

Protocol for the Determination of Optimal Concentration of DEAE-Dextran (December 2011)

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 Assay for HIV-1 in M7-Luc Cells; Neutralizing Antibody Assay for HIV-1 in A3R5 Cells) and uses 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 the necessary amount of time for incubation for each assay.

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

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

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 Research and Reference Reagent Program

A3R5 Cells

Vendor: Colonel Jerome Kim and Dr. Robert McLinden (USMHRP)

M7-Luc Cells

Vendor: Dr. Nathaniel R. Landau, Salk Institute, La Jolla, CA

CEM-NKR-CCR5-Luc Cells

Vendor: NIH AIDS Research and Reference Reagent Program

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 for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in M7-Luc Cells and A3R5 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

Trypan Blue (0.4%)

Vendor: Sigma

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

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 pipette, 5-50 μ l
12-channel pipette, 30-300 μ l
Single channel pipette, 5-50 μ l
Single channel pipette, 30-200 μ l

Manufacturer: Drummond Scientific Co.
PipetteAid XP

Manufacturer: BioHit
12 channel, 50-1200 μ l Electronic Pipette
Single channel, 10-300 μ l Electronic Pipette
Single channel, 5-120 μ l

Light Microscope
Manufacturer: Olympus

Fluorescence Microscope
Manufacturer: Olympus

Hemocytometer
Manufacturer: INCYTO

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 for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells, M7-Luc Cells and A3R5 Cells and Protocols for Preparation and Titration of HIV-1 Env-pseudotyped Viruses and Env.IMC.LucR 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 2: 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. Cell Toxicity Test via Titration of DEAE-Dextran in TZM-bl Cells

NOTE 3: All incubations are performed in a humidified 37°C, 5% CO₂ 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 4: 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 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 μ l of the first virus to all wells in columns 1-11, rows A through D.
- 3.5. Dispense 50 μ l of the second virus to all wells in columns 1-11, rows E through H.
- 3.6. Dispense 160 μ l 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 5: 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.

- 3.7. Cover the plate and incubate for 48 hours.

NOTE 6: 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.

- 3.8. After 48 hrs, remove 150 μ l of culture medium from each well, leaving approximately 100 μ l.
- 3.9. Dispense 100 μ l 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 μ l to a corresponding 96-well black plate. Read the plate immediately in a luminometer.

4. Cell Toxicity Test via 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 7: This format is designed to measure DEAE-Dextran concentrations in the range of 48 $\mu\text{g/ml}$ down to 5.2 $\mu\text{g/ml}$. 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 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$. 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 Env.IMC.LucR 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 8: 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 9: 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, remove 90 μ l of supernatant from all wells of the plate.

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

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

4.11. Add 30 μ l 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. Cell Toxicity Test via 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 μ l of GM in all wells in the entire plate. Place an additional 148 μ l of GM in all wells of column 1 (to receive DEAE-Dextran). Place an additional 50 μ l to column 12 (cell control).

5.2. Add 12 μ l 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 μ l 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 μ l 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 10: This format is designed to measure DEAE-Dextran concentrations in the range of 48 μ g/ml down to 5.2 μ g/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 μ g/ml and 7.5 μ g/ml. This format is designed to assay two viruses, 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 Env.IMC.LucR Viruses for measurement of TCID and selection of 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 11: 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 12: 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 assays.

- 5.8. For M7-Luc cell assays using Env.IMC.LucR viruses using ViviRen

- 5.8.1. After 4 days, remove 90 µl of supernatant from all wells of the plate.

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

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

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

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

NOTE 13: For M7-Luc assays using Env.IMC.LucR viruses, one may use either Britelite Plus or ViviRen substrate. For M7-Luc assays using Env.IMC or uncloned viruses, Britelite Plus must be used.

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).

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

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).