Protocol for the Determination of Optimal Concentration of DEAE-Dextran

(April 2017)

I. Introduction

Serum and plasma samples are tested for the presence of neutralizing antibodies by using specific assays that are described in various Protocols (Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells; Neutralizing Antibody Screening Assay for HIV-1 in TZM-bl Cells; Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells; Neutralizing Antibody Assay for HIV-1 in A3R5 Cells; and Neutralizing Antibody Screening Assay for HIV-1 in A3R5 Cells) and use supporting procedures as described in various Protocols (Heat-inactivation of Serum and Plasma Samples; Preparation and Titration of HIV-1 Env-pseudotyped Viruses and Preparation and Titration of HIV-1 IMC Viruses).

In order to achieve optimal levels of virus infection, it is recommended to supplement the assay medium with DEAE-Dextran. This polycation counters the repulsive electrostatic forces between the virus and cells surface without affecting antibody binding and neutralization. However, DEAE-Dextran from different sources and different lots may exhibit substantial variability in potency and cell toxicity. For this reason, each new batch of DEAE-Dextran, regardless of lot, should be titrated by performing serial dilutions in a 96-well plate in each respective assay referenced above. The optimal concentration of DEAE-Dextran is determined from the dilution that yields the highest RLU and has no detrimental effects on the cell as observed by light microscopy after incubation.

II. Definitions

DEAE-Dextran:	Diethylaminoethyl-Dextran
GM:	Growth Medium
Luc:	Luciferase
RLU:	Relative Luminescence Unit
DPBS:	Dulbecco's Phosphate Buffered Saline
TCID:	Tissue Culture Infectious Dose
IMC:	Infectious Molecular Clone

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.

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

TZM-bl Cells
Vendor: NIH AIDS Reagent Program

A3R5 Cells
Vendor: NIH AIDS Reagent Program

M7-Luc Cells
Vendor: Dr. Nathaniel R. Landau, Salk Institute, La Jolla, CA

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

RPMI-1640 Growth Medium (see Protocols: Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in A3R5 Cells; and Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells)

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

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 lyophilized Britelite Plus Substrate is not classified as hazardous.

*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.

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

Microliter pipettor tips, sterile
Vendor: ICN

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
Vendor: Costar/Fisher

Flat-bottom white solid plates, 96-well
Vendor: Costar/VWR

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

15 ml Conical tubes
Vendor: Fisher Scientific

Reagent reservoirs, 50 ml capacity
Vendor: Costar

Trypan Blue (0.4%)
Vendor: Sigma

Distilled Water
Vendor: Generic

IV. Instrumentation

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

Luminometer
Manufacturer: PerkinElmer Life Science

Biological Safety Cabinet
*Manufacturer:*Baker CO.

Incubator
Manufacturer: Forma Scientific

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

Light Microscope
Manufacturer: Olympus

Fluorescence Microscope
Manufacturer: Olympus

Hemacytometer
Manufacturer: INCYTO

NOTE2: An automated cell counting device (i.e. Countess, Manufacturer: Invitrogen) may be used in lieu of a light microscope / hemacytometer for the cell counting and viability calculation.

Low Temperature Freezer
Manufacturer: Harris
Manufacturer: Puffer Hubbard

4°C Refrigerator
Manufacturer: Sci-Cool

-20°C Freezer
Manufacturer: Sci-Cool

Water bath
Manufacturer: Precision Scientific

V. Specimens

Cells and viruses listed in Protocols: Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells; Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells; Neutralizing Antibody Assay for HIV-1 in A3R5 Cells; Preparation and Titration of HIV-1 Env-pseudotyped Viruses; and Preparation and Titration of HIV-1 IMC Viruses.

VI. Protocol

1. Criteria for Deciding When the Optimal DEAE-Dextran Concentration Needs to be Determined

1.1. The optimal concentration of DEAE-Dextran for use in assays should be determined via a toxicity test each time a new batch of DEAE-Dextran is prepared, regardless of lot or receipt information.

2. Stock Solution Preparation

2.1. Reconstitute 5 grams of DEAE-Dextran in one liter of distilled sterile water.

2.2. Filter through a 0.45-micron filter and aliquot 10 ml in 15 ml conical tubes.

2.3. Store tubes at -80°C.

NOTE 3: Make sure to freeze tubes in open racks. Styrofoam racks are unsuitable for freezing because the freezing process starts at the top of a tube which may crack the bottom of the tube.

3. Titration of DEAE-Dextran in TZM-bl Cells

NOTE 4: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

3.1. Using the format of a 96-well flat bottom culture plate as illustrated in Appendix A, place 40 µl of GM in all wells in the entire plate. Place an additional 148 µl of GM in all wells of column 1 (to receive DEAE-Dextran). Place an additional 50 µl to column 12 (cell control).

3.2. Add 12 µl of test DEAE-Dextran (5 mg/ml stock solution) to each well in column 1 (rows A-H). Mix the samples in column 1 and transfer 160 µl to column 2. Repeat the transfer and dilution of DEAE-Dextran through column 11 (these are serial 1.25-fold dilutions). After the final transfer and mixing is complete, discard 160 µl from the wells in column 11 (rows A-H) into waste container. Wells in column 12 will serve as cell controls for background luminescence (no virus added).

NOTE 5: This format is designed to measure DEAE-Dextran concentrations in the range of 48 µg/ml down to 5.2 µg/ml. Appropriate adjustments may be made to test a different range of dilutions. Previous validation experiments have shown that the possible DEAE-Dextran concentration optimal for use in the neutralization assay is between 30 µg/ml and 7.5 µg/ml. This format is designed to assay two pseudoviruses in quadruplicate wells at each DEAE-Dextran concentration per plate (Appendix A).

3.3. Thaw the required number of vials of each virus by placing in an ambient temperature water bath. When completely thawed, appropriately dilute each virus/pseudovirus in GM in two separate reservoirs. (See Protocols for Preparation and Titration of HIV-1 Env-pseudotyped Viruses and Env.IMC.LucR Viruses for measurement of TCID and selection of virus dose.)

3.4. Dispense 50 µl of the first virus/pseudovirus to all wells in columns 1-11, rows A through D.

3.5. Dispense 50 µl of the second virus/pseudovirus to all wells in columns 1-11, rows E through H.

3.6. Dispense 160 µl of prepared TZM-bl cell suspension (10,000 cells per well) (see Protocol for Trypsin-EDTA Treatment for Disruption of Cell Monolayers) to each well in columns 1-12, rows A through H.

NOTE 6: To minimize carry over, always add cells and virus/pseudovirus from the column that contains the smallest concentration of DEAE-Dextran and proceed to the column that contains the greatest concentration of DEAE-Dextran.

3.7. Cover the plate and incubate for 48 hours.

NOTE 7: Examine all wells for normal cell morphology and viability by microscopic examination. It is important to note the presence of unhealthy cells and/or toxicity as certain doses of DEAE-Dextran can cause detrimental effects to the cells and thus the validity of assays will be compromised. If cell stress and/or toxicity are present at any given concentration, this particular dose of DEAE-Dextran should not be used in the assays.

3.8. For TZM-bl cell assays with pseudovirus using Britelite Plus

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

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

3.8.3. 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 immediately in a luminometer.

4. Titration of DEAE-Dextran in A3R5 Cells

4.1. Using the format of a 96-well flat bottom culture plate as illustrated in Appendix A, place 40 µl of GM in all wells in the entire plate. Place an additional 148 µl of GM in all wells of column 1 (to receive DEAE-Dextran). Place an additional 50 µl to column 12 (cell control).

4.2. Add 12 µl of test DEAE-Dextran (5 mg/ml stock solution) to each well in column 1 (rows A-H). Mix the samples in column 1 and transfer 160 µl to column 2. Repeat the transfer and dilution of DEAE-Dextran through column 11 (these are serial 1.25-fold dilutions). After the final transfer and mixing is complete, discard 160 µl from the wells in column 11 (rows A-H) into

waste container. Wells in column 12 will serve as cell controls for background luminescence (no virus added).

NOTE 8: This format is designed to measure DEAE-Dextran concentrations in the range of 48 µg/ml down to 5.2 µg/ml. Appropriate adjustments may be made to test a different range of dilutions. Previous validation experiments have shown that the possible DEAE-Dextran concentration optimal for use in the neutralization assay is between 30 µg/ml and 7.5 µg/ml. This format is designed to assay two Env.IMC.LucR viruses in quadruplicate wells at each DEAE-Dextran concentration per plate (Appendix A).

4.3. Thaw the required number of vials of each virus by placing in an ambient temperature water bath. When completely thawed, appropriately dilute each virus in GM in two separate reservoirs. (See Protocol for Preparation and Titration of HIV-1 IMC Viruses for measurement of TCID and selection of virus dose.)

4.4. Dispense 50 µl of the first virus to all wells in columns 1-11, rows A through D.

4.5. Dispense 50 µl of the second virus to all wells in columns 1-11, rows E through H.

4.6. Dispense 160 µl of prepared A3R5 cell suspension (90,000 cells per well) (see Protocol for Neutralizing Antibody Assay for HIV-1 in A3R5 Cells) to each well in columns 1-12, rows A through H.

NOTE 9: To minimize carry over, always add cells and virus from the column that contains the smallest concentration of DEAE-Dextran and proceed to the column that contains the greatest concentration of DEAE-Dextran.

4.7. Cover the plate and incubate for 4 days for Env.IMC.LucR viruses.

NOTE 10: Examine all wells for normal cell morphology and viability by microscopic examination. It is important to note the presence of unhealthy cells and/or toxicity as certain doses of DEAE-Dextran can cause detrimental effects to the cells and thus the validity of assays will be compromised. If cell stress and/or toxicity is present at any given concentration, this particular dose of DEAE-Dextran should not be used in the assays.

4.8. After 4 days, carefully remove 90 µl of supernatant from all wells of the plate.

4.9. Suspend cells in each well and transfer 75 µl of cell suspension to a flat-bottom white plate.

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

4.11. Add 30 µl of diluted ViviRen substrate to each well of cell suspension. Tap the plate lightly to mix.

4.12. Incubate at room temperature for 4 minutes and read the plate using the 0.5 sec/well protocol on the luminometer.

5. Titration of DEAE-Dextran in M7-Luc Cells

5.1. Using the format of a 96-well flat bottom culture plate as illustrated in Appendix A, place 40 µl of GM in all wells in the entire plate. Place an additional 148 µl of GM in all wells of column 1 (to receive DEAE-Dextran). Place an additional 50 µl to column 12 (cell control).

5.2. Add 8 µl of test DEAE-Dextran (5 mg/ml stock solution) to each well in column 1 (rows A-H). Mix the samples in column 1 and transfer 160 µl to column 2. Repeat the transfer and dilution of DEAE-Dextran through column 11 (these are serial 1.25-fold dilutions). After the final transfer and mixing is complete, discard 160 µl from the wells in column 11 (rows A-H) into waste container. Wells in column 12 will serve as cell controls for background luminescence (no virus added).

> ***NOTE 11:*** This format is designed to measure DEAE-Dextran concentrations in the range of 32 µg/ml down to 5.2 µg/ml. Appropriate adjustments may be made to test a different range of dilutions. Previous validation experiments have shown that the possible DEAE-Dextran concentration optimal for use in the neutralization assay is between 30 µg/ml and 7.5 µg/ml. This format is designed to assay two Env.IMC, or Env.IMC.LucR viruses, in quadruplicate wells at each DEAE-Dextran concentration per plate (Appendix A).

5.3. Thaw the required number of vials of each virus by placing in an ambient temperature water bath. When completely thawed, appropriately dilute each virus in GM in two separate reservoirs. (See Protocol for Preparation and Titration of HIV-1 IMC Viruses for measurement of TCID and selection of a replication competent virus dose.)

5.4. Dispense 50 µl of the first virus to all wells in columns 1-11, rows A through D.

5.5. Dispense 50 µl of the second virus to all wells in columns 1-11, rows E through H.

5.6. Dispense 160 µl of prepared M7-Luc cell suspension (60,000 cells per well) (see Protocol for Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells) to each well in columns 1-12, rows A through H.

NOTE 12: To minimize carry over, always add cells and virus from the column that contains the smallest concentration of DEAE-Dextran and proceed to the column that contains the greatest concentration of DEAE-Dextran.

5.7. Cover the plate and incubate for 4 days for Env.IMC.LucR viruses.

> ***NOTE 13:*** Examine all wells for normal cell morphology and viability by microscopic examination. It is important to note the presence of unhealthy cells and/or toxicity as certain doses of DEAE-Dextran can cause detrimental effects to the cells and thus the validity of assays will be compromised. If cell stress and/or toxicity are present at any given concentration, this particular dose of DEAE-Dextran should not be used in assays.

5.8. For M7-Luc cell assays using uncloned, Env.IMC, or Env.IMC.LucR viruses using Britelite Plus Reagent.

5.8.1. Following an incubation period specified by the TCID data, carefully remove 150 µl of culture medium from all wells of the plate, leaving 100 µl.

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

5.8.3. 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 white plate. Read the plate immediately in a luminometer.

6. Determination of Optimal DEAE-Dextran Concentration

6.1. The optimal concentration of DEAE-Dextran is determined from the dilution that yields the highest RLU and has no detrimental effects on the cells as observed by light microscopy after the respective incubation time period.

NOTE 14: If the optimal DEAE-Dextran concentration is 10 µg/ml in an assay plate, use 50 µl of the 5 mg/ml stock solution per one neutralization assay plate (DEAE-Dextran concentration in the cell suspension is 25 µg/ml). For TCID assays, use 40 µl of 5 mg/ml stock solution per one TCID plate (DEAE-Dextran concentration in the cell suspension is 20 µg/ml).

7. Testing the newly determined optimal DEAE-Dextran concentration

7.1 After determining the optimal concentration of DEAE-Dextran, test the dilution in a neutralization assays test plate before distributing the reagent.

7.2 Using the format of a 96-well flat bottom culture plate, place 150 µl of the appropriate GM in all wells of column 1 (cell control). Place 100 µl in all wells of columns 2-12.

7.3 Dispense 50 µl of cell-free virus (optimally the same viruses used for the DEAE-Dextran titration) to all wells in columns 2-12 as described in Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells, Protocol for Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells, and Protocol for Neutralizing Antibody Assay for HIV-1 in A3R5 Cells.

7.4 Cover the plates and incubate for 45-90 minutes.

7.5 During the incubation, prepare a suspension of cells using the newly determined optimal concentration of DEAE_Dextran as described in Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells, Protocol for Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells, and Protocol for Neutralizing Antibody Assay for HIV-1 in A3R5 Cells.

7.6 Cover the plates and incubate for the respective amount of time as described in Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells, Protocol for Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells, and Protocol for Neutralizing Antibody Assay for HIV-1 in A3R5 Cells.

7.7 After incubation, observe by light microscopy at least 10 wells that contain virus and cells for detrimental effects. Choose a lower dilution of DEAE-Dextran or repeat the titration if cells are not healthy.

VII. Appendix A: Plate Layout

Assay template for measuring cell toxicity via titration of DEAE-Dextran, 2 viruses per plate

Virus One

	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
	48µg/ml	38.4µg/ml	30.7µg/ml	24.6µg/ml	19.7µg/ml	15.7µg/ml	12.6µg/ml	10.1µg/ml	8.1µg/ml	6.4µg/ml	5.2µg/ml	

Virus Two

Note: The concentrations listed below the table are the final concentrations of DEAE-Dextran in each well.

CC, Cell control wells (cells only).