Protocol for the Preparation and Titration of HIV-1 Env-pseudotyped Viruses
(December 2011)

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 Env-pseudotyped viruses has advantages over uncloned virus in terms of reagent stability and affording greater reproducibility and precision in neutralization assays. This protocol describes the production of molecularly cloned Env-pseudotyped viruses in 293T/17 cells by co-transfection with an Env-expressing plasmid plus a backbone plasmid containing a defective Env gene. Co-transfection generates pseudovirus particles that are able to infect cells, but due to the absence of a complete genome, they are generally unable to produce infectious progeny virions. This single round of infection is readily detectable in genetically engineered cell lines that contain a Tat-responsive reporter gene, such as luciferase. Neutralization assays that are based on a single-round of infection should use viruses that are titrated in a similar single-round infection format.

It is important to note that Env-pseudotyped viruses are handled and treated the same as live HIV-1 cultures as 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

Luc: Luciferase

RLU: Relative Luminescence Units

FBS: Fetal Bovine Serum

ID: Identification

DPBS: Dulbecco's Phosphate Buffered Saline

TCID: Tissue Culture Infectious Dose

CO_2: Carbon Dioxide

RCV: Replication Competent Virus

DEAE-Dextran: Diethylaminoethyl-Dextran

III. Reagents and Materials

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

293T/17 cells
Vendor: American Tissue Culture Collection

TZM-bl cells
Vendor: NIH AIDS Research and Reference Reagent Program

Growth Medium (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells**)**

DEAE-Dextran, hydrochloride, average Mol. Wt. 500,000 (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)
Vendor: Sigma

Trypsin-EDTA (0.25% trypsin, 1 mM EDTA)
Vendor: Invitrogen
Sterile

DPBS
Vendor: Invitrogen
Sterile

FuGENE 6 Transfection Reagent
Vendor: Promega

Britelite Plus Reporter Gene Assay System (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)
Vendor: Perkin Elmer Life and Analytical Sciences

NOTE 1: The lyophylized Britelite Plus substrate is not classified as hazardous.

NOTE 2: 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. Britelite and Bright Glo are classified as hazardous. Personal Protective Equipment (PPE) is required when working with these reagents.

Microliter pipettor tips
Vendor: ICN
Sterile

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

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

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

Cryogenic vials, 1.5 ml sterile screw cap, frosted label
Vendor: Sarstedt Brand Products

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

Reagent reservoirs, 50 ml capacity
Vendor: Costar

Trypan Blue (0.4%)
Vendor: Sigma

IV. Instrumentation

Recommended manufacturers are listed. Unless otherwise specified, equipment of equal or better quality than the recommended ones can be used whenever necessary.

Biological Safety Cabinet
Manufacturer: Baker Co.

CO_2 Incubator
Manufacturer: Forma Scientific
Water-jacketed (37^{o}C, 5% CO2 standard requirements)

Centrifuge and Microcentrifuge
Manufacturer: Jouan
(low speed capable of up to 500 x g)
50 ml tube holder
Microtitration plate holder

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

Luminometer
Manufacturer: PerkinElmer Life Sciences

Water bath
Manufacturer: Precision Scientific

Hemacytometer
Manufacturer: INCYTO

Light Microscope
Manufacturer: Olympus

Pipettor
Manufacturer: ThermoLabsystems
12-channel pipetteman, 5-50 µl
12-channel pipetteman, 30-300 µl
Single channel pipetteman, 5-50 µl
Single channel pipetteman, 30-200 µl

Manufacturer: Drummond Scientific Co.
PipetteAid XP

Manufacturer: BioHit
12-channel electronic pipettor, 50-1200 µl
Single channel electronic pipettor, 10-300 µl

Manufacturer: Rainin
12-channel pipettor, 20-200 µl

V. Specimens

Molecularly cloned Env-pseudotyped viruses are generated by using a two plasmid system:

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 for Env.

VI. Protocol

NOTE 3: All incubations are performed in a humidified 37°C, 5% CO_2 / 95% air environment incubator unless otherwise specified.

1. Preparation of Cells

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.

1.1 Trypsinize the 293T/17 cells (see Protocol for Trypsin-EDTA Treatment for Disruption of Cell Monolayers) and check for an acceptable level of cell viability.

1.2 Cell Viability Procedure

a. Dilute the cell suspension 1:10 (or a dilution that yields approximately 500,000 cells/ml) in sterile DPBS or GM.

b. Mix a portion of the cell suspension (e.g., 100 µl) with an equal volume of 0.4% Trypan Blue staining solution.

c. Load a hemacytometer and count viable cells (viable cells are clear, dead cells are blue).

d. At least 80% of the cells must be viable in order to use the cells for transfection.

2. Transfection of 293T/17 Cells

2.1 Seed 3-5 x 10^6 293T/17 cells in a T-75 flask containing 12 ml of GM. Incubate overnight (20-24 hours). Monolayers should be 50-80% confluent on the day of transfection.

NOTE 5: The following steps pertain to 1 x T75 flask. Additional flasks can be prepared by multiplying each volume by an appropriate factor per flask.

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

NOTE 6: The amounts of Env plasmid and backbone plasmids shown above are a recommendation. The optimal ratio of Env and backbone plasmids used to prepare each env-pseudotyped virus strain may vary. If it is necessary to improve the virus yield, the optimal ratio for a particular virus may be determined by transfecting 293T/17 cells using different Env and backbone plasmid ratios. A different ratio may be used if it generates higher virus yield than the recommended ratio.

2.3 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 with a pipettor.

2.4 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.

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

2.6 Mix the entire contents of the transfection complexes immediately prior to adding them to the T-75 flask of 293T/17 cells.

2.7 Gently 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.

2.8 Incubate for 3 to 8 hours at 37°C to allow the plasmids to enter the cells.

2.9 Carefully remove the medium containing DNA FuGENE complexes and replace with 15 ml of fresh GM. Incubate for 24-48 hours.

2.10 Harvest virus-containing culture supernatants by collecting the medium from the flask using a pipette. Adjust the FBS concentration in the virus-containing culture medium to 20% (i.e. for each 1 ml of virus harvested, add 0.125 ml of FBS) and mix using a pipette. Filter the virus-containing culture medium through a 0.45-micron filter. Aliquot the virus to sterile screw-cap cryogenic vials that have been labeled to identify the isolate name and date of harvest. The harvest date becomes the specific lot number. Store the aliquots at −80°C.

2.11 Record the harvest date information and location of the vials as well as the information regarding the Env plasmid DNA, backbone plasmid DNA, and cells used in the transfection in a laboratory virus log book.

2.12 Add 5-12 ml of fresh GM to the cells in each flask. Incubate overnight and harvest the virus-containing culture supernatants once more, as indicated above, and discard the cells. Record the appropriate harvest information as before.

3. Titrating Pseudovirus in TZM-bl Cells (TCID Assay)

NOTE 7: Assays with replication-competent viruses are incubated for 48 hours to keep virus replication at a minimum.

3.1 Place 100 µl of GM per well in all wells of a 96-well flat-bottom culture plate. Transfer 25 µl of virus to the first 4 wells of a dilution series (column 1, rows A-D for one virus and rows E-H for a second virus), 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 11^{th} dilution. Wells in column 12 will serve as cell controls for background luminescence (no virus added).

3.2 Plates may be incubated in the incubator or at room temperature while cells are prepared.

3.3 Trypsinize the TZM-bl cells (see Protocol for Trypsin-EDTA Treatment for Disruption of Cell Monolayers) and check for an acceptable level of cell viability.

3.4 Cell Viability Procedure

- **a.** Dilute the cell suspension 1:10 (or a dilution that yields approximately 500,000 cells/ml) in sterile DPBS or GM.
- **b.** Mix a portion of the cell suspension (e.g., 100 ul) with an equal volume of 0.4% Trypan blue staining solution.
- **c.** Load a hemacytometer and count viable cells (viable cells are clear, dead cells are blue).
- **d.** At least 80% of the cells must be viable in order to use the cells for the TCID assay.

3.5 Add 100 µl of TZM-bl cells (10,000 cells/100 µl GM containing 25 µg DEAE-Dextran/ml) to all wells starting with the cell control and the most dilute virus. The cell/GM/DEAE-Dextran suspension should be prepared as follows: addition of GM, addition of cells, and finally addition of DEAE-Dextran. The cell suspension should be thoroughly mixed. Rinse the pipettor tips in a reagent reservoir containing sterile DPBS or change the pipettor tips between each plate to minimize carry-over. The final concentration of DEAE-Dextran is 10 µg/ml.

NOTE 8: The concentrations of DEAE-Dextran shown above are approximations. The actual optimal concentration should be determined for each new batch of DEAE-Dextran prepared by performing a titration assay. (see Protocol for Determination of Optimal Concentration of DEAE-Dextran)

NOTE 9: The use of DEAE-Dextran is optional. When the TCID of the virus is measured in the absence of DEAE-Dextran, the TZM-bl assay must also be conducted in the absence of DEAE-Dextran.

3.6 Cell Calculations:

To calculate the cell concentration, count the total number of cells in a predetermined number of quadrants in a hemacytometer and obtain the average cell count per quadrant. Multiply this number by the dilution to yield the cell concentration, "C_1", in cells x 10^4. To calculate the total cell mixture volume, "V_2", that you need, multiply the number of plates by the total volume of cell mixture needed per plate. The concentration of cells desired is 100,000 cells/ml, "C_2". Thus, using the equation $C_1V_1 = C_2V_2$, one can solve for "V_1", the volume of cells needed.

For example:

Total number of cells counted = 60
Number of quadrants counted = 4
Dilution = 1:10
Number of plates = 1
Cell mixture needed per plate = 10 ml

60 cells ÷ 4 quadrants = 15 cells/quadrant

15 X 10 = 150 x 10^4 cells/ml = 15 x 10^5 cells/ml = C_1

1 X 10 ml = 10 ml = V_2

Optimum final concentration of cells = 100000 = C_2

Therefore: $C_1V_1 = C_2V_2$

(100000 X 10) ÷ 1500000 = 0.67 ml of cells

To calculate the amount of DEAE-Dextran to use, first multiply the optimal concentration of DEAE-Dextran (derived from the titration experiment) by 0.2 ml (the final volume in each well) to get the amount of DEAE-Dextran per well. Multiply the amount of DEAE-Dextran per well by 100 wells/plate (96 wells rounds to 100) to derive the amount of DEAE-Dextran per plate. Divide the amount of DEAE-Dextran needed per plate by the stock concentration of the DEAE-Dextran to yield the volume of DEAE-Dextran stock needed. Multiply this number by the number of plates to yield the total volume of DEAE-Dextran stock needed.

For example:

If the optimal concentration of DEAE-Dextran to use is 10 µg/ml and the DEAE-Dextran stock is at 25 mg/ml

10 X 0.2 ml (volume in well) = 2.0 µg of DEAE-Dextran needed in each well

2.0 µg X 100 wells/plate = 200 µg of DEAE-Dextran needed per plate = 0.2 mg of DEAE-Dextran

0.2 mg of DEAE-Dextran per plate ÷ 25 mg/ml stock concentration = 0.008 ml of DEAE-Dextran stock needed

To calculate the amount of Growth Medium to add, subtract the total volume of cells needed and the total volume of DEAE-Dextran stock needed from the total volume of cell mixture needed.

So, the total volume needed is 10 ml

10 ml – 0.67 ml cells – 0.008 ml DEAE-Dextran = 9.322 ml of GM

3.7 Incubate for 48 – 72 hours if Env-pseudotyped viruses are being assayed. Replication-competent viruses require a 48 hour incubation period.

3.8 After incubation, remove plates from incubator. Plates should not stay out of the incubator longer than one hour before running the luciferase reaction.

NOTE 10: Check the plates for syncitia and cell toxicity via light microscopy.

3.9 Remove 100 µl of culture medium from each well, leaving approximately 100 µl. Dispense 100 ml of Britelite Plus reagent to each well.

3.10 Incubate at room temperature for 2 minutes to allow complete cell lysis. Mix by pipettor action (at least two strokes) and transfer 150 µl l to a corresponding 96-well black plate. Read the plate immediately in a luminometer (if possible, the luminometer should be connected to a secure network file server). The electronic data should be backed up on a routine basis.

3.11 Calculate the TCID using the "TCID" macro (if possible, this macro should be stored on a secure network file server). 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 11*:** 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 12: TCID assays should be performed for pseudovirus stocks that have been thawed one time. These vials are marked with a "1X".