Standard Operating Procedure for

Measuring Neutralizing Antibodies against HIV-1 Pseudovirus using a Luciferase Reporter Gene Assay in TZM-bl Cells

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SOP: **Awaiting approval**

Version: **DRAFT**

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Revision History:

Version	**Description**	**Effective Date**
DRAFT		

This SOP has been read and understood by:

Name	**Date**

PURPOSE

This document describes how to measure HIV-1 pseudovirion neutralisation in with TZM-bl cells as a function of a reduction in luciferase (Luc) reporter gene expression.

SCOPE

This SOP applies to the assay stocks of pseudoviruses used for the measurement of neutralizing antibodies present in HIV-1 patient samples.

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1.0 Authority and Responsibility

The Director (or his/her designee) and the Principal Investigator of the HVTN Central Endpoint Assay Laboratory performing this procedure have the authority to establish this procedure. The Quality Assurance (QA) Unit is responsible for the control of SOP documentation.

The Principal Investigator/Laboratory Manager is responsible for the implementation of this procedure and for ensuring that all appropriate personnel are trained.

All research assistants/technicians working on HVTN studies are responsible for reading and understanding this SOP prior to performing the procedures described.

2.0 Introduction

The use of molecularly cloned pseudoviruses has advantages over uncloned virus in terms of reagent stability and affording greater reproducibility and precision in neutralisation assays. This SOP describes the production of molecularly cloned pseudoviruses in 293T 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 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. Neutralisation assays that are based on a single-round of infection should use viruses that are titrated in a similar singe-round infection format.
The TZM-bl cell line is a HeLa cell clone that was engineered to express CD4 and CCR5 (8.2) and contains integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV-1 LTR (8.3), 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 pseudoviruses. DEAE dextran is used in the medium during neutralisation 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.

3.0 Definitions

GM: Growth Medium
DMEM: Dulbecco's Modified Eagle Medium
Luc: Luciferase
RLU: Relative Luminescence Units
FCS: Fetal Calf Serum
ID: Identification

4.0 Reagents, Material and Equipment

Recommended vendors are listed. Products of equal or better quality may be used when necessary.

4.1 293T cell line

American Type Culture Collection; Catalogue No. CRL 11268

4.2 TZM-bl cell line

NIH AIDS Research and Reference Reagent Program; Catalogue No. 8129

4.3 Growth Medium

4.3.1 D-MEM, with L-glutamine, sodium pyruvate, glucose and pyridoxine, sterile. Gibco BRL Life Technologies, cat. no. 11995-065, store refrigerated at 4ºC

4.3.2 Fetal calf serum (FCS), heat-inactivated 56ºC for 45 minutes, 500 ml bottle, sterile. (Adcock Ingram Scientific, Catalogue No. 14-501A1) (Store at -20ºC. Once thawed, store at 4ºC for up to 1 month).

4.3.3 Complete GM consists of D-MEM containing 10% heat-inactivated FCS. Warm medium to room temperature prior to use.

4.4 DEAE dextran, hydrochloride, average Mol. Wt. 500,000
Sigma; Catalogue No. D9885
Stock concentration: 7.5 mg/ml (0.075g in 10mls DMEM)
(See Appendix 1 – DEAE dextran solution)

4.5 Trypsin-EDTA (0.25% trypsin, 1 mM EDTA), sterile.
Gibco; Catalogue No. 25200-056

4.6 PolyFect Transfection Reagent
QIAGEN; Catalogue No. 301107

4.7 Bright Glo substrate solution
Promega; Catalogue No. E2650

Reconstitute one vial of lyophylized Luciferase Assay Substrate (part #E263B) with 400 ml of Luciferase Assay Buffer (part #E264B). After the substrate has dissolved completely (about 1 minute), mix gently and distribute 10 ml to 15 ml conical polypropylene tubes and store at -80^{o}C immediately. Thaw in a room temperature water bath in the dark immediately before each use. Mix gently prior to use. Use within 5 minutes of thawing. Excess reagent may be stored at -80^{o}C and used once more.

Caution: *The lyophylized Bright-Glo substrate contains dithiothreitol and is classified as hazardous. The concentration of dithiothreitol after reconstitution in assay buffer is less hazardous. Latex gloves, surgical gown and eye protection are required when working with these reagents*.

4.8 Hemocytometer for counting cells (see PLT 0046 Cell counts and Viability)
Sigma

4.9 12-channel pipetteman, 5-50 μl (see SOP 0004 and SOP 0119)
ThermoLabsystems; AEC Amersham

4.10 12-channel pipetteman, 50-300 μl (see SOP 0004 and SOP 0119)
ThermoLabsystems; AEC Amersham

4.11 Single channel pipetteman, 5-50 μl (see SOP 0004 and SOP 0119)
Gilson; Laboratory & Scientific Equipment

4.12 Single channel pipetteman, 50-200 μl (see SOP 0004 and SOP 0119)
Gilson; Laboratory & Scientific Equipment

4.13 Pipette tips, sterile
Laboratory & Scientific Equipment

4.14 PipetteAid XP (annual external maintenance)
Labotec

4.15 Disposable pipettes, sterile, individually wrapped
Adcock Ingram Scientific
1 ml pipettes
5 ml pipettes
10 ml pipettes
25 ml pipettes
50 ml pipettes

4.16 Flat-bottom culture plates, 96-well, low evaporation, sterile
Costar; AEC Amersham

4.17 Flat-bottom black solid plates, 96-well, Nunc
AEC Amersham

4.18 1.8 ml cryotubes, sterile
NUNC; AEC Amersham

4.19 Culture flasks with vented caps, sterile
Costar/Adcock Ingram Scientific
T-25 flask
T-75 flask

4.20 Reagent reservoirs, 50 ml capacity
Costar/Adcock Ingram Scientific

4.21 Surgical Gowns
Vitamed

4.22 Latex Gloves
Profmedical Supplies

5.0 Instrumentation

5.1 Biological Laminar Flow Cabinet (annual certification required) (see SOP 0092)
BioFlow II; Labotec

5.2 Incubator, water-jacketed (37ºC, 5% CO2 standard requirements) (see SOP 0113)
Forma Scientific, Model 3111

5.3 Luminometer equipped to read 96-well plates (SOP IN PROCESS)
PerkinElmer Life Sciences, Seperation Scientific, Model Victor2

5.4 Controlled temperature water bath (see SOP 0111)
Labotec

5.5 Microcentrifuge (maximum rotational speed = 14,000 rpm) (see SOP 0061)
Hettich, Mikro 20, D78532; Labotec
24 place standard rotor for 1.5 ml microcentrifuge tubes

6.0 Specimens

HIV-1 negative or positive serum or plasma samples can be used in this assay

7.0 Protocol

All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

7.1 Thawing Cells

Wear a full-face shield during handling of frozen samples.

TZM-bl and 293T 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.

7.1.1 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.

7.1.2 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.

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

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

7.2 Splitting Cells

7.2.1 Decant the culture medium and remove residual fetal calf serum by rinsing monolayers with 5 ml of sterile PBS/DMEM.

7.2.2 Slowly add 3 ml of an 0.25% Trypsin-EDTA solution to cover the cell monolayer. Incubate at room temp for 30 seconds. Remove the trypsin

solution and incubate at 37°C for 5 minutes in the case of TZM-bl cells. Incubate at room temperature for 1 minute in the case of 293T cells. Do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.

7.2.3 Add 10 ml of GM and suspend the cells by gentle pipette action. Count cells.

7.2.4 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% CO_2/95% air environment. Cells should be split approximately every 3 days.

7.3 Transfection of 293T Cells

7.3.1 Seed 3 x 10^6 293T cells in a T75 flask containing 15 ml GM. Incubate overnight.

7.3.2 Dispense 5 μg of Env plamid DNA and 10 μg of backbone plasmid DNA into a 15 ml conical tube containing 300 μl D-MEM, mix.

7.3.3 Add 120 μl of PolyFect Transfection Reagent, vortex for 10 seconds, incubate at room temperature for 5-10 minutes to allow complex formation.

7.3.4 Add 1.5 ml of GM, mix gently and transfer the entire content to the T75 flask of 293T cells in which the medium was replaced with 10 ml fresh GM immediately before transfer. Mix gently for thorough distribution.

7.3.5 Incubate for 6-12 hours.

7.3.6 Decant the medium containing DNA-PolyFect complexes and replace with 15 ml fresh GM. Incubate for 2 days.

7.3.7 Harvest virus-containing culture supernatants by collecting the medium from the flask using a pipette. Collect as much as possible without drawing cells into the pipette. Filter the virus-containing culture fluid through a 0.45-micron filter. Adjust the FCS concentration to 20%, mix.

7.3.8 Distribute 1 ml aliquots to 1.8 ml cryotubes 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. Keep one aliquot out – this is to perform the $TCID_{50}$. Record the harvest and location of the vials. Include the identification of the seed stock when logging this information.

7.4 Titrating Virus in TZM-bl ($TCID_{50}$ Assay)

It is necessary to determine the TCID50 of each virus stock in a single-cycle infection assay (2-day incubation) in TZM-bl cells prior to performing neutralisation assays. A cut-off value of 2.5-times background RLU when quantifying positive infection in TCID50 assays.

Too much virus in the neutralisation assay can result in strong virus-induced cytopathic interfering with accurate measurements. Most virus stocks must be diluted at least 50-fold to avoid cell-killing. A standard inoculum of 200 TCID50 is used for the neutralisation assay to minimize virus-induced cytopathic effects while maintaining an ability to measure a 2-log reduction in virus infectivity. It should be noted that different strains vary significantly in their cytopathicity. It may be necessary to use a lower TCID50 in the neutralisation assay for highly cytopathic strains of virus. Virus-induced cytopathic effects may be monitored by visual inspection of syncytium formation under light microscopy. Cytopthic effects may also be observed as reductions in luminescence at high virus doses in the TCID50 assay.

7.4.1 Place 150 μl of GM per well in all wells of a 96-well flat-bottom culture plate. Transfer 50 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 4-fold dilutions (i.e., transfer 50 μl, mixing each time) for a total of 11 dilutions. Discard 50 μl from the 11th dilution. Wells in column 12 will serve as cell controls (no virus added).

7.4.2 Add 100 μl of TZM-bl cells (10,000 cells/100 μl GM-DEAE dextran 7.5 mg/ml) to all wells. Rinse your pipette tips in a reservoir of RPMI between each step to minimize carry-over.

7.4.3 Incubate for 48 hours.

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

7.4.5 Calculate the $TCID_{50}$ according to the method of Reed and Muench as described (8.1) using the "LotXTZM-bl $TCID_{50}$" calculation sheet. A cut-off value of 2,5-times background RLU is used when quantifying positive infection in $TCID_{50}$ assays.

7.5 Neutralizing Antibody Assay Protocol

7.5.1 Heat-Inactivation of samples

Clinical specimens may be serum or plasma, although serum is preferred. Anticoagulants (heparin, EDTA, ACD) in plasma are problematic in the assay. They are toxic to cells at plasma dilutions lower than 1:60. Samples should be heat-inactivated at 56°C for 1 hour prior to assay, to destroy complement activity. Complement in the serum of humans and nonhuman primates may enhance virus infection and mask neutralizing activity in cells that express complement receptors. Complement activation may lead to lysis and inactivation of the virus. This is a concern for serum from small animals such as mice, guinea pigs and rabbits.

Samples should be mixed thoroughly by gentle agitation after thawing. Multiple freeze-thaw cycles should be avoided. Centrifuge the samples briefly after mixing to assure that no sample remains adhered to the inside cap or sides of the tube. Place tubes in a pre-calibrated 56°C water bath, allowing the water level to reach the top of the sample volume but not touching the rim of the cap of the tube (sterility purposes). Incubate for 1 hour. Remove and gently mix the sample to collect condensation that accumulated on the sides and top of the tube. Centrifuge briefly to pellet any insoluble materials. Store at 4°C in cases where assays are to performed within two weeks. Store at -80°C in cases where assays will be delayed for more than two weeks.

7.5.2 Using the format of a 96-well flat-bottom culture plate as illustrated in Figure 1; place 150 µl of GM in all wells of column 1 (cell control). Place 100 µl in all wells of columns 2-11 (column 2 - virus control). Depending on dilution of test sample (see Standard Dilution Chart): place an additional **X** µl of GM in all wells of columns 3-11, row H. Place 250 µl in all wells of column 12 (blank).

This format is designed to assay 3 samples in triplicate at each serum dilution (Figure 1, Template A). Adjustments may be made to test a larger number of samples per plate (6 samples, Figure 1, Template B). Alternatively, samples may be screened at a single dilution. This latter option is advantageous when neutralizing activity is to be determined against a large number of strains. Due to possible nonspecific activity at low sample dilutions, it is recommended that corresponding pre-immune samples be included when performing screening assays.

A positive control with a known neutralisation titer against the target virus should be included on at least one plate in series each time assays are performed. Also, at least one negative control sample is strongly advised. Ideally, negative controls consist of corresponding pre-immune or pre-infection samples from either test animals or study subjects. In the case of clinical vaccine trials, a sufficient number of post-inoculation samples from placebo recipients may serve to provide adequate information on negative control values.

7.5.3 Thaw the required number of vials of virus by placing in an ambient temperature water bath. When completely thawed, dilute the virus in GM to achieve a concentration of 4,000 $TCID_{50}$/ml.

7.5.4 Add **X** µl of test sample to each well in columns 3-5, row H. Add **X** µl of a second test sample to each well in columns 6-8, row H. Add **X** µl of a third test sample to each well in columns 9-11, row H. 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 3-11, row A into a waste container of disinfectant.

7.5.5 Dispense 50 µl of cell-free virus (200 $TCID_{50}$) to all wells in columns 2-11, rows A through H. Mix by pipette action after each transfer. Rinse pipette tips in a reagent reservoir containing sterile PBS between each transfer to avoid carry-over.

7.5.6 Cover plate and incubate for 1 hour.

7.5.7 Prepare a suspension of TZM-bl cells at a density of 1 x 10^5 cells/ml in GM containing DEAE dextran (7.5 mg/ml) (See Appendix 1). Dispense 100 µl of cell suspension (10,000 cells per well) to each well in columns 1-11, rows A though H. Rinse pipette tips in a reagent reservoir containing sterile PBS between each transfer to avoid carry-over. The final concentration of DEAE dextran is 30 µg/ml.

7.5.8 Cover plates and incubate for 48 hours.

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

7.5.10 Percent neutralisation is determined by calculating the difference in average RLU between test wells (cells + serum sample + virus) and cell control wells (cells only, column 1), dividing this result by the difference in average RLU between virus control (cell + virus, column 2) and cell control wells (column 1), subtracting from 1 and multiplying by 100. Neutralizing antibody titers are expressed as the reciprocal of the serum dilution required to reduce RLU by 50%.

7.6 Analysing Results

7.6.1 Results and data print-out from each read/experiment must include: 1) experiment number; 2) all LOT numbers and dates of reagents, cells, virus stock; 3) ID of each sample used, plasma/serum, visit number, bleed date etc., 4) name and signature of person responsible for assay.

Template A

	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	BLK
B	CC	VC	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	BLK
C	CC	VC	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	BLK
D	CC	VC	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	BLK
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	BLK
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	BLK
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	BLK
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	BLK
				Sample 1			Sample 2			Sample 3		

Template B

	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	BLK
B	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	BLK
C	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	BLK
D	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	BLK
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	BLK
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	BLK
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	BLK
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	BLK
				Sample 1&2			Sample 3&4			Sample 5&6		

Figure 1: Template for measuring the titer of neutralising antibodies. Template A is for three samples per plate. Template B is for six samples per plate.
CC, cell control wells (cells only); VC, virus control wells (virus and cells but no serum); BLK, blank wells that contain only GM.

STANDARD DILUTION CHART

STANDARD DILUTION CHART FOR 2-FOLD 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 3-11. Add the extra amount of growth medium listed above, then add the desired sample volume to the first 3 wells and do 2-fold dilutions (i.e., serial transfers of 100 μl).

STANDARD DILUTION CHART FOR 3-FOLD 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 3-11. Add the extra amount of growth medium listed above, then add the desired sample volume to the first 3 wells and do 3-fold dilutions (i.e., serial transfers of 50 μl).

Preparation of solution of DEAE-Dextran:

DEAE-DEXTRAN (chloride form)
SIGMA Cat.# D9885; Lot # 013K0753

0.075 g Dextran powder reconstitute in 10 ml of distilled water to make 7.5 mg/ml solution. Filter. Each new stock of Dextran needs to be tested for toxicity.

Test of Dextran toxicity

Setting a plate with new stock of **DEAE-Dextran batch # D6 – 7.5 mg/ml** stock solution:

- Place 100 µl of GM in the whole plate.
- Add 92 µl of GM to upper 4 wells in first column, and 50 µl to a last one.
- Add 8 µl of D6-dextran [7.5 mg/ml] to a first column (4 wells); do 2-fold dilution lengthwise (transfer 100µl); last column is cell control.

- Add 50 µl of appropriately diluted virus to the plate.
- Add 100 µl of TZM-bl cells (10^4cells/well)

- Read Luminescence after **2 days**, compare luminescence in wells with both batches of dextran. For assays choose concentration of dextran with highest RLU reading or little lower.

Dextran start concentration: 120µg/ml – 2fold

Results will determine at what concentration the working stock of DEAE-dextran should be used at.

NOTE: This concentration of working stock DEAE-dextran may vary from stock to stock

8.0 References

8.1 Johnson, V.A., and R.E. Byington. 1990. Infectivity assay (virus yield assay). In : Techniques in HIV Research (Aldovani, A., and Walker, B.D., eds.). Stockton Press, New York, N.Y., pp71-76.

8.2 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.

8.3 Wei, X., J.M. Decker, H. Lui, 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.