Preparation and Titration of HIV-1 Env-Pseudoviruses (Montefiori Lab) July 2008

I. INTRODUCTION

The performance of neutralizing antibody assays under properly standardized, optimized and validated conditions requires accurate titration of virus infectivity. Moreover, use of molecularly cloned pseudoviruses has advantages over uncloned virus for greater reagent stability and assay reproducibility and precision. This protocol describes the production of molecularly cloned Env-pseudotyped HIV1 in 293T/17 cells by co-transfection with an Env-expressing plasmid plus a backbone plasmid lacking Env. Co-transfection generates pseudovirus particles that are able to infect cells but, due to the absence of a complete genome, are generally unable to produce infectious progeny virions. This single round of infection is readily detectable in genetically engineered cells lines that contain a Tat-responsive reporter gene, such as luciferase. Neutralization assays based on a single-round of infection should use viruses that are titrated in a similar singe-round infection format.

It is important to note that Env-pseudotyped viruses are handled and treated the same as live HIV-1 cultures since replication-competent virus could be present. Sequence analysis indicates that recombination can occur that repairs the defect in the backbone plasmid(s) lacking Env, thus rendering the virus replication-competent. The recombination event occurs at a relatively low frequency and the proportion of the pseudovirus that is replication-competent is low. When recombination occurs that renders RCV, the virus generated is predicted to behave identically to wild-type HIV-1.

II. DEFINITIONS

GM, Growth Medium
DMEM, Dulbecco's Modified Eagle Medium
HEPES, N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
Luc, Luciferase
RLU, Relative Luminescence Units
FBS, Fetal Bovine Serum
DPBS, Dulbecco’s Phosphate Buffered Saline
ID, Identification
RCV, Replication competent virus

III. REAGENTS AND MATERIALS

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality may be used when necessary.

293T/17 cells
ATCC

TZM-bl cells
NIH AIDS Research and Reference Reagent Program

Growth Medium*
DMEM, with L-glutamine, sodium pyruvate, glucose and pyridoxine, sterile. Store refrigerated at 4ºC.
Gibco BRL Life Techonologies, cat no. 11995-065

Fetal bovine serum, heat-inactivated 56°C for 30 minutes, 500 ml bottle, sterile. Store at -20°C. Once thawed, store at 4°C for up to 1 month.
Hyclone

Gentamicin solution, 10 mg/ml, sterile
Sigma, store at 4ºC

HEPES Buffer, 1M, store at 4°C
Sigma

*Complete GM consists of DMEM containing 10% heat-inactivated FBS, 50 μg gentamicin/ml and 25mM HEPES. To make 500 ml of GM, combine 435 ml DMEM, 50 ml FBS , 2.5 ml of gentamicin, and 12.5 ml HEPES in a sterile bottle, mix, store at 4°C for up to 2 months. Warm medium to 25°C – 37°C prior to use.

DEAE-Dextran, hydrochloride, average Mol. Wt. 500,000
Sigma

Prepare a 5 mg/ml solution by dissolving 5 gm in 1 L of sterile water. Store at -80°C in 10 ml aliquots in 15 ml sterile polypropylene tubes.

Trypsin-EDTA (0.25% trypsin, 1 mM EDTA), sterile
Gibco cat. no. 25200-056

DPBS
Dulbecco's Phosphate Buffered Saline

FuGENE 6 Transfection Reagent
Roche Applied Science cat no. 11988387001

Britelite Plus Luminescence Reporter Gene Assay System
Perkin Elmer Life and Analytical Sciences cat no. 6016769

Reconstitute one vial of lyophylized Britelite Plus Substrate Solution with 250 ml of Britelite Plus Substrate Buffer Solution. After the substrate has dissolved completely (about 1 minute), mix gently and distribute 10.5 ml to 15 ml conical polypropylene tubes and store at -80°C immediately. Thaw in a room temperature water bath in the dark immediately before each use. Mix gently prior to use. Use within 60 minutes of thawing. Excess reagent may be stored at -80°C and used once more.

Caution: The lyophylized Britelite substrate is classified as hazardous. Latex gloves, surgical gown and eye protection are required when working with these reagents.

NOTE 1: Bright Glo substrate solution from Promega and Britelite substrate solution from Perkin Elmer Life and Analytical Sciences are acceptable substitutes for Britelite Plus. Please follow manufacturer's guidelines for preparation and use.

Hemocytometer
Hausser Scientific, Horsham, PA

12-channel pipetteman, 5-50 µl
ThermoLabsystem

12-channel pipetteman, 30-300 µl
ThermoLabsystem

Single channel pipetteman, 5-50 µl
ThermoLabsystem

Single channel pipetteman, 30-200 µl
ThermoLabsystem

Microliter pipet tips, sterile
ICN

PipetteAid XP
Drummond Scientific Co.

Disposable pipettes, sterile, individually wrapped
Falcon/VWR
1 ml pipets
5 ml pipets
10 ml pipets
25 ml pipets
50 ml pipets

Flat-bottom culture plates, 96-well, low evaporation, sterile
Costar/VWR

Flat-bottom black solid plates, 96-well, Costar brand
Fisher

15 ml conical polypropylene tubes, screw-cap, sterile
Corning

Culture flasks with vented caps, sterile
Costar/VWR
T-25 flask
T-75 flask

Reagent reservoirs, 50 ml capacity
Costar

Instrumentation:

Biological Laminar Flow Cabinet (annual certification required)
NuAIRE, Plymouth, MN; Model NU-425-600

Incubator, water-jacketed (37°C, 5% CO_2 standard requirements)
Forma Scientific, Steri-Cult 200, Model 3033

Desk-top centrifuge (low speed capable of up to 500 x g)
Jouan, Model C412
50 ml tube holder
15 ml tube holder
Microtitration plate holder

Luminometer equipped to read 96-well plates
PerkinElmer Life Sciences, Model Victor2

Controlled temperature water bath
Precision Scientific, Model 182

Microcentrifuge (maximum rotational speed = 14,000 rpm)
Eppendorf, Model 5415C
18 place standard rotor F-45-18-11 for 1.5 ml microcentrifuge tubes

Specimens:

Molecularly cloned pseudoviruses are generated by using a two plasmid system: 1) an Env expression plasmid (e.g., pcDNA 3.1D/V5-His-TOPO-Env) and a backbone vector (e.g., pSG3ΔEnv) that expresses the entire HIV-1 genome except Env. Uncloned viruses for titration may be cell-free stocks that are produced in either PBMC or human T cell lines.

IV. PROTOCOL

NOTE 2: All incubations are performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified.

Thawing Cells

NOTE 3: Be sure to wear a full-face shield during handling of frozen samples.

NOTE 4: TZM-bl and 293T/17 are adherent cell lines that are maintained in T-75 culture flasks. Cell monolayers are disrupted and removed by treatment with trypsin/EDTA at confluency.

1. Transfer cryovials containing frozen cells from liquid nitrogen to a room temperature water bath in the biosafety hood. If liquid nitrogen has seeped into the cryovial, loosen the cap slightly to allow the nitrogen to escape during thawing. Hold the cryovial on the surface of the water bath with an occasional gentle "flick" during thawing. Do not leave the cryovial unattended during the thawing process. (It is important for cell viability that the cells are thawed and processed quickly - thawing only takes a few seconds). Dry off the outside of the cryovials and wipe with alcohol solution before opening to prevent contamination.

2. Transfer the contents of one vial of cells to a T-75 culture flask containing 30 ml of GM. Note: It is important to dilute the DMSO at least 30-fold at this point to avoid cell toxicity.

3. Incubate the cells at 37°C for 1 day.

4. Remove the medium and replace with 15 ml of fresh GM. Change the medium every 2-3 days until the cell monolayers are confluent.

Splitting Cells

1. Decant the culture medium and remove residual serum by rinsing monolayers with 5 ml of sterile DPBS.

2. Slowly add 2.5 ml of an 0.25% Trypin-EDTA solution to cover the cell monolayer. Incubate at room temp for 30 seconds. Decant the trypsin solution and incubate at 37°C for 4 minutes in the case of TZM-bl cells. Incubate at room temperature for 1 minute in the case of 293T/17 cells. Do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.

3. Add 10 ml of GM and suspend the cells by gentle pipet action. Count cells.

4. Seed new T-75 culture flasks with approximately 10^6 cells in 15 ml of GM. Cultures are incubated at 37°C in a 5% CO2/95% air environment. Cells should be split approximately every 3 days.

Transfection of 293T/17 Cells

1. Seed 3 x 10^6 293T/17 cells in a T75 flask containing 12 ml GM. Incubate overnight. Monolayers should be 50-80% confluent on the day of transfection.

2. Based on the volume of Env plasmid DNA and backbone plasmid DNA to be dispensed, add the appropriate volume of DMEM, such that the total volume of the mixture is 100 µl, into one sterile tube. Dispense 4 µg of Env plasmid DNA and 8 µg of backbone plasmid DNA to the tube containing DMEM and mix well.

3. To a second sterile tube, add 652 µl DMEM. Pipet 48 µl of FuGENE 6 reagent directly into medium without contacting the sides of the plastic tube. Mix well.

4. Transfer the entire contents of the plasmid DNA mixture from the first tube to the second tube containing the FuGENE solution. Mix by pipetting or briefly vortexing.

5. Incubate for 30 minutes at room temperature to allow complex formation.

6. Add the entire contents of the transfection complexes to a T-75 flask of 293T/17 cells. Gently swirl the flask for uniform distribution of the complexes.

7. Incubate for 3 to 8 hours at 37°C in a 5% incubator to allow the plasmids to enter the cells.

8. Decant the medium containing DNA-FuGENE complexes and replace with 15 ml fresh GM. Incubate for 24-48 hours.

9. Harvest virus-containing culture supernatants by collecting the medium from the flask using a pipet. Collect as much as possible without drawing cells into the pipet. Adjust the FBS concentration in the virus-containing culture medium to 20% and mix using a pipette. Filter the virus-containing culture fluid through a 0.45-micron filter. Distribute aliquots of appropriate volume to polypropylene screw-cap tubes that have been labeled to identify the isolate name and the date of harvest. The harvest date becomes the specific lot number. Store the aliquots at -80°C. Record the harvest and location of the vials. Include the identification regarding the Env plasmid DNA, backbone DNA, and

cells used in the transfection when logging this information.

10. Add 12 ml of fresh GM to the cells in each flask. Incubate overnight and harvest the virus-containing culture supernatants once more, as previously indicated, and discard the cells. Record appropriate harvest information as before.

Titrating Pseudovirus in TZM-bl Cells (TCID Assay)

1. Place 100 µl of GM per well in all wells of a 96-well flat-bottom culture plate. Transfer 25 µl of pseudovirus to the first 4 wells of a dilution series (column 1, rows A-D for one pseudovirus and rows E-H for a second pseudovirus), mix, do serial 5-fold dilutions (i.e., transfer 25 µl, mixing each time) for a total of 11 dilutions. Discard 25 µl from the 11th dilution. Wells in column 12 will serve as cell controls (no virus added).

2. Add 100 µl of TZM-bl cells (10,000 cells/100 µl DMEM containing 25 µg DEAE dextran/ml) to all wells. Rinse your pipet tips in a reservoir containing sterile DPBS or change pipettor tips between each plate to minimize carry-over. The final concentration of DEAE-Dextran is 10 µg/ml.

NOTE 5: The concentrations of DEAE-Dextran shown above are approximations. The actual optimal concentration should be determined for each new batch of dextran by performing a titration assay (see Protocol for Determination of Optimal Concentration of DEAE-dextran).

NOTE 6: The use of DEAE-Dextran is optional. When the TCID of the pseudovirus is measured in the absence of DEAE-Dextran, the TZM-bl assay must also be conducted in the absence of DEAE-Dextran.

3. Incubate for 48-72 hours if Env-pseudotyped viruses are being assayed.

NOTE 7: Replication-competent viruses require a 48 hour incubation period.

4. Remove 100 µl of culture medium from each well, leaving approximately 100 µl. Dispense 100 µl of Britelite Plus Reagent to each well. Incubate at room temperature for 2 minutes to allow complete cell lysis. Mix by pipet action (two strokes) and transfer 150 µl to a corresponding 96-well black plate. Read the plate immediately in a luminometer.

5. Calculate the TCID using the "TCID" macro. Select the pseudovirus dilution that yields 150,000 RLU equivalents (+/- 15,000 RLU). For pseudoviruses that do not reach 150,000 RLU, select a dose of virus that yields at least 15,000 RLU but is not toxic to the cells via light microscopy.

NOTE 8: The RLU equivalents measured in the TCID assay may not match the RLUs in the virus control of the neutralization plate. This difference is acceptable provided that the virus control is greater than or equal to 10 times the background (cell control) and the virus is not toxic to the cells based on light microscopy.

NOTE 9: See Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells for virus dose determination for uncloned virus stocks.