

**Protocol for Preparation of Cell-Free Stocks of TCLA HIV-1 in Cell Lines
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I. INTRODUCTION

During the course of investigating the neutralizing antibody response in recipients of candidate HIV-1 vaccines, it is necessary and important to establish the extent to which those antibodies neutralize genetically and antigenically diverse strains of the virus. HIV-1 exhibits an unusually high degree of genetic variability throughout its genome. This variation is most pronounced in the envelope glycoproteins, which are the major targets for neutralizing antibodies. Sequence comparisons have identified three genetic groups of HIV-1, designated M, O and N. Group M is further divided into ten phylogenically related genetic subtypes (or clades), designated A, B, C, D, F1, F2, G, H, J and K. Together with circulating inter-subtype recombinant forms (CRFs), group M comprises the majority of HIV-1 variants in the world today [1, 2]. It is a major goal to develop a vaccine that generates broadly cross-reactive neutralizing antibodies.

The study of neutralizing antibodies is complicated by a dichotomy in neutralization-sensitivity between T cell line adapted (TCLA) variants and primary isolates of HIV-1 *in vitro*. By simple definition, TCLA variants have been passaged multiple times in T cell lines whereas primary isolates are low-passaged variants produced exclusively in human PBMC. In general, TCLA strains are much more sensitive to neutralization by sera from infected individuals compared to primary isolates. This dichotomy is thought to be due to structural features in the envelope glycoproteins involving N-linked glycans and tertiary folds that effect epitope exposure. Since it cannot be certain which category of HIV-1 (i.e., TCLA or primary isolate) is more likely to predict a protective neutralizing antibody response generated by candidate HIV-1 vaccines, it is important to measure neutralizing antibodies with both categories of the virus.

II. DEFINITIONS

TCLA: T Cell Line adapted

GM: Complete Growth Medium

FBS: Fetal Bovine Serum

PBS: Phosphate Buffered Saline

ID: Identification

D-MEM: Dulbecco's Modified Eagle Medium

RPMI Medium: Roswell Park Memorial Institute Growth Medium

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

III. REAGENTS AND MATERIALS

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality may be used when necessary.

TZM-bl cells

NIH AIDS Research and Reference Reagent Program

H9 cells

NIH AIDS Research and Reference Reagent Program

Growth medium*

RPMI 1640 with 2 mM L-glutamine and 25 mM HEPES, sterile. Store refrigerated at 4°C.

Gibco BRL Life Technologies

Fetal bovine serum (FBS), heat-inactivated 56°C for 30 minutes, sterile. Store at -20°C. Once thawed, store at 4°C for up to two months.

Hyclone

Gentamicin solution, (10 mg/ml), sterile. Store at 4°C.

Sigma

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

Gibco

***Complete growth medium consists of RPMI-1640 containing 12% heat-inactivated FBS and 50 µg gentamicin/ml, referred to as GM.** To make 500 ml of GM, combine 437.5 ml RPMI-1640, 60 ml FBS and 2.5 ml of gentamicin into a sterile bottle, mix, store at 4°C for up to 2 months. Warm medium to 20°C - 37°C prior to use.

D-MEM Growth Medium*

D-MEM, with L-glutamine, sodium pyruvate, glucose, and pyrooxidine. Store at 4°C.

Gibco BRL Life Technologies

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

DEAE dextran, hydrochloride, average Mol. Wt. 500,000

Sigma

Prepare a 7.5 mg/ml solution by dissolving 3.75 g of DEAE dextran in 500 ml of sterile water. Store at -80°C in 10 ml aliquots in 15 ml sterile conical polypropylene tubes. Titrate each batch of dextran to determine the best concentration to use. Too much dextran is toxic to cells. Too little dextran will not enhance infectivity.

p24 Immunoassay kit, NEN™ Life Science Products HIV-1 p24 ELISA

Perkin Elmer Life Sciences, Inc.

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

Invitrogen

Britelite™ Substrate Solution

Perkin Elmer Life and Analytical Sciences

Reconstitute one bottle of lyophilized Britelite Substrate Solution with 250 ml of Britelite Substrate Buffer Solution.

Mix the contents of the vial by inversion until the substrate is completely dissolved (about 1 minute). 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 substrate is classified as hazardous. Latex gloves, surgical gown, and eye protection are required when working with these reagents.*

Phosphate-buffered saline solution, sterile. Dulbecco's Phosphate-Buffered Saline solution w/o Ca⁺⁺ and Mg⁺⁺. Store at room temperature.

Gibco BRL Life Technologies

Microliter pipettor tips, sterile

ICN

Disposable pipettes, sterile, individually wrapped

Falcon/VWR

1 ml pipets

5 ml pipets

10 ml pipets

25 ml pipets

50 ml pipets

Flat-bottom culture plates, 96-well, low evaporation, sterile

Costar/VWR

Culture flasks with vented caps, sterile

Costar/VWR

T-25 flask

T-75 flask

15 ml conical polypropylene tubes, screw-cap, sterile

Corning

Reagent reservoirs, 50 ml capacity

Costar

Filter bottle system (500 ml), sterile, individually wrapped

Corning

Instrumentation:

Biological Safety Cabinet

NuAIRE

Incubator

Forma Scientific

Light Microscope

Olympus

Centrifuge, low speed capable of up to 500 x g.

Manufacturer: Jouan

Buckets

50 ml tube holder

15 ml tube holder

Microtitration plate holder

Luminometer

Perkin Elmer Life Sciences

WW004 Wellwash 4 Microplate Washer

MTX LabSystems, Inc.

Vmax Kinetic Microplate Reader (Thermomax Microplate Readers may be substituted)

Molecular Devices Corp.

Multiskan Plus plate reader

TiterTek

Water bath

Precision Scientific, Model 182

Hemocytometer

Hausser 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

PipetteAid XP

Drummond Scientific Co.

Specimens:

Seed stocks of TCLA strains of HIV-1 may be grown in any cell type, although T cell lines are preferred. The TCID₅₀ of the virus should be at least 1×10^3 /ml. Log all documentation relating to each isolate, contributing laboratory, passage history, infectious titer, genetic subtype and co-receptor usage.

IV. PROTOCOL

Maintenance of cell cultures for infection

NOTE 2: Be sure to wear a full-face shield during handling of frozen samples.

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.
2. Transfer the contents of one vial of cells to a T-25 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.
3. Incubate the cells at 37°C for 1 day.
4. Remove the medium and replace with 20 ml of fresh GM. Change the medium every 2 days until the cells are readily dividing. Expand the cells in a T-75 flask containing 100 ml of GM at this time. Replace the medium completely and reduce the cell density to 1×10^6 cells/ml every 2-3 days as needed for 2-4 weeks. To prepare the cells for infection, suspend 5×10^8 cells in 20 ml of GM.

Infect Cells

1. Quickly thaw a vial of frozen virus by immersing the vial in a room-temperature water bath, taking care not to submerge the cap of the vial.
2. Transfer 1 ml of virus to the flask containing 20 ml of exponentially growing cells that are at a density of approximately 2.5×10^7 cells/ml. Incubate overnight at 37°C.
3. Remove all medium with a pipet and add 20 ml of RPMI-1640. Pellet the cells at low speed (1400 rpm). Decant supernatant and repeat the washing procedure. These washes are done to remove the virus inoculum.
4. Resuspend the washed cell pellet in 100 ml of fresh GM. Replace the medium completely and reduce the cell density to 1×10^6 cells/ml every 2-3 days as needed for 14 - 20 days. Monitor virus production by performing p24 ELISA. When the p24 is 2-5 ng, remove 90 ml of culture fluid. Suspend the cells in the remaining 10 ml of culture fluid and distribute 2 ml to each of five T-75 flasks containing 100 ml of GM each. High titer virus should be present in 2-3 days. Harvest virus when p24 concentration is >20 ng/ml.

Harvesting Virus

1. Harvest virus-containing culture supernatants by collecting the medium from the flask using a pipet. Collect as much as possible without drawing cells into the pipet. Filter the virus-containing culture fluid

through a 0.45-micron filter. Adjust the FBS concentration to 20%, mix. Distribute 10 ml aliquots to 15 ml conical polypropylene 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. Record the harvest and location of the vials. Include the identification of the seed stock when logging this information.

Titration of the Virus in TZM-bl Cells (TCID₅₀ Assay)

***NOTE 3:** Assays with replication-competent viruses are incubated for 48 hours to keep virus replication at a minimum.*

1. Place 100 µl of D-MEM GM per well in all wells of a 96-well flat-bottom culture plate. Transfer 25 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 5-fold dilutions (i.e., transfer 25 µl, mixing each time) for a total of 11 dilutions. Discard 25 µl from the 11th dilution. Wells in column 12 will serve as cell controls (no virus added).
2. Add 100 µl of TZM-bl cells (10,000 cell/100 µl D-MEM GM containing 30 µg DEAE dextran/ml) to all wells. Rinse your pipettor tips in a reservoir of sterile PBS or change the pipettor tips between each step to minimize carry-over. The final concentration of DEAE dextran is 15 µg/ml.
3. Incubate for 48 hours.
4. Remove 100 µl of culture medium from each well, leaving approximately 100 µl. Dispense 100 µl of Britelite™ Substrate to each well.
5. Incubate at room temperature for 2 minutes to allow complete cell lysis. Mix by pipettor action (two strokes) and transfer 150 µl to a corresponding 96-well black plate. Read the plate immediately in a luminometer.
6. Calculate the TCID₅₀ according to the method of Reed and Muench as described [8.1] using the “TCID₅₀” macro on the computer. Wells with RLU <2.5 times background are considered negative for the calculation.

V. REFERENCES

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