Cell Fractionation and Quantitative Analysis of HIV-1 Reverse Transcription in Target Cells

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[Abstract] This is a protocol to detect HIV-1 reverse transcription products in cytoplasmic and nuclear fractions of cells infected with VSV-G-pseudotyped envelope-defective HIV-1. This protocol can also be extended to HIV-1 with regular envelope.

Materials and Reagents

1. HEK 293T cells
2. HeLa cells
3. Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Cellgro®, catalog number: 10-013-CV)
4. R9-ΔE plasmid ((Zhou and Aiken, 2001), an HIV-1 proviral DNA clone created by introducing a frameshift mutation in envelope of the wild-type infectious R9 clone. Virions produced by this clone are non-infectious but can be made infectious by pseudotyping with envelopes from VSV or other viruses)
5. pHCMV-G (VSV-G) plasmid ((Yee *et al*., 1994), a retrovirus-derived plasmid in which the retroviral envelope glycoprotein is replaced with glycoprotein from vesicular stomatitis virus [VSV]).
6. p24 ELISA kit (in-house)
7. Phosphate-buffered saline (PBS) (Mediatech, Cellgro®, catalog number: 21-0310-CV)
8. VSV-G-pseudotyped envelope-defective HIV-1 (R9-ΔE) virus particles
9. Efavirenz (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, catalog number: 11680)
10. DNase I (Roche, catalog number: 10104159001)
11. 0.25% Trypsin/2.21 mM EDTA (Mediatech, Cellgro®, catalog number: 25-053-CI)
12. Triton X-100 (Mallinckrodt, catalog number: 9002-93-1)
13. DNeasy blood and tissue kit (QIAGEN, catalog number: 69506)
14. cOmplete, Mini, EDTA-free protease-inhibitor cocktail tablet (Roche, catalog number: 11836170001)
15. 4 to 20% Polyacrylamide gradient Tris-glycine gels (Bio-Rad Laboratories)

16. Nitrocellulose membrane (General Electric Company)
17. Mouse monoclonal anti-GAPDH antibody (Santa Cruz, catalog number: sc-47724)
18. Mouse monoclonal anti-LaminB1 antibody (Life Technologies, catalog number: 33-2000)
19. SYBR green (ABI, catalog number : 4309155)
20. DpnI (New England Biolabs, catalog number: R0176L)
21. DTT
22. Yeast tRNA (Roche, catalog number : 10109541001)
23. Forward primer MH531 (5'-TGTGTGCCCGTCTGTTGTGT-3')
24. Reverse primer MH532 (5'-GAGTCCTGCGTCGAGAGAGC-3')
25. DNase/RNase-free water
26. SDS-PAGE sample buffer
27. Sodium deoxycholate (Sigma-Aldrich, catalog number: 30970)
28. *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) (Sigma-Aldrich, catalog number: B4554)
29. Hypotonic buffer (see Recipes)
30. Radioimmunoprecipitation buffer (see Recipes)
31. 2x BES-buffered saline (BBS) (see Recipes)

Equipment

1. 10 cm cell culture dish
2. 0.45-µm-pore-size syringe filters (Thermo Fisher Scientific, catalog number: 190-2545)
3. 0.20- µm-pore-size syringe filters (Thermo Fisher Scientific, catalog number: 190-2520)
4. 1.5 ml screw-cap tube
5. Tabletop centrifuge (Thermo Fisher Scientific, Sorvall®)
6. Tabletop refrigerated centrifuge (Thermo Fisher Scientific)
7. Mx-3000p thermocycler (Stratagene)
8. CO_2 incubator

Procedure

A. Production of VSV-G-pseudotyped envelope-defective HIV-1 (R9-ΔE clone) virus particles (Aiken, 1998)
   1. Culture 293T cells in DMEM containing 10% v/v fetal bovine serum (FBS) and supplemented with antibiotics [Penicillin (100 IU/ml) and Streptomycin (100 µg/ml)] at 37 °C, 5% CO_2.

2. Detach cells from a nearly confluent culture dish with the help of 0.25% Trypsin/2.21 mM EDTA and seed 2 x 10^6 cells in 9 ml medium per 100 mm culture dish and incubate at 37 °C.
3. Transfect of 293T cells next day using the calcium phosphate-BBS method (Chen and Okoyama, 1987).
   a. Mix 15 µg of R9-ΔE and 5 µg of pHCMV-G (VSV-G) plasmids in a tube.
   b. Add 0.2 µm filtered water to the tube to make up the volume to 450 µl.
   c. Add 50 µl of 2.5 M $CaCl_2$ to the tube.
   d. Add 500 µl of 2x BBS to the tube dropwise.
   e. Gently mix the contents of the tube by pipetting few times.
   f. Incubate the tube at room temperature for 20 to 30 min.
   g. Add the mixture to 293T cells with gentle swirling and incubate cells at 35 °C and 3% CO_2.
4. Aspirate media from the transfected dish ~16 h after transfection, wash cells with 5 ml PBS, replenish with 5 ml of fresh cell culture media and incubate at 37 °C, 5% CO_2.
5. Two days after transfection, harvest culture supernatant containing virus particles, centrifuge at 1,500 *x g* for 5 min to pellet cells and debris.
6. Filter the supernatant through 0.45-µm-pore-size syringe filters, aliquot and freeze at -80 °C.

B. Infection of HeLa cells with VSV-G-pseudotyped envelope-defective HIV-1 (R9-ΔE)
1. Plate HeLa cells at a density of 1.5 x 10^5 cells/well in 12-well plates (1 ml total culture volume per well).
2. 24 h later treat virus inocula with DNase I (20 µg/ml) plus $MgCl_2$ (10 mM) and incubate in a water bath at 37 °C for 1 h.
3. Infect cells with DNase I-treated inocula equivalent to 15 ng of p24 (determined by p24 ELISA using in-house kit (Wehrly and Chesebro, 1997)).
4. Perform parallel infection in the presence of efavirenz (1 µM) to define the residual plasmid DNA levels carried over from transfection.
5. Incubate infected cells at 37 °C for 8 h.
   Note: One can also analyse time course of reverse transcription by harvesting infected cells at different time intervals after infection.

C. Cell fractionation of HIV-1 infected HeLa cells
1. After incubation for desired time, aspirate culture media and wash cells once with PBS.
2. Dislodge adherent cells by incubation with 500 µl of 0.25% Trypsin-EDTA at 37 °C for 2 min.

3. Collect trypsinized cells in a 1.5 ml screw-cap tube. Centrifuge at 300 *x g* for 5 min to pellet cells.
4. Lyse cell pellets in 200 µl of hypotonic buffer containing 0.1% Triton-X-100 and incubate on ice for 15 min.

   Note: Concentration of Triton-X-100 was optimized for HeLa cells. The concentration of Trition X-100 represents the lowest concentration at which about 95% of the cells counted under the microscope had intact nuclei but no plasma membrane.
5. Centrifuge at 17,000 *x g* for 5 min at 4 °C and collect the supernatant as cytoplasmic fraction.
6. Wash the nuclear pellet with 1 ml hypotonic buffer without Triton-X-100 thrice. After each wash centrifuge at 17,000 *x g* for 5 min at 4 °C to pellet the nuclei and aspirate off supernatant.
7. Isolate DNA from nuclear pellet using DNeasy blood and tissue kit as per manufacturer's protocol. Elute DNA in the last step in a fresh collection tube using 100 µl DNase/RNase-free water. Eluted DNA can be stored at -80 °C or used directly to perform qPCR.
8. In parallel, prepare whole-cell, cytoplasmic and nuclear lysates from uninfected cells to check for cytoplasmic contamination of nuclear fractions.
9. To prepare whole cell lysate, lyse cells in radioimmunoprecipitation (RIPA) buffer (Follow steps C2-C5 except the use of RIPA buffer instead of hypotonic buffer). Add equal volume of 2x SDS-PAGE sample buffer for gel electrophoresis and heat at 95 °C in a heat block for 5 min.
10. Prepare cytoplasmic lysate as described above (steps C2-C5). Add equal volume of 2x SDS-PAGE sample buffer for gel electrophoresis and heat at 95 °C in a heat block for 5 min.
11. To prepare nuclear lysate, follow steps 2 to 6, and then lyse the nuclear pellet in 1x SDS-PAGE sample buffer. Heat at 95 °C in a heat block for 5 min. Resolve equal volumes of whole cell, cytoplasmic and nuclear lysates on a 4-20% polyacrylamide gradient Tris-glycine gel.
12. Transfer resolved proteins onto a nitrocellulose membrane.
13. Block the membrane with 5% non-fat milk solution in PBS and probe with anti-GAPDH and anti-LaminB1 antibodies (concentrations recommended by manufacturer) followed by appropriate secondary antibodies (concentrations recommended by manufacturer) as cytoplasmic and nuclear markers respectively.

D. SYBR green-based Quantitative PCR for quantitation of viral reverse transcription products

1. Treat isolated DNA from step C7 with DpnI (17 µl DNA + 2 µl buffer + 1 µl of DpnI-20 units) by incubation at 37 °C for 1 to 2 h. Inactivate DpnI by incubation at 80 °C for 20 min.
2. Quantitation of viral reverse transcription products.
   a. Prepare reaction mixture by mixing DNA (5 µl), PCR mix containing SYBR green (12.5 µl), forward primer (150 nM), reverse primer (150 nM) and tRNA (1 µg/µl) containing DNase/RNase-free water up to 25 µl.
   b. Prepare standards ranging from 10 to 10^9 copies/reaction of R9-ΔE plasmid. Dilutions of standards should be made in 1 µg/µl tRNA-containing water.
   c. Set PCR reaction using the following thermal profile:

50 °C – 2 min
95 °C – 10 min } 1st cycle

95 °C – 15 sec
60 °C – 90 sec } 40 cycles
72 °C – 05 sec

95 °C – 60 sec
55 °C – 30 sec } last cycle
95 °C – 30 sec

Recipes

1. Hypotonic buffer
   10 mM Tris pH 8.0
   10 mM KCl
   1.5 mM $MgCl_2$
   1 mM DTT
   Protease inhibitor cocktail (one tablet per 10 ml of buffer)
2. Radioimmunoprecipitation buffer
   50 mM Tris pH 7.5
   1% Triton-X-100
   250 mM NaCl
   5 mM EDTA
   0.1% SDS
   1% sodium deoxycholate
   Protease inhibitors cocktail (one tablet per 10 ml of buffer)

3. 2x BES-buffered saline (BBS)
   50 mM BES (*N, N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid)
   1.5 mM Na_2HPO_4
   280 mM NaCl
   pH 6.95

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