Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells

(December 2011)

I. Introduction

This assay measures neutralization in TZM-bl cells as a function of a reduction in Tat-induced luciferase (Luc) reporter gene expression after a single round of virus infection. TZM-bl cells (also called JC57BL-13) may be obtained from the NIH AIDS Research and Reference Reagent Program. This is a HeLa cell clone that was engineered to express CD4 and CCR5 [1] and contains integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV-1 LTR [2], permitting sensitive and accurate measurements of infection. The cells are highly permissive to infection by most strains of HIV, SIV and SHIV, including primary HIV-1 isolates and molecularly cloned Env-pseudotyped viruses. DEAE dextran is used in the medium during neutralization assays to enhance infectivity. Expression of the reporter genes is induced in trans by viral Tat protein soon after infection. Luciferase activity is quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum. The assay is performed in 96-well culture plates for high throughput capacity. Use of a clonal cell population provides enhanced precision and uniformity. The assay has been validated for single-round infection with either uncloned viruses grown in human lymphocytes or molecularly cloned Env-pseudotyped viruses produced by transfection in 293T/17 cells.

II. Definitions

GM: Growth Medium

Luc: Luciferase

RLU: Relative Luminescence Units

DPBS: Dulbecco's Phosphate Buffered Saline

ID: Identification

TCID: Tissue Culture Infectious Dose

DEAE-Dextran: Diethylaminoethyl-Dextran

EDTA: Ethylenediaminetetraacetic acid

III. Reagents

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

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) (Protocol for Trypsin-EDTA Treatment for Disruption of Cell Monolayers)
Vendor: Invitrogen
Sterile

Trypan Blue (0.4%)
Vendor: Sigma

DPBS
Vendor: Invitrogen
Sterile

Britelite Plus Reporter Gene Assay System (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 lyophilized 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 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, sterile
Vendor: ICN
Vendor: Rainin

Disposable pipettes, sterile, individually wrapped
Vendor: Fisher
1 ml pipettes
2 ml pipettes
5 ml pipettes
10 ml pipettes
25 ml pipettes
50 ml pipettes

100 ml pipettes

Flat-bottom culture plates, 96-well, low evaporation, sterile
Vendor: Fisher

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

Culture flasks with vented caps, sterile
Vendor: Fisher
T-25 flask
T-75 flask

Reagent reservoirs, 50 ml, 100 ml capacity
Vendor: Costar
Vendor: VWR

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: NuAIRE

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
15 ml tube holder
Microtitration plate holder

*Manufacturer:*Eppendorf
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

Pipettor
Manufacturer: ThermoLabsystem
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, 50-1200 µl Electronic Pipette
Single channel, 10-300 µl Electronic Pipette
Single channel, 5-120 µl

Manufacturer: Rainin
12 channel pipettor, 20-200 µl

Light Microscope
Manufacturer: Olympus

Low Temperature Freezer (−70°C or lower)
Manufacturer: Harris
Manufacturer: Puffer Hubbard

4°C Refrigerator
Manufacturer: Sci-Cool

−20°C Freezer
Manufacturer: Sci-Cool

V. Specimens

Samples should be heat-inactivated at 56°C for 30-60 min prior to assay as described in Protocol for Heat-Inactivation of Serum and Plasma Samples. Samples may be serum or plasma, although serum is preferred. Anticoagulants in plasma are problematic in the assay, especially when heparin is used. For example, some forms of heparin have potent and strain-specific antiviral activity. Also, all anticoagulants are toxic to the cells at plasma dilutions lower than 1:60.

VI. Protocol

1 Neutralization Assay

NOTE 3: All incubations are performed in a humidified 37ºC, 5% CO_2 incubator unless otherwise specified.

1.1 Using the format of a 96-well flat bottom culture plate as illustrated in Appendix A, place 150 µl of GM in all wells of column 1 (cell control). Place 100 µl in all wells of columns 2-12 (column 2 will be the virus control). Place an additional 40 µl in all wells of columns 3-12, row H (to receive test samples).

1.2 Spin test samples in a centrifuge at 14,000 RPM for one minute prior to adding them to the test plate.

1.3 Add 11 µl of test sample in duplicate to row H, columns 3-12 in following order: sample 1 – wells H3-H4, sample 2 – wells H5-H6, sample 3 – wells H7-H8, sample 4 – wells H9-H10, and sample 5 – wells H11-H12. Mix the samples in row H and transfer 50 µl to row G. Repeat the transfer and dilution of samples through row A (these are serial 3-fold dilutions). After final transfer and mixing is complete, discard 50 µl from the wells in columns 3-12, row A into waste container.

NOTE 4: This format is designed to measure neutralizing antibody titers in the range of 1:20 to 1:43,740. The above description is for a dilution of 1:20. Appropriate adjustments may be made to test a different range of dilutions (refer to "Sample dilution charts:" in Appendix C). This format is designed to assay 5 samples in duplicate wells at each serum dilution per plate (Appendix A). Adjustments may be made to test a larger number of samples per plate. For example, 10 samples may be assayed at 4 dilutions in duplicate per plate by simply dividing the plate in half (Appendix B).

NOTE 5: A positive control with a known neutralization titer against the target virus should be included on at least one plate in series each time assays are performed.

1.4 Thaw the required number of vials of virus by placing in an ambient temperature water bath placed in a biological safety cabinet. When completely thawed, dilute the virus in GM to achieve a TCID of approximately 150,000 RLU equivalents (+/- 15,000 RLU). For pseudoviruses that do not reach 150,000 RLU, pick a dose of virus that gives at least 15,000 RLU but is not toxic to the cells via light microscopy. See Protocol for Preparation and Titration of HIV-1 Env-pseudotyped Viruses for measurement of TCID in TZM-bl cells.

NOTE 6: 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 ≥10X the background and the virus control is not toxic to the cells based on light microscopy.

NOTE 7: Leftover virus may be refrozen in the -80°C Freezer and marked with a "1X" on the lid and label of the vial. The "1X" notes that that particular vial has been thawed one time. When using "1X" vials of virus in the TZM-bl assay, the technician must consult the virus database to obtain the optimal virus dilution for viruses that have been thawed one time. No pseudovirus should be used in the TZM-bl assay if it has been thawed and refrozen more than once.

1.5 Dispense 50 µl of cell-free virus to all wells in columns 2-12.

Virus Calculations:

To calculate the total volume (vol.) of virus/GM mixture needed for the assay, multiply the total number of plates by the volume of virus/GM mixture to be used per plate. Then divide the total volume of virus/GM mixture by the optimal virus dilution to use (based on the TCID assay) to derive the volume of undiluted virus needed. Then subtract the volume of undiluted virus needed from the total volume of virus/GM mixture to derive the volume of GM needed.

Total number of plates X Vol. of virus/GM per plate = Total vol. virus/GM needed

Total vol. virus/GM needed ÷ Optimal virus dilution = Vol. of undiluted virus needed

Total vol. of virus/GM needed – Vol. of undiluted virus needed = Vol. of GM needed

1.6 Cover the plates and incubate for 45 – 90 minutes.

1.7 Prepare a suspension of trypsinized TZM-bl cells at a density of 1 x 10^5 cells/ml in GM containing DEAE-Dextran (25 µg/ml). Trypsinize the cells 10-15 minutes prior to use as described in Protocol for Trypsin-EDTA Treatment for Disruption of Cell Monolayers. 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. Dispense 100 µl of the prepared cell suspension (10,000 cells per well) to each well in columns 1-12, rows A through H. The final concentration of DEAE-Dextran is 10 µg/ml.

NOTE 8: Cells must be checked for viability during counting. Trypan Blue is recommended but other viability dyes are acceptable.

NOTE 9: The concentrations of DEAE-Dextran shown above are approximations. The actual optimal concentration should be determined for each new batch of DEAE-Dextran prepared in accordance with Protocol for Determination of Optimal Concentration of DEAE-Dextran.

NOTE 10: The use of DEAE-Dextran is optional. When omitted, the TCID of the virus needs to have been measured in the absence of DEAE-Dextran

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 factor 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 factor = 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 plate X 10 ml/plate = 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 (see Protocol for Determination of Optimal Concentration of DEAE-Dextran) by 0.250 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 0.25 ml (volume in well) = 2.5 µg of DEAE-Dextran needed in each well

2.5 µg X 100 wells/plate = 250 µg of DEAE-Dextran needed per plate = 0.25 mg of DEAE-Dextran

0.25 mg of DEAE-Dextran per plate ÷ 5 mg/ml stock concentration = 0.05 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

10ml – 0.67 ml cells – 0.05 ml DEAE-Dextran = 9.28 ml of GM

1.8 Cover plates and incubate for 48 – 72 hours if Env-pseudotyped viruses are used. If replication-competent virus is used, the plates should be incubated for 46-50 hours to minimize virus replication.

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

NOTE 11: Examine at least 2 virus control wells for the presence of syncytia by microscopic examination. It is important to note the presence of syncytia as too many syncytia indicate cell killing and thus the validity of the assay is compromised. If cell killing is present, the assays should be repeated using a lower dose of the virus. Also check the bottom row of the plate for the presence of toxicity. Cell toxicity could be erroneously interpreted as neutralization.

1.10 Thaw Britelite Plus directly before use in an ambient temperature water bath away from light.

1.11 Remove 150 µl of culture medium from each well, leaving approximately 100 µl.

1.12 Dispense 100 µl of Britelite Plus Reagent to each well.

1.13 Incubate at room temperature for 2 minutes to allow complete cell lysis. Mix by pipettor action (at least two strokes) and transfer 150 µl to a corresponding 96-well black plate. Read the plate after the two minute incubation time (but no longer than fifteen minutes) in a luminometer.

2 Analyzing and printing results

2.1 Prior to reading the plates in the luminometer, enter the assay protocol information in the Wallac Software of the luminometer.

2.2 Read the plates in a luminometer interfaced to a dedicated computer in the laboratory.

2.3 Use the software program associated with the luminometer to save the raw data onto the desired location, after each plate is read, using a unique file identification number (ID) for each plate.

2.4 Analyze and print the data using the appropriate Microsoft Excel "Luminescence" macro (provided by the Central Reference Laboratory). The data print-out must include: i) experiment number, ii) protocol and/or study number, iii) cells used in the assay, iv) length of incubation in days, v) name, lot number and dilution of the virus stock used, vi) ID, visit number and bleed date of each sample and vii) signature of technician who performed the assay.

NOTE 12: The "Luminescence" macro calculates the percent neutralization provided by each serum dilution. Percent neutralization is determined by calculating the difference in average RLU between virus control (cells + virus, column 2) and test wells (cells + serum sample + virus), dividing this result by the difference in average RLU between virus control (cell + virus, column 2) and cell control wells (column 1), and multiplying by 100. Neutralizing antibody titers are expressed as the reciprocal of the serum dilution required to reduce RLU by 50%. Failure to score at least 50% reduction of RLU at any serum dilution constitutes a negative test.

NOTE 13: Percent neutralization may also be determined by calculating the difference in average RLU between test wells containing post-immune sample and test wells containing

pre-immune sample from the same individual. The pre-immune and post-immune samples must be assayed on the same assay plate.

3 Pass/Fail Criteria

3.1 The average RLU of virus control wells is >10 times the average RLU of cell control wells.

3.2 The standard deviation of RLU in the virus control wells is ≤30%.

3.3 The standard deviation for duplicate wells is ≤30% for sample dilutions that yield at least 40% neutralization.

3.4 The neutralization curves are smooth and linear around the 50% neutralization cut-off.

3.5 The value of the positive control agrees with previous values for that particular control-virus combination.

VII. References

1. Platt, E.J., K. Wehrly, S.E. Kuhmann, B. Chesebro, and D. Kabat. 1998. Effects of CCR5 and CD4 cell surface concentrations on infection by macrophage tropic isolates of human immunodeficiency virus type 1. J. Virol. 72:2855-2864.

2. Wei, X., J.M. Decker, H. Liu, Z. Zhang, R.B. Arani, J.M. Kilby, M.S. Saag, X. Wu, G.M. Shaw, and J.C. Kappes. 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. 46:1896-1905.

VIII. Appendix

A: Assay template for measuring neutralization titers, 5 samples per plate

Assay template for measuring neutralization titers, 5 samples per plate

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

CC, Cell control wells (cells only). VC, virus control wells (virus and cells but no serum sample are added here.

B. Assay template for measuring neutralization titers, 10 samples per plate

Assay template for measuring neutralization titers, 10 samples per plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
B	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
C	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
D	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
			Sample 1 (bottom)		*Sample 3(bottom)*		*Sample 5 (bottom)*		*Sample 7 (bottom)*		*Sample 9 (bottom)*	
			Sample 2 (top)		*Sample 4 (top)*		*Sample 6 (top)*		*Sample 8 (top)*		*Sample 10 (top)*	

CC, Cell control wells (cells only). VC, virus control wells (virus and cells but no serum sample are added here

C: Sample dilution charts

STANDARD DILUTION CHART FOR 2-FOLD SAMPLE DILUTIONS:

DESIRED START DILUTION	GM VOLUME (µl)	SAMPLE VOLUME (µl)
1:5	40	60
1:10	70	30
1:15	80	20
1:20	85	15
1:25	90	12
1:30	90	10
1:50	95	6

First place 100 µl of growth medium in all wells of columns 3-12. Add the extra amount of growth medium listed above, then add the desired sample volume to the first 2 wells and do 2-fold dilutions (i.e., serial transfers of 100 µl).

STANDARD DILUTION CHART FOR 3-FOLD SAMPLE DILUTIONS

DESIRED START DILUTION	GM VOLUME (µl)	SAMPLE VOLUME (µl)
1:5	5	45
1:8	25	28
1:10	30	22
1:15	35	15
1:20	40	11
1:24	50	10
1:45	45	5

First place 100 µl of growth medium in all wells of columns 3-12. Add the extra amount of growth medium listed above, then add the desired sample volume to the first 2 wells and do 3-fold dilutions (i.e., serial transfers of 50 µl).