

Protocol for the Determination of Optimal Concentration of DEAE-Dextran (August 2010)

I. Introduction

Serum and plasma samples are tested for the presence of neutralizing antibodies by using a specific assay that is described in Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells and uses supporting procedures as described in Protocol for Heat-inactivation of Serum and Plasma Samples, and Protocol for Preparation and Titration of HIV-1 Env-pseudotyped Viruses.

In order to achieve optimal levels of pseudovirus 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 and adding a representative Env-pseudotyped virus and TZM-bl cells as described for the neutralization assay (Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells). 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 48 hour 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

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)
Sigma

TZM-bl Cells

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)

Trypsin-EDTA (0.25% trypsin, 1 mM EDTA)

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)

Perkin Elmer Life and Analytical Sciences

NOTE 1: 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.

Microliter pipettor tips, sterile

ICN

Disposable pipettes, sterile, individually wrapped

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

Costar/VWR

Flat-bottom black solid plates, 96-well

Costar/Fisher

Culture flasks with vented caps, sterile

Costar/VWR

T-25 flask

T-75 flask

Reagent reservoirs, 50 ml capacity

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

PerkinElmer Life Science

Biological Safety Cabinet

NuAIRE

Incubator

Forma Scientific

Pipettor

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

Drummond Scientific Co.

PipetteAid XP

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

Olympus

Hemocytometer

INCYTO

Low Temperature Freezer

Harris

Puffer Hubbard

4°C Refrigerator

Sci-Cool

-20°C Freezer

Sci-Cool

Water bath

Precision Scientific

V. Specimens

Cells and viruses listed in Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells and Protocol for Preparation and Titration of HIV-1 Env-pseudotyped 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. Cell Toxicity Test via Titration of DEAE-Dextran

NOTE 2: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

2.1 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).

2.2 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 3: 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).

2.3 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-pseudotyped Viruses for measurement of TCID and selection of virus dose in TZM-bl cells.)

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

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

2.6 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 4: 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.

2.7 Cover the plate and incubate for 48 hours.

NOTE 5: 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.

2.8 Remove 150 μ l of culture medium from each well, leaving approximately 100 μ l.

2.9 Dispense 100 μ l of Britelite Plus Reagent to each well.

2.10 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.

3. Determination of Optimal DEAE-Dextran Concentration

3.1 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 a 48 hour incubation.

NOTE 6: If the optimal DEAE-Dextran concentration is 10 μ g/ml in an assay plate, use 50 μ l of the 5 mg/ml stock solution per one neutralization assay plate (DEAE-Dextran concentration in the cell suspension is 25 μ g/ml). For TCID assays, use 40 μ l of 5 mg/ml stock solution per one TCID plate (DEAE-Dextran concentration in the cell suspension is 20 μ g/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 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).