

**Protocol for Determination of Optimal Concentration of DEAE-Dextran  
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**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

DMEM: Dulbecco's Modified Eagle Medium

HEPES: N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid

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 may be used when necessary.

**DEAE-Dextran**, hydrochloride, average Mol. Wt. 500.  
Sigma

\* To prepare a 5 mg/ml solution, dissolve 5 gm of DEAE-Dextran in 1 L of sterile H<sub>2</sub>O. Create 10 ml aliquots in 15 ml sterile conical polypropylene tubes. Store aliquots at -80°C. DEAE-Dextran from some manufacturers does not have a listed expiration date. Contact the manufacturer for the stability of each DEAE-Dextran lot. It is important to note that the conical tubes with DEAE-Dextran solution should not be placed in Styrofoam racks for storage until after contents are completely frozen. The freezing process begins at the exposed part of the tube. Consequently, the shielded bottom of the tube will crack rendering the contents of the vial unusable.

### **TZM-bl Cells**

NIH AIDS Research and Reference Reagent Program

### **Complete Growth Medium\***

**DMEM**, with L-glutamine, sodium pyruvate, glucose and pyridoxine. Sterile, store refrigerated at 4°C.  
Gibco BRL Life Technologies

**Fetal Bovine Serum**. Heat-inactivated at 56°C for 30 minutes, sterile. Store at -20°C. Once thawed, store at 4°C.

Hyclone

**Gentamicin** solution, 10 mg/ml. Sterile, store at 4°C.

Sigma

**HEPES** Buffer, 1 M. Sterile, store at 2°C – 8°C

Gibco

\*Complete GM consists of DMEM containing 10% heat-inactivated FBS, 50 µg gentamicin/ml, and 25 mM HEPES. To make a 500 ml of complete GM, combine 435 ml DMEM, 50 ml FBS, 2.5 ml gentamicin and 12.5 ml HEPES in a sterile bottle, mix, store at 4°C for up to 2 months.  
Warm medium to 20-37°C prior to use.

### **Trypsin-EDTA (0.25% trypsin, 1 mM EDTA, sterile**

Invitrogen

### **Britelite Plus Reporter Gene Assay System**

Perkin Elmer Life and Analytical Sciences

Reconstitute one vial of lyophilized Britelite Plus Substrate Solution with 250 ml of Britelite Plus Substrate Buffer Solution. After the substrate has dissolved completely (about 1 minute), mix gently and distribute 10.5 ml to 15 ml conical polypropylene tubes and store at -80°C immediately. Thaw in a room temperature water bath in the dark immediately before each use. Mix gently prior to use. Use within 60 minutes of thawing. Excess reagent may be stored at -80°C and used once more.

**NOTE 1: Caution!** The lyophilized Britelite Plus Substrate Solution is classified as hazardous. Personal Protective Equipment (PPE) is required when working with these reagents.

\*Britelite substrate solution from Perkin Elmer Life and Analytical Sciences or BrightGlo substrate solution from Promega are acceptable substitutes for Britelite Plus. Please follow manufacturer's guidelines for preparation and use.

**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/VWR

**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  $\mu$ l

12-channel pipette, 30-300  $\mu$ l

Single channel pipette, 5-50  $\mu$ l

Single channel pipette, 30-200  $\mu$ l

Drummond Scientific Co.

PipetteAid XP

**BioHit**

12 channel, 50-1200  $\mu$ l Electronic Pipette  
Single channel, 10-300  $\mu$ l Electronic Pipette  
Single channel, 5-120  $\mu$ l

**Light Microscope**

Olympus

**Hemocytometer**

Hausser Scientific

**Low Temperature Freezer**

Harris  
Puffer Hubbard

**4°C Refrigerator**

Sci-Cool

**-20°C Freezer**

Sci-Cool

**Water bath**

Precision Scientific

**-80°C Freezer**

Harris

**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**

**Protocol for Determination of Optimal Concentration of DEAE-Dextran**

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

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% CO<sub>2</sub> incubator unless otherwise specified.

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

2.2 Add 12  $\mu$ 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  $\mu$ 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  $\mu$ 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 3:** This format is designed to measure DEAE-Dextran concentrations in the range of 48  $\mu$ g/ml down to 5.2  $\mu$ 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  $\mu$ g/ml and 7.5  $\mu$ g/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  $\mu$ l of the first virus to all wells in columns 1-11, rows A through D.

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

2.6 Dispense 160  $\mu$ l of prepared TZM-bl cell suspension (10,000 cells per well) (see Protocol: Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells) 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  $\mu$ l of culture medium from each well, leaving approximately 100  $\mu$ l.

2.9 Dispense 100  $\mu$ 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  $\mu$ 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  $\mu\text{g/ml}$  in an assay plate, use 50  $\mu\text{l}$  of the 5 mg/ml stock solution per one neutralization assay plate (DEAE-Dextran concentration in the cell suspension is 25  $\mu\text{g/ml}$ ). For TCID assays, use 40  $\mu\text{l}$  of 5 mg/ml stock solution per one TCID plate (DEAE-Dextran concentration in the cell suspension is 25  $\mu\text{g/ml}$ ).

**VII. Appendix**

**Appendix A: Plate Lay Out**

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).*