Protocol for Preparation and Titration of HIV-1 IMC 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 IMC 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 IMC viruses by transfection in 293T/17 cells. These viruses are replication competent as they are generated utilizing one full length plasmid DNA with or without an Env-expressing, molecular clone that carries a Renilla luciferase gene. Neutralization assays, such as the A3R5 Assay and M7-Luc Assay, that are based on multiple rounds of infection should use viruses that are titrated in a similar multiple round of infection format.

It is important to note that IMC viruses are handled and treated the same as live HIV-1 cultures as replication-competent virus is present. The virus generated is predicted to behave identically to wild-type HIV-1.

II. Definitions

GM: Growth Medium

DMEM: Dulbecco's Modified Eagle Medium

RPMI-1640: Roswell Park Memorial Institute

Luc: Luciferase

RLU: Relative Luminescence Units

Env.IMC.LucR: Env-expressing infectious molecular clones carrying a Renilla luciferase reporter gene

IMC: Infectious Molecular Clone

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

M7-Luc Cells
Provided by: Nathaniel R. Landau, The Salk Institute

A3R5 Cells
Provided by: Colonel Jerome Kim, US MHRP

DMEM-Growth Medium (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells) used for maintenance of TZM-bl and 293T/17 cells

RPMI-12%-Growth Medium (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells) used for maintenance of M7-Luc cells

RPMI-10%-Growth Medium (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in A3R5 Cells) used for maintenance of A3R5 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

RPMI-1640
Vendor: Invitrogen
Sterile

FuGENE 6 Transfection Reagent
Vendor: Roche Applied Science
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

ViviRen Live Cell Substrate (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in A3R5 Cells**)**
Vendor: Promega

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
Vendor: Fisher

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

Flat-bottom white 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% CO_2 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

Countess Automated Cell Counter
Manufacturer: Invitrogen

Hemacytometer
Manufacturer: INCYTO

Light Microscope
Manufacturer: Olympus

Fluorescence 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

1. Molecularly cloned Env.IMC.LucR viruses are generated by using one full length Env-expressing infectious molecular clone that carries a Renilla Luciferase reporter gene.

2. Molecularly cloned IMC viruses are generated using one full length plasmid DNA that does not carry a Renilla Luciferase reporter gene.

2.1 These viruses are prepared utilizing the same procedure as when preparing Env.IMC.LucR viruses but must be assayed in a cell line that has been engineered to carry a luciferase reporter gene, such as M7-Luc cells. In this case, the Britelite Plus Reporter Gene Assay System, or comparable product, must be used to measure luminescence.

VI. Protocol

NOTE 1: All incubations are performed in a humidified 37°C, 5% CO_2 / 95% air environment incubator unless otherwise specified.

1. Preparation of Cells

NOTE 2: 293T/17 is an adherent cell line that is 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 (see [1.2.4]).

1.2 Cell Viability Procedure

1.2.1 Dilute the cell suspension (dilution that yields approximately 500,000 cells/ml) in sterile DPBS or GM.
1.2.2 Mix a portion of the cell suspension (e.g., 50 ul) with an equal volume of 0.4% Trypan Blue staining solution.
1.2.3 Load a hemacytometer and count viable cells (viable cells are clear, dead cells are blue).
1.2.4 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 cm^2 flask containing 12 ml of DMEM-GM. Incubate overnight (20-24 hours). Monolayers should be 50-80% confluent on the day of transfection**.**

NOTE 3: The following steps pertain to one T-75 flask. Additional flasks can be prepared by multiplying each volume by an appropriate factor per flask.

2.2 Based on the volume of IMC 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 12 µg of IMC plasmid DNA into the tube containing DMEM and mix well with a pipettor.

NOTE 4: The amount of IMC plasmid DNA shown above is a recommendation. The optimal amount of IMC plasmid DNA used to prepare each IMC virus strain may vary. If it is necessary

to improve the virus yield, the optimal amount for a particular virus may be determined by transfecting 293T/17 cells using various IMC plasmid DNA amounts. A different amount may be used if it generates a higher virus yield than the recommended amount.

2.3 To a second sterile tube, add 652 µl DMEM. Pipet 48 µl of FuGENE 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 cm^2 flask of 293T/17 cells.

2.7 Gently add the entire contents of the transfection complexes to a T-75 cm^2 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 1ml of harvested virus, add 0.125 ml of FBS). 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 IMC plasmid DNA and cells used in the transfection in a designated laboratory virus log book.

2.12 Add 8-12 ml of fresh GM to the cells in the 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 Virus in M7-Luc and A3R5 Cells (TCID Assay)

NOTE 5: The procedures for titrating virus in either M7-Luc or A3R5 cells are identical except for the treatment of the cells, concentration of cells/well and the viral infectivity check.

3.1 Place 100 µl of appropriate GM (for M7-Luc - RPMI-12%-GM, for A3R5 - RPMI-10%-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 Cell Viability Procedure

3.3.1 Remove cell culture media containing a selective antibiotic from the culture flask leaving only 2-3 ml. Dilute 100 µl of cell suspension 1:10 (or a dilution that yields approximately 500,000 cells/ml) in GM.

3.3.2 Perform viability procedure for cells as described for 293T/17 cells in [1.2] above.

3.4 Add 100 µl of cells (M7-Luc - 60,000 cells/100 µl RPMI-12%-GM containing 16 µg DEAE-Dextran/ml, A3R5 - 90,000 cells/100 µl RPMI-10%-GM containing 20 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 in M7-Luc cells is 8 µg/ml and in A3R5 cells is 10 µg/ml.

NOTE 6: 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 7: The use of DEAE-Dextran is optional. When the TCID of the virus is measured in the absence of DEAE-Dextran, the M7-Luc or A3R5 assay must also be conducted in the absence of DEAE-Dextran.

3.5 Cell Calculations:

3.5.1 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 factor to yield the cell concentration, "C_1", in suspension 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. Thus, using the equation $C_1V_1 = C_2V_2$, one can solve for "V_1", the volume of cells needed. The concentration of cells desired, "C_2" is 600,000 cells/ml for M7-Luc or 900,000 cells/ml for A3R5 (shown below).

For example:

Total number of cells counted = 60
Number of quadrants counted = 4
Dilution factor = 20 (10 x 2 = 20 from Trypan Blue dilution)
Number of plates = 1
Cell mixture needed per plate = 10 ml

60 cells ÷ 4 quadrants = 15 cells/quadrant

15 x 20 x 10^4 cells/ml = 30 x 10^5 cells/ml = C_1

1 plate x 10 ml/plate = 10 ml = V_2

Optimum final concentration of cells = 900,000/ml = C_2

Therefore: $C_1V_1 = C_2V_2$

V_1 = (900,000 X 10) ÷ 3,000,000 = 3.0 ml of cells

3.5.2 To calculate the amount of DEAE-Dextran to use, first multiply the optimal concentration of DEAE-Dextran (see Protocol for Determination of Optimal Concentration of DEAE-Dextran) 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 5 mg/ml

10 µg/ml 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 = 0.25 mg of DEAE-Dextran

0.2 mg of DEAE-Dextran per plate ÷ 5 mg/ml stock concentration = 0.04 ml of DEAE-Dextran stock needed per plate

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 for one plate is 10 ml

10 ml – 3.0 ml cells – 0.04 ml DEAE-Dextran = 6.96 ml of GM

3.6 Incubate for 6-7 days. Keep the cells in good condition by splitting cells or refeeding when necessary.

4. Virus Infectivity Check in A3R5 cells

4.1 After 4 days incubation, remove plates from incubator. Plates should not stay out of the incubator longer than one hour prior to running the luciferase reaction.

NOTE 8: Check the plates for the presence of syncytia and cell toxicity via light microscopy.

4.2 Carefully remove 40 µl of supernatant from the plate. Suspend cells in each well and transfer 75 µl to corresponding wells of a 96-well white solid plate.

NOTE 9: When transferring the cells to the white plate, begin transferring the cells in the cell control wells and proceed across the plate to the wells with the highest virus concentration.

4.3 Dilute 10 µl of ViviRen Live Cell Substrate in 3.5 ml of GM. Substrate should be thawed immediately prior to use.

4.4 Dispense 30 µl of ViviRen Reagent to each well in the white plate. Tap the plate lightly to mix.

4.5 Incubate at room temperature for 4 minutes to allow for maximum luminescence. Read the plate in a luminometer using the 0.5 sec/well protocol on the Wallac Software.

4.6 Select the virus dilution that yields 50,000 RLU equivalents (± 10,000 RLU). For viruses that do not reach 50,000 RLU, select a dose of virus that yields at least 10,000 RLU but is not toxic to the cells via light microscopy.

4.7 Refeed the plates with 120 µl of fresh GM with DEAE-Dextran and return the plates to the incubator. Repeat reading procedure on day 6 or 7.

NOTE 10: Plates may be read on day 7 if day 6 occurs on a weekend or a holiday.

5. Virus Infectivity Check in M7-Luc cells

5.1 After 4 days incubation, remove plates from incubator. Plates should not stay out of the incubator longer than one hour prior to running the luciferase reaction.

NOTE 11: Check the plates for the presence of syncytia and cell toxicity via light microscopy. Check M7-Luc cells for fluorescence via fluorescence microscopy.

5.2 Thaw Britelite Plus immediately before use in an ambient temperature water bath away from light.

5.3 Suspend cells in each well and transfer 100 µl to corresponding wells of a 96-well white solid plate.

5.4 Dispense 100 µl of Britelite Plus to each well. Tap the plate lightly to mix.

5.5 Incubate at room temperature for 2 minutes to allow for maximum luminescence. Read the plate in a luminometer using the 1.0 sec/well protocol on the Wallac Software.

5.6 Select the virus dilution that yields 50,000 RLU equivalents (±10,000 RLU). For viruses that do not reach 50,000 RLU, select a dose of virus that yields at least 10,000 but it not toxic to the cells via light microscopy.

5.7 Refeed the plates with 100 µl of fresh GM with DEAE-Dextran and return the plates to the incubator. Repeat the reading procedure on day 6 or 7 (See NOTE 10).

6. Final TCID reading in M7-Luc and A3R5 cells

6.1 A final TCID reading should be made on day 6 or 7 (see NOTE 10). Repeat the reading procedure from day 4, see [5.1] to [5.7] for A3R5 cells and [5.1] to [5.7] for M7-Luc cells.

6.2 Calculate the TCID using the "TCID" macro (if possible, this macro should be stored on a secure network file server). Use 5x background control (cell control) cut-off for selecting positive wells

***NOTE 12*:** 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 13: TCID assays should also be performed for virus stocks that have been thawed one time. These vials are marked with a "1X".

VII. Appendix A:

Assay template for TCID Assay, 2 viruses per plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
B	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
C	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
D	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
E	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
F	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
G	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
H	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC

Rows A-D are for Virus 1. Rows E-H are for Virus 2.

CC, Cell control wells (cells only).