear translocation is a necessary step in understanding mechanisms of cytoplasmic maturation and nuclear import of HIV-1 RTCs. Here, we review methods allowing to quantitatively assess the dynamics of the maturation of HIV-1 RTCs and transformations of RTC protein composition associated with nuclear import of the complexes.

Keywords

HIV-1; reverse transcription complex; cell fractionation; real-time PCR; RTC protein composition

1. Introduction

The early (pre-integration) phase of the HIV life cycle includes virus entry into a target cell, conversion of its genome from RNA to DNA (reverse transcription), delivery of viral DNA to the site of integration and integration itself. After fusion with the cellular plasma membrane and entry into the target cell, virus particles transform into multimolecular complexes termed reverse transcription complexes (RTCs). They provide protection for the viral genome and reverse transcription machinery, and perform transportation of the viral genetic material towards the site of integration (reviewed in (1;2)). Technical difficulties associated with observation, isolation and analysis of RTCs at different steps of their trafficking in the cytoplasm and especially in the nucleus explain our poor understanding of the molecular events in the early phase of the retroviral life cycle.

HIV-1 RTCs isolated from the cytoplasm of infected cells contain viral proteins, such as reverse transcriptase (RT), integrase (IN), matrix protein (MA) and Vpr (3-5), as well as host cell proteins, such as non-homologous DNA end joining proteins Ku70 and Ku80 (6), lens epithelium-derived growth factor (LEDGF/p75) (7), nonhistone chromosomal protein HMGA (8), integrase interactor 1 (Ini1) and PML protein (4;5;9). The capsid protein (CA) was detected in virus-specific complexes early after infection, but it was absent in cRTCs analyzed at later time points and in nuclear RTCs (3;10;11). Nuclear import of RTCs

associates with loss of RT and CA proteins (10;11). Composition of the cytoplasmic and nuclear HIV-1 RTCs, interaction of RTC proteins with proteins of infected cell, as well as quantitative analysis of dynamics of the RTC maturation and nuclear import may be accomplished using co-immunoprecipitation of RTCs with antibodies against viral and host cell proteins and subsequent real-time PCR analysis with primers specific for HIV-1 DNA (6;11-17).

2. Materials

2.1. Plasticware and hardware

- 2.1.1. Tissue culture flasks, 250 ml, 75 cm² with vented plug seal caps (Becton Dickinson, Franklin Lakes, NJ, Cat. No. 353110).
- 2.1.2. 0.45- μ m-pore-size syringe filter (Fisher Scientific, Cat. No. 09-719B).
- 2.1.3. Ultra-clear centrifuge tubes (14 $\times$ 89 mm) (Beckman, Palo Alto, CA, Reorder. No. 344059).
- 2.1.4. Beckman SW-41 rotor.
- 2.1.5. Polypropylene cryogenic vials, 2.0 ml (Corning, Cat. No. 430489).
- 2.1.6. Six-well polystyrene cell culture plates (Corning, Cat. No. 3506).
- 2.1.7. Microplate adaptors for low-speed centrifuge suitable for 6-well tissue culture plates.
- 2.1.8. Polyethylene cell lifters (Corning, Corning, NY, Cat. No. 3008).
- 2.1.9. Dounce tissue homogenizer type B, 2 ml (Fisher Scientific, Cat. No. NC9781184).
- 2.1.10. Safe-lock Eppendorf tubes 2.0 ml (Brinkman Instruments, Cat. No. 22 60 004-4).
- 2.1.11. Polyallomer centrifuge tubes (11 $\times$ 60 mm) (Beckman, Reorder. No. 328874).
- 2.1.12. Beckman SW-60 rotor.
- 2.1.13. Rotation shaker.
- 2.1.14. 96-Well low profile polypropylene microplates, white (MJ Research, Waltham, MA, Cat. No. MLL-9651) and Flat cap PCR tube strips (BioRad, Cat. No. TCS0803), or other plates and cap strips for real-time RCP dependent on the type of PCR thermocycler/fluorescence detector.

2.2. Reagents and supplies

- 2.2.1. Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA, Cat. No. 10-013-CV) supplemented with 2 mM glutamine (Cambrex, Walkersville, MD, Cat. No. 17-605E), 10% (v/v) fetal bovine serum (Cambrex, Cat. No. 14-472F), 100 units/ml penicillin, and 100 units/ml streptomycin (Cambrex, Cat. No. 17-602E) (complete DMEM) is filtered through 0.22- μ m-pore-size filter and stored at 4°C.
- 2.2.2. Trypsin/EDTA, 0.05% Trypsin/0.53 mM EDTA in HBSS (Mediatech, Herndon, VA, Cat. No. 25-052-CI) is aliquoted and stored at -20°C.

- 2.2.3. Dulbecco's phosphate buffered saline (PBS), 1X prepared from 10X solution (Mediatech, Cat. No. 20-031-CV) is filtered through 0.22- μ m-pore-size filter and stored at 4°C.
- 2.2.4. Metafectene Transfection Reagent for Mammalian Cells (Biontex, Germany, Cat. No. T020) (see **Note 1**) is stored at 4°C.
- 2.2.5. MgCl_2 is dissolved as 1M solution, filtered through 0.22- μ m-pore-size filter and stored at 4°C.
- 2.2.6. DNase I, RNase-free, 10 U/ μ l (Roche, Indianapolis, IN, Cat. No. 10 776 785 001) is stored at -20°C.
- 2.2.7. Sucrose 99+% (Sigma, Cat. No. S-0389) is dissolved at 30% in PBS, filtered through 0.22- μ m-pore-size filter and stored at 4°C.
- 2.2.8. HEPES buffer (1 M), pH 7.4 (Sigma, St. Louis, MO, Cat. No. H-0887) is dissolved at 20 mM in DMEM, filtered through 0.22- μ m-pore-size filter and stored at 4°C.
- 2.2.9. HIV-1 p24 ELISA Kit (PerkinElmer Life Sciences, Boston, MA, Cat. No. NEK050B) is stored at 4°C.
- 2.2.10. Ethylenediamine tetraacetic acid (EDTA) 0.5 M, pH 8.0 (Gibco BRL, Grand Island, NY, Cat. No. 15575-020) is dissolved at 0.5 mM in PBS, filtered through 0.22- μ m-pore-size filter and stored at 4°C.
- 2.2.11. Hypotonic buffer (buffer H): 10 mM HEPES (pH 7.9), 1.5 mM MgCl_2 , 10 mM KCl, 5 mM dithiothreitol (DTT), and 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail (Roche, Cat. No. 1 836 170) per 10 ml. Filter through 0.22- μ m-pore-size filter and store at 4°C.
- 2.2.12. Liquid nitrogen.
- 2.2.13. Triton X-100 (Sigma, Cat. No. T-9284) dissolved at 5% in buffer H, filtered through 0.22- μ m-pore-size filter and stored at room temperature.
- 2.2.14. Isotonic buffer A (buffer I^A): 10 mM Tris HCl (pH 7.4), 160 mM KCl, 5 mM MgCl_2 , 1 mM DTT, and 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail (Roche) per 10 ml. Filter through 0.22- μ m-pore-size filter and store at 4°C.
- 2.2.15. Isotonic buffer B (buffer I^B): 20 mM Tris HCl (pH 7.4), 150 mM KCl, and 5 mM MgCl_2 . Filter through 0.22- μ m-pore-size filter and store at 4°C.
- 2.2.16. Iodixanol 60% (W/V) solution in water (Sigma, Cat. No. D1556) is dissolved at 25 and 30% in buffer I^B, filtered through 0.45- μ m-pore-size filter and stored at room temperature.
- 2.2.17. EZ-Grind Kit (G Biosciences, St. Louis, MO, Cat. No. 786-139) is stored at room temperature.
- 2.2.18. Sucrose 99% (Sigma, Cat. No. S-0389) is dissolved at 50% in buffer H and buffer I^A, filtered through 0.45- μ m-pore-size filter and stored at 4°C.

- 2.2.19. Buffer K: 20 mM HEPES (pH 7.3), 150 mM KCl, 5 mM MgCl₂, 1 mM DTT, and 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail (Roche) per 10 ml. Filter through 0.22- μ m-pore-size filter and store at 4°C.
- 2.2.20. Protein G sepharose 4 fast flow (Amersham Biosciences, Uppsala, Sweden, Cat. No. 17-0618—01) is stored at 4°C.
- 2.2.21. Albumin, bovine fraction V (BSA) (Sigma, Cat. No. A-9418) is dissolved at 100 mg/ml in ddH₂O, filtered through 0.22- μ m-pore-size filter, aliquotd by 0.5 ml and stored at -20°C.
- 2.2.22. Sheared salmon sperm DNA (10 mg/ml) (5 Prime-3 Prime, Boulder, CO, Cat. No. IK197A), stored at -20°C.
- 2.2.23. Non-immune sera: Normal Mouse Serum (Jackson ImmonoResearch, Cat. No. 015-000-001), Normal Rabbit Serum (Jackson ImmonoResearch, Cat. No. 011-000-001), stored at 4°C.
- 2.2.24. Isotype controls: ChromPure mouse IgG, whole molecule (Jackson ImmonoResearch, Cat. No. 015-000-003), and ChromPure rabbit IgG, whole molecule (Jackson ImmonoResearch, Cat. No. 011-000-003), stored at 4°C.
- 2.2.25. Antibodies against HIV-1 and host-cell RTC proteins. The following antibodies react with RTC proteins in IP assay (11): mouse monoclonal antibodies to MA, RT and IN (ABI, Columbia, MD), CA (AIDS Research and Reference Reagent Program (18) and PML (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit antiserum to Ini1 (Santa Cruz Biotechnology).
- 2.2.26. Triton X-100 (Sigma, Cat. No. T-9284) dissolved at 0.1% in buffer K, filtered through 0.22- μ m-pore-size filter and stored at room temperature.
- 2.2.27. IsoQuick Nucleic Acid Extraction Kit (ISC BioExpress, Kaysville, UT, Cat. No. G-3120-1), stored at room temperature.
- 2.2.28. Glycogen for molecular biology (20 mg/ml solution) (Roche, Mannheim, Germany, Cat. No. 901 393) is dissolved in IsoQuick extraction matrix (Reagent 2) to final concentration 150 μ g/ml, stored at room temperature.
- 2.2.29. Isopropanol, DNase, RNase, protease-free (Acros Organics, NJ, Cat. No. 327270010).
- 2.2.30. Ethanol anhydrous, molecular biology certified (Shelton Scientific, Shelton, CT, Cat. No. IB15720) is dissolved at 70% in ddH₂O.
- 2.2.31. Molecular grade water (distilled, deionized), DNase, RNase and protease tested, sterile (Mediatech, Cat. No. 46-000-CM).
- 2.2.32. iQ SYBR Green Supermix (BioRad, Hercules, CA, Cat. No. 170-8880), stored at -20°C.
- 2.2.33. Purified DNA from 8E5 cells, dissolved in ddH O, aliquotd and stored at -80° 2 C.
- 2.2.34. Primers specific for the negative-strand “strong-stop” HIV-1 DNA (the early reverse transcription product): M667 (5'-GGCTAACTAGGGAACCCACTG-3') and AA55 (5'-CTGCTAGAGATTTTCCACACTGAC-3') (19), dissolved at 10 μ M in ddH₂O and stored at -20°C.

2.2.35. Primers specific for the late reverse transcription products: FOR-LATE (5'-TGTGTGCCCCGTCTGTTGTGT-3') and REV-LATE (5'-GAGTCCTGCGTCGAGAGATC-3') (19), dissolved at 10 μ M in ddH₂O and stored at -20°C.

2.3. Cell cultures, viruses

2.3.1. 293T/17 cell line, a derivative of the 293T (293tsA1609neo) line of embryonic kidney epithelial human cells, transformed with adenovirus 5 DNA (ATCC, Manassas, VA, Item No. CRL-11268).

2.3.2. HeLa cell line, human epithelial cervical adenocarcinoma cells (ATCC, Item No. CCL-2).

2.3.3. 8E5 cell line, a derivative of the CEM line of the lymphoblastoid human cells from peripheral blood, containing a single copy of HIV-1 LAV provirus per cell (ATCC, Item No. CRL-8993).

2.3.4. Purified DNA of HIV-1 molecular clone (plasmid encoding complete HIV-1 cDNA). For instance, pNLHXB – HIV-1 HXB2 molecular clone (20) is dissolved in ddH₂O and stored at -20°C.

2.3.5. Purified plasmid encoding the Env protein of the amphotropic MLV, for instance pcDNA-Env(MLV) vector containing sequence of MLV Env under control of CMV promoter (e.g. as described in (11)) is dissolved in ddH₂O and stored at -20°C.

3. Methods

The main problem in isolation of HIV-1 RTCs is low quantity of the complexes in infected cells and their low stability. To increase the efficiency of HIV-1 infection and RTC yield, we recommend infection of HeLa cells with HIV-1 pseudotyped with Env glycoprotein of the amphotropic murine leukemia virus (MLV). This approach provides a ten-fold increase in the level of infection when compared to infection of CD4-positive HeLa cells with the wild type HIV-1. The cells can be infected using spinoculation, centrifugation of the cells with virus suspension at 1,000 $\times$ g for 2 h, described in (21). This method allows infection of 70-80% of the cells (11). To avoid distortion of the protein composition of RTCs, they should be isolated from cytoplasmic and nuclear fractions without detergents. Analysis of protein composition of the RTCs can be accomplished using immunoprecipitation (IP) followed by real-time PCR analysis of HIV-1 DNA. Sensitivity of the Western blot assay is not sufficient for adequate analysis, especially when nuclear RTC specimens are concerned. If interaction between an RTC protein and its specific antibody correlates with the content of this protein in the reverse transcription complex (which is often the case), real-time PCR analysis of cDNA isolated from the immunoprecipitates can be used as a quantitative measure of this protein. This approach is handy in comparing levels of certain proteins in cytoplasmic and nuclear RTCs, or changes in protein content upon maturation of RTCs. However, this method does not allow comparison between different proteins because of differences in affinity of antibodies used for the IP.

The experimental procedures described below are based on the methods published in (11;13;19;21-24).

3.1. Preparation of infectious virus

3.1.1. Collect approximately 5×10^6 293T/17 cells from maintained culture with trypsin/EDTA, wash with 20 ml PBS once and transfer to a 75 cm² tissue culture flask in 12 ml of

complete DMEM to provide culture for transfection with viral molecular clone. Incubate cells overnight at 37°C in atmosphere containing 5% CO₂ to 70-80% confluence ($\sim 1 \times 10^7$ cells).

3.1.2. Carefully remove culture medium from cell monolayer, add 10 ml of pre-warmed complete DMEM and incubate cells for 1-2 h at 37°C and 5% CO₂.

3.1.3. Prepare transfection mix containing 24 µg of HIV-1 molecular clone DNA (e.g. NLHXB (20)) and 6 µg of pcDNA-Env(MLV), 800 µl of serum and antibiotic-free DMEM and 90 µl of Metafectene transfection reagent according to manufacturer protocol (see **Note 1**). Add transfection mix drop wise into flask with 293T/17 cells, incubate cells at 37°C and 5% CO₂ for 6 h or overnight.

3.1.4. Carefully remove culture medium containing transfection complexes from cell monolayer, wash cells with 15 ml of pre-warmed PBS (see **Note 2**). Add 15 ml of complete DMEM, maintain culture at 37°C and 5% CO₂ for 48-72 h.

3.1.5. Harvest culture medium from transfected cells, spin down floating cells by low-speed centrifugation (~ 800 - $1000 \times g$) at room temperature for 5 min. Filter supernatants through a 0.45-µm-pore-size filter into 15 or 50 ml plastic centrifuge tubes. To filtered supernatant (virus suspension) add 1 M MgCl₂ (to final concentration of 10 mM) and RNase-free DNase I (60 U/ml), incubate on a water bath for 1 h at 37°C.

3.1.6. Dispense virus suspension in 11 ml aliquots into Ultra-Clear centrifuge tubes (14 × 89 mm) (see **Note 3**). For balancing the tubes, virus suspension can be diluted by DMEM to final volume of 11 ml. Underlie 1 ml of 30% sucrose in PBS, balance tubes and spin at 24,000 RPM in a Beckman SW-41 rotor for 2 h at 4°C. Completely remove supernatants after centrifugation and resuspend the virus pellets in 1 ml of Dulbecco's modified Eagle medium containing 20 mM HEPES (pH 7.4). Place suspension of concentrated virus into 2 ml polypropylene cryogenic vials - 1 ml/vial - seal and keep at -70°C.

3.2. Infection, fractionation of infected cells and isolation of HIV-1 RTCs

3.2.1. Collect 24×10^6 HeLa cells from maintained culture with trypsin/EDTA, wash with 40 ml of PBS twice, resuspend in 48 ml of complete DMEM and transfer into four 6-well tissue culture plates (0.5×10^6 cells/2 ml/well) to provide culture for infection. Incubate cells overnight at 37°C in atmosphere containing 5% CO₂.

3.2.2. Measure viral titer using p24 ELISA kit according to manufacturer's protocol (see **Note 4**) and normalize viral load to 0.5 pg of p24 per cell. Since virus stocks comprise high level of p24 protein, we recommend the following dilutions of virus suspension for p24 ELISA: $\times 10^{-3}$, $\times 10^{-4}$, $\times 10^{-5}$, $\times 10^{-6}$. Each sample should be duplicated. Then dilute virus stocks with complete DMEM to final p24 concentration of 0.25 µg/ml. Virus suspension can be kept on ice at 4°C for up to 24 h until infection.

3.2.3. Wash HeLa monolayer with cold PBS (2 ml/well) once, then add 2 ml of virus suspension per well. Cover plates by lids, seal with two layers of Parafilm and place them in microplate centrifuge adaptors for spinoculation. Centrifuge using low-speed centrifuge for 2 h at $1,000 \times g$ and 18°C (to prevent viral internalization by the cells during spinoculation).

3.2.4. Completely remove virus-containing supernatants after centrifugation, wash cell monolayers with 2 ml/well of cold PBS twice and add 2 ml per well of complete DMEM at room temperature. Incubate cells at 37°C in atmosphere containing 5% CO₂ for different times depending on the purposes of the experiment (see **Note 5**).

3.2.5. Remove supernatant from cell monolayer, wash cells twice with 2 ml per well of room temperature PBS containing 0.5 mM EDTA, add trypsin/EDTA (0.5 ml per well) and incubate at 37°C for 3 min. Then lift cells using polyethylene cell lifters. Wash cells in 50 ml centrifuge tubes with 40 ml of ice-cold PBS twice. Completely remove PBS and resuspend cellular pellet in 5 volumes (~1.2 ml) of ice-cold hypotonic buffer (buffer H). Place cell suspension into 2 ml Eppendorf microtubes and incubate on ice for 20 min.

3.2.6. Centrifuge suspension of HeLa cells in buffer H on table-top microcentrifuge at 5,000 rpm and 4°C for 5 min, resuspend cell pellet in 3 volumes (~0.7 ml) of buffer H and incubate on ice for 10-15 min. Pre-chill Dounce homogenizer type B, place cell suspension into the barrel of the homogenizer and destroy cytoplasm of the cells by 10-15 strokes of the pestle (see **Note 6**). Immediately place the lysate into Eppendorf microtubes and pellet the nuclear fraction at 5,500 rpm and 4°C for 10 min. Place the supernatant (cytoplasmic lysate) into new microtubes, keep nuclear pellet on ice.

3.2.7. Clarify cytoplasmic lysate by centrifugation at 10,000 rpm ($9,500 \times g$), 4°C for 10 min. Place clarified supernatant (cytoplasmic extract) into new microtubes. Cytoplasmic extract and nuclear pellet (see step 6) can be supplemented with sucrose solution in buffer H to a final concentration of sucrose equal 8%, snap-frozen in liquid nitrogen and stored at -80°C.

3.2.8. For isolation of nuclear RTCs, nuclei should be washed from components of the cytoplasm to avoid cytoplasmic contamination of the nuclear complexes (see **Note 7**). To eliminate cytoplasmic contamination, resuspend nuclear pellet in 10 volumes of ice-cold buffer H and add 0.1 volume of 5% Triton X-100 in buffer H to a final concentration of 0.5%. Incubate suspension on ice for 10 min, shake on vortex for 10 s and spin down nuclei using table-top microcentrifuge at 6,000 rpm, 4°C for 10 min. Wash nuclei with 0.5-1 ml of ice-cold buffer H, pellet at the same speed and 4°C for 5 min and completely remove supernatant.

3.2.9. Resuspend nuclear pellet in 0.9 ml of isotonic buffer (buffer I^B) supplemented with 25% iodixanol (Sigma) and place into 2 ml Eppendorf tubes. Underlie suspension with 30% iodixanol in buffer I^B and centrifuge at 10,500 rpm ($10,000 \times g$) and 4°C for 20 min as described in (25). Carefully remove and discard supernatant and resuspend nuclear pellet in 0.5 ml of ice-cold isotonic buffer A (buffer I^A) to wash from iodixanol.

3.2.10. Pellet nuclei again at 6,000 rpm, 4°C for 5 min and resuspend in 50 µl of buffer I^A. Prepare grinding-resin tube from EZ-Grind kit according to manufacturer's protocol (wash resin with 200 µl of buffer I^A) and place the nuclear suspension into the tube. Destroy nuclei by 80-100 strokes of matching pestle. Place suspension into Eppendorf tube and clarify the nuclear extract by centrifugation at 13,000 rpm ($16,000 \times g$) and 4°C for 10 min. Collect supernatant into new microtube. Nuclear extracts can be snap-frozen with 8% sucrose in liquid nitrogen and stored at -80°C as described for cytoplasmic extracts (step 6), or directly used for ultracentrifugation. RTCs can be purified from cytoplasmic and nuclear extracts using ultracentrifugation through 50% sucrose cushion. According to Stremlau et al. (24), a 50% sucrose cushion is optimal for discriminating between intracellular capsids associated with entry-competent viruses and those nonspecifically associated with cells.

3.2.11. Dilute cytoplasmic and nuclear extracts to 3.5 ml with ice-cold buffers H and I^A, respectively. Place diluted suspensions into Beckman polyallomer centrifuge tubes 11 × 60 mm and underlie by 0.5 ml of 50% sucrose in buffer H for cytoplasmic and in buffer I^A for nuclear extracts. Centrifuge samples using SW-60 bucket rotor in a Beckman ultracentrifuge at 34,000 RPM ($100,000 \times g$) for 3 h at 4°C.

3.2.12. Remove and discard supernatants, resuspend RTC pellets in 50-100 μ l of buffer K and aliquot by 10-20 μ l to Eppendorf microtubes,. Snap-freeze RTC suspensions in liquid N₂ and store at -80°C .

3.3. Purification of HIV-1 cDNA and quantification of RTCs using real-time PCR

For quantitative analysis of protein composition of HIV-1 RTCs, complexes should be normalized according to the cDNA content. Thus, real-time PCR analysis of DNA isolated from cytoplasmic and nuclear RTCs is optimal for HIV-1 DNA quantification and subsequent normalization of RTC for the immunoprecipitation assays.

3.2.1. Thaw aliquots of cytoplasmic and nuclear RTCs, dilute by Sample Buffer (Reagent A) from IsoQuick Nucleic Acid Extraction Kit to final volume 50 μ l (see **Note 8**). Incubate suspensions at room temperature for 5 min and add equal volume (50 μ l) of Lysis Solution (Reagent 1). Then extract DNA according to Rapid DNA extraction protocol from the manufacturer. Since quantity of DNA in RTC samples is very low, we recommend adding glycogen solution (20 mg/ml) to IsoQuick Extraction Matrix (Reagent 2) to final concentration of glycogen 150 μ g/ml.

3.2.1. Following extraction, air dry DNA pellets and resuspend in 20 μ l of DNase, RNase and protease-free ddH₂O (RTC DNA from $4-4.5 \times 10^6$ cells). Use this cDNA solution for real-time PCR assay.

Quantitative analysis of HIV-1 cDNA can be accomplished using SYBR Green or TaqMan real-time PCR assay. Here we present protocol for quantitative analysis using iQ SYBR Green Supermix (BioRad).

Serial dilutions of DNA from 8E5 cells can be used as the quantitative standards. Total DNA from non-activated 8E5 cells can be isolated using IsoQuick Nucleic Acid Extraction Kit. Since each cell contains a single copy of HIV-1 LAV provirus (19), the number of HIV-1 DNA copies is equal to the cell count.

Real-time PCR should be performed in triplicate for each standard and experimental sample. Since the purpose of this analysis is to assess the count of cytoplasmic and nuclear HIV-1 RTCs per cell, we recommend using primers specific for the negative-strand “strong-stop” DNA (the early reverse transcription product): M667 and AA55 (see **Materials**).

3.2.1. Prepare serial dilutions of cDNA from cytoplasmic and nuclear RTCs with the following coefficients of dilution: $\times 1$, $\times 10^{-1}$, $\times 10^{-2}$, $\times 10^{-3}$, $\times 10^{-4}$. Use 5 μ l from each dilution for each PCR sample with total volume 50 μ l. Prepare the following serial dilutions of DNA from 8E5 cells: 1,000, 500, 250, 125, 62, 31 and 15 HIV-1 DNA copies/5 μ l/sample.

3.2.1. Prepare PCR premix containing $\frac{1}{2}$ volume of 2X iQ SYBR Green Supermix and 15 pmoles of each primer per 45 μ l of mix. ddH₂O should be added to make up to the final reaction volume. Dispense 45 μ l of premix into wells of 96-well low profile polypropylene white microplate and add 5 μ l of standard or unknown DNA into each well.

3.2.1. Place the PCR plate in the thermal cycler and start the cycling program. For M667 and AA55 primers the cycling protocol is the following:

(1) Pre-incubation	50°C for 2 min
(2) Initial denaturation	95°C for 10 min
(3) Denaturation	94°C for 10 s

(4) Annealing	62°C for 20 s
(5) Extension	72°C for 20 s
(6) Fluorescence data acquisition	
(7) Cycle number	45 cycles (steps 3-6)
(8) Final extension	72°C for 5 min
(9) Fluorescence data acquisition	
(10) Melting curve	65-95°C, 0.2°C 1 s hold
(11) Re-annealing	72°C for 5 min.

3.2.1. Analyze melting curve for each sample to control the specificity of an amplified product. Determine absolute HIV-1 RTC cDNA count using a standard curve generated from dilutions of standard DNA from 8E5 cells.

3.4. Co-immunoprecipitation of HIV-1 RTCs and isolation of viral cDNA

While sensitivity of a real-time PCR method of quantitative DNA analysis is very high, for representative results the quantity of RTCs in each specimen for immunoprecipitation (IP) should be higher than 1×10^6 .

3.2.1. Wash appropriate volume of protein G sepharose (5 µg of beads is required for each RTC sample) three times with 1-1.5 ml of buffer K. After each wash, pellet the beads by centrifugation on a table-top microcentrifuge at 5,000 RPM and 4°C for 5 min. Resuspend protein G beads in 0.5 ml of buffer K supplemented with 10 mg/ml BSA and 1 mg/ml salmon sperm DNA. Incubate suspension of beads on rotation shaker at 10 RPM and 4°C for 3 h or overnight.

3.2.1. Pellet protein G beads as described above, wash with 1.5 ml of ice-cold buffer K once and finally resuspend in the volume of buffer K equal to the initial one.

3.2.1. Dispense cytoplasmic and nuclear RTCs into Eppendorf microtubes; the amount of RTCs should be such as to create a large excess of the antibody to be used for IP. For a comparison of protein composition of cytoplasmic and nuclear RTCs, these RTCs should be added in equal amounts to IP reactions. Adjust volume of each aliquot of RTC suspensions to 250 µl by ice-cold buffer K. Add 4 µl of non-immune serum and 2.5 µg of protein G-Sepharose in buffer K to each sample. Incubate suspensions on rotation shaker for 2 h at 4°C and 10 RPM.

3.2.1. Clarify samples by centrifugation on a table-top microcentrifuge at 5,000 RPM and 4°C for 5 min. Place supernatants into new Eppendorf tubes and add 4 µg of appropriate antibody or purified mouse, rabbit or other IgG as isotype control. Incubate overnight on rotation shaker at 4°C and 10 RPM, then add 2.5 µg of protein G-sepharose to each sample and continue incubation for an additional 2 h.

3.2.1. Pellet immune complexes by centrifugation at 5,000 RPM and 4°C for 10 min and completely remove supernatants. Wash pellets three times with 1 ml of buffer K supplemented with 0.1% Triton X-100, and once with 1 ml of buffer K without Triton.

3.2.1. Pellet immune complexes as described above, resuspend in 50 µl of Sample Buffer (Reagent A) from IsoQuick Nucleic Acid Extraction Kit (see **Note 8**). Incubate suspension at room temperature for 10-15 min and add equal volume (50 µl) of Lysis Solution (Reagent 1). Then extract DNA as described in 3.3 steps 1, 2.

3.5. Real-time PCR analysis of DNA from immune-precipitates of HIV-1 RTCs

For examination of RTC protein composition, cDNA isolated from RTC immune precipitates is quantitatively analyzed by real-time PCR as described in 3.3, steps 3-6. Analysis can be performed using SYBR Green and TaqMan real-time PCR assay. Here we present protocol for iQ SYBR Green Supermix (BioRad).

Real-time PCR should be performed in triplicate for each standard and unknown specimen dilution; DNA counts in isotype immunoprecipitates should be subtracted from the data obtained with specific antibody.

M667 and AA55 primers described in 3.3 are specific for the negative-strand “strong-stop” DNA and allow analysis of protein composition of RTCs comprising both incomplete and complete reverse transcripts (immature and mature RTCs). To recognize proteins in mature RTCs (with complete cDNA), the FOR-LATE and REV-LATE primers specific for the late reverse transcripts (19) should be used.

3.2.1. Complete step 3 from 3.3. Then prepare appropriate volumes of PCR premixes for primers specific for the early and late HIV-1 cDNA. Each premix should contain ½ volume of 2X iQ SYBR Green Supermix and 15 pmoles of each primer (M667 and AA55 for early reverse transcription product, and FOR-LATE and REV-LATE-NL43 for late reverse transcription product detection) per 45 µl of mix. ddH₂O should be added to make up to the final reaction volume. Dispense 45 µl of premix into wells of 96-well low profile polypropylene white microplate and add 5 µl of standard or unknown DNA dilution into each well.

3.2.1. Perform real-time PCR reaction using cycling protocol described in step 5 of 3.3. The same protocol can be applied to PCR reaction with primers specific for early and late HIV-1 cDNA.

3.2.1. Determination of absolute cDNA counts from IP specimens allows to calculate values of cDNA recovery in immunoprecipitated RTCs as a percentage of total HIV-1 DNA detected in the cRTCs (see 3.3).

4. Notes

3.2. Various transfection reagents such as FuGene-6 (Roche) or Lipofectamine 2000 (Invitrogen) can be used for co-transfection of 293T/17 cells with molecular clone of HIV-1 and Env (MLV)-encoding plasmid.

3.2. 293T/17 cells form very unstable monolayers. Change of medium and wash of cells should be performed with 37°C pre-warmed DMEM or PBS very carefully to avoid detaching the cells.

3.2. Polyallomer centrifuge tubes (Beckman Coulter) for purification of viral stocks can be used as well as ultra-clear tubes.

3.2. Any HIV-1 p24 ELISA kits can be used for normalization of viral titers for subsequent infection.

3.2. For the isolation of reverse transcription complexes from infected cells, long-time incubation of cells after infection is not recommended. Since RTCs are not stable and are actively destroyed by proteasomes, duration of incubation should not exceed 24 h if cells are not treated with proteasomal inhibitors.

3.2. Cell lysis can be assessed using phase-contrast microscopy (use 10 μ l of samples after cell homogenization).

3.2. Contamination of nuclear fractions with cytoplasmic components can be assessed using PCR with primers specific for mitochondrial DNA (forward primer, Mito1: 5'-GAA TGT CTG CAC AGC CAC TT-3'; reverse primer, Mito2: 5'-AGA AAG GCT AGG ACC AAA CC-3'). PCR analysis of serial dilutions of cytoplasmic and nuclear extract samples allows assessing the level of contamination.

4.8. We use IsoQuick Nucleic Acid Extraction Kit to extract the HIV-1 cDNA from suspensions of cytoplasmic and nuclear RTCs. However, other methods and kits for DNA extraction are acceptable.

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