Protocol for Preparation of Cell-Free Stocks of HIV-1 in PBMC (January 2014)

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 [8.1, 8.2]. 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

PBMC:	**Peripheral Blood Mononuclear Cells**
IL-2:	**Human Interleukin 2**
IL-2-GM:	**Complete Growth Medium containing 5% v/v human IL-2**
FBS:	**Fetal Bovine Serum**
PBS:	**Phosphate Buffered Saline**
PHA-P:	**Phytohemagglutinin-P**

III. REAGENTS AND MATERIALS

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

IL-2-GM*

RPMI 1640 with 2 mM L-glutamine and 25 mM HEPES, sterile. Store at 4°C.
Gibco BRL Life Technologies.

Fetal bovine serum (FBS), sterile. Heat-inactivated 56°C for 30 minutes. Store at -20°C. Once thawed, store at 4°C for up to 1 month.
Hyclone

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

Human T-Cell Growth Factor Interleukin 2 (IL-2), purified. Store at -80ºC in 5 ml or 10 ml aliquots. Advanced Biosciences, Inc (ABI).

> ***Complete IL-2-growth medium consists of RPMI-1640 containing 20% heat-inactivated FBS, 5% IL-2 and 50 µg gentamicin/ml. This is referred to as IL-2-GM.** To make 100 ml of IL-2-GM, combine 75 ml RPMI-1640, 20 ml FBS, 5 ml IL-2 and 0.5 ml of gentamicin into a sterile bottle, mix, store at 4°C for up to 5 days. Warm medium to 25°C - 37°C prior to use.

Phosphate-Buffered Saline (PBS) solution, Dulbecco's Phosphate- Buffered Saline solution w/o Ca^{++} and Mg^{++}, sterile, store at room temperature.
Gibco BRL Life Technologies

PHA-P (from Phaseolus vulgaris), lyophilized, mitogenic potency 10 µg/ml
Sigma

> Prepare PHA by dissolving 2 mg in 2 ml of sterile water for a concentration of 1 mg/ml. Store at -20°C in 300 µl aliquots in 2 ml sterile tubes. Discard unused contents after thawing.

Triton X-100, store at room temperature
Sigma

> Prepare a 0.5% v/v solution by dissolving 0.5 ml in 100 ml of distilled water. Store at room temperature.

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

U-bottom culture plates, 96-well, low evaporation, sterile
Falcon/VWR

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

P24 immunoassay kit, NEN™ Life Science Products HIV-1 p24 ELISA
PerkinElmer Life Sciences, Inc.

Filter bottle system, 250 ml, sterile individually wrapped
Corning

Cryogenic vials, 1.5 ml sterile screw cap, frosted label
Sarstedt Brand Products

Reagent reservoirs, 50 ml capacity
Costar

Flat-bottom culture plates, 96-well, low evaporation, sterile
Costar / VWR

Instrumentation:

Biological Safety Cabinet
NuAire

Incubator, 37°C, 5% CO_2 standard requirements
Forma Scientific

Light Microscope
Olympus

Centrifuge, Low speed capable of up to 500 x g
Jouan
Buckets
50 ml tube holder
15 ml tube holder
Microtitration plate holder

WW004 Wellwash 4 Microplate Washer
MTX Lab Systems, Inc.

Vmax® Kinetic Microplate Reader (Thermomax Microplate Reader may be substituted)
Molecular Devices Corporation

Water Bath
Precision Scientific

Hemacytometer
Hausser Scientific

Pipettor
ThermoLabsystem
12-channel pipetteman (5-50 µl, 20-200 µl)
Single channel pipetteman (5-50 µl, 30-300 µl)

PipetteAid XP
Drummond Scientific Co.

Specimens:

All seed stocks of HIV-1 should be of a low-passage PBMC exclusively (e.g., no more than 3 passages). It is preferred to use virus-containing culture fluids obtained during the original isolation. The TCID50 of the virus should be at least 1 x 10^3/ml. Log all documentation relating to each isolate, including patient source, contributing laboratory, passage history, infectious titer, genetic subtype and co-receptor usage.

IV. PROTOCOL

Thawing and stimulating PBMCs

NOTE 1: Be sure to wear a full-face shield during the handling of frozen samples.

1. Transfer cryovials containing frozen PBMC (2.5 x 10^7 cells/ml/vial) from liquid nitrogen to a room temperature water bath in the laminar flow 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 cells to a T-25 or T-75 culture flask (depending on the volume needed) that contains 30 ml of IL-2-GM + PHA-P (5 μg/ml) for every 1 ml of thawed cell suspension.

NOTE 2: It is important to dilute a cryoprotectant DMSO present in the cryovial at least 30-fold at this point to avoid cell toxicity.

3. Incubate the cells at 37°C for 1 day. The cells typically divide once during this incubation.

4. Remove the medium and replace with 5 ml of fresh IL-2-GM (no PHA-P) for every vial of cells used. The PBMC are now at a density of 5 x 10^6 cells/ml and are ready for use. The cells should be used for infection within 3 days.

Infecting PBMCs

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 the freshly-stimulated PBMC. Incubate overnight at 37°C.

3. Remove all medium with a pipette and add 20 ml of RPMI-1640. Pellet the cells at low speed (1400 rpm). Decant supernatant and repeat the washing procedure.

4. Resuspend the washed cell pellet in 10 ml of IL-2-GM. Incubate at 37°C.

Harvesting Virus

NOTE 3: Monitor virus production every 2 days by measuring p24 concentration in the culture fluid starting on day 5. Follow the p24 ELISA kit instruction manual. Change the medium completely every 2 days starting on day 5 after completing the p24 measurement. Save the

culture fluid if the p24 concentration is >10 ng/ml. Aim for 3 or more harvests that contain >10 ng p24/ml. Continue to harvest every 2 days if the p24 concentration continues to increase.

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

2. Filter the virus-containing culture fluid through a 0.45-micron filter.

3. Distribute 1 ml aliquots to 1.5 ml sterile screw-cap cryovials that have been labeled to identify the isolate name and the date of harvest. The harvest date and computer issued ID become the specific lot number.

4. Store the aliquots at -80^{o}C.

5. Record the harvest and location of the vials. Include the identification of the seed stock when logging this information.

Titrating the Virus (TCID50 Assay)

1. Thaw and stimulate PBMC's (follow section 'Thawing and stimulating PBMCs' above)

2. Place 100 µl of IL-2-GM per well in all wells of columns 1-11 of all rows of a 96-well U-bottom 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.

3. Add 100 µl of stimulated PBMCs (2.5 x 10^5 cells, you will have 200 µl total volume at this point). Rinse the pipettor tips in a reagent reservoir containing sterile PBS or change the pipettor tips between each plate to minimize carry-over.

4. Incubate overnight at 37°C in a humidified incubator chamber (the chamber must be humidified to minimize evaporation in the outside wells. Some evaporation will still occur and may be adjusted by adding more medium to the affected wells).

5. Remove the virus inoculum by removing approximately 190 µl of culture supernatant from all wells. This is easily accomplished by angling your pipettor tips to touch the bottom of the well just adjacent to the border of the cells. Draw the medium up slowly and evenly. The cells are not disturbed as easily as one might expect, and the drawing action need not be laboriously slow.

6. Replace with 190 µl of fresh IL-2-GM. Rinse the pipettor tips in a reagent reservoir containing sterile PBS or change the pipettor tips between each plate to minimize carry-over.

Infectivity Check

1. On day 4, and if necessary on day 5, transfer 25 µl of culture supernatant to the corresponding wells of a fresh 96-well flat-bottom plate. Rinse the pipettor tips in a reagent reservoir containing sterile PBS or change the pipettor tips between each plate to minimize carry-over. Replace fresh 25µl of IL-2-GM and return the original plate to the incubator.

2. Add 225 µl of 0.5% Triton X-100 to the plate containing the harvested culture supernatants, mix, store at 4°C in zip-lock plastic bags.

3. Using HIV-1 p24 ELISA kit, perform p24 immunoassays on all wells of the plate containing the Triton X-100 lysates. Use the wells in column 12 for the standard curve.

4. Read plates at A450 on Vmax® Kinetic or Thermomax Microplate Reader. Choose the dilution which will be used in neutralization assay which yields approximately 2-5ng/ml.

5. Change 150 µl of the medium on days 5 and 9 of incubation.

Final TCID50 measurement

1. On day 11, transfer 25 µl of culture supernatant to the corresponding wells of a fresh 96-well flat-bottom plate. Rinse the pipettor tips in a reagent reservoir containing sterile PBS or change the pipettor tips between each plate to minimize carry-over.

2. Add 225 µl of 0.5% Triton X-100 to the plate containing the harvested culture supernatants, mix, store at 4^{o}C in zip-lock plastic bags.

3. Using HIV-1 p24 ELISA kit, perform p24 immunoassays on all wells of the plate containing the Triton X-100 lysates. Use the wells in column 12 for the standard curve.

4. Read plates at A_{450} on Vmax® Kinetic or Thermomax Microplate Reader.

5. Assign each raw data file an experiment number and file identification number (ID) corresponding to the date and assay number. For example, the ID for February 4, 2002 of plate #3 would be: 0204023 X/Y/Z.

6. Save the raw data electronically to the secure access file server.

7. Analyze and print the data using the Softmax Pro software. The data print-out must include: i) experiment number, ii) cells used in the assay, iii) length of incubation in days, iv) name, lot number and starting dilution of the virus stock used, v) signature of technician who performed the assay.

8. Calculate the TCID50 according to the method of Reed and Muench as described [8.3] using the "TCID50" macro on the computer. Wells with <0.2 ng p24/ml are considered negative for the calculation.

V. REFERENCES

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