

**Protocol for HIV-1 Isolation by PBMC Co-Culture
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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.

Primary isolates of HIV-1 are most readily isolated by mixing PBMC from infected subjects with mitogen-stimulated PBMC from healthy, uninfected donors. The coculture is incubated in growth medium containing the T cell growth factor cytokine, IL-2. Mitogen stimulation of the donor PBMC acts to upregulate the IL-2 receptor, adding to the responsiveness of the cells to exogenously added cytokine.

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 Interleukin 2 (IL-2), delectinized. Store at -80°C in 5 ml or 10 ml aliquots

Advanced Biotechnologies, 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 20°C - 37°C prior to use.**

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

Gibco BRL Life Technologies

Phytohemagglutinin P (PHA-P)

Sigma

Prepare PHA by dissolving 2 mg in 2 ml of sterile water for a concentration of 1 mg/ml. Aliquot 300 µl into 2 ml sterile vials. Store at -20°C. 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 with 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

Sigma

Cryogenic vials, 1.5 ml sterile screw cap, frosted label

Sarstedt Brand Products

Reagent reservoirs, 50 ml capacity

Costar

Instrumentation:

Biological Safety Cabinet

NuAIRE

Incubator, 37°C, 5% CO₂ 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 Readers may be substituted)

Molecular Devices Corp.

Water bath

Precision Scientific

Hemocytometer

Hausser Scientific

Pipettor

ThermoLabsystem

12-channel pipette, 5-50 μ l

12-channel pipette, 20-200 μ l

Single channel pipette, 5-50 μ l

Single channel pipette, 30-300 μ l

PipetteAid XP

Drummond Scientific Co.

Specimens:

PBMC from normal donors and infected individuals are separated from either whole blood or buffy coats and cryopreserved.

IV. PROTOCOL

Thaw and stimulate PBMC

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

1. Transfer cryovials containing normal donor PBMC (2.5×10^7 cells/ml/vial) from liquid nitrogen to a room temperature water bath in the laminar flow hood. Use 4 vials of normal donor PBMC per virus isolation. 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 μ g/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 30 ml of fresh IL-2-GM (no PHA-P). The PBMC are now at a density of about 3.3×10^6 cells/ml and are ready for use. The cells should be used for infection within 3 days.

Add PBMC from Infected Subject

1. Thaw a single cryovial of PBMC from each infected subject as described above.
2. Transfer the PBMC to a 30 ml culture of PHA-P-stimulated normal donor PBMC in IL2-GM.
3. Incubate overnight at 37°C.

4. Remove all medium with a pipette and add 20 ml of RPMI-1640.
5. Pellet the cells at low speed (1400 rpm).
6. Decant supernatant and resuspend the washed cell pellet in 30 ml of fresh IL2-GM.
7. 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 becomes the specific lot number.
4. 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.

Titrating the Virus (TCID₅₀ Assay)

1. Place 100 µl of IL2-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.
2. Add 100 µl of PBMC (500,000 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.
3. 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).
4. 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.
5. 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.

6. Change the medium again on days 5 and 9 of incubation.
7. On day 11, transfer 25 μ l 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.
8. Add 225 μ l of 0.5% Triton X-100 to the plate containing the harvested culture supernatants, mix, and store at 4°C in zip-lock plastic bags.
9. 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.
10. Read plates at A₄₅₀ on Vmax® Kinetic or Thermomax Microplate Reader that is interfaced to a dedicated computer in the BSL-3 laboratory and linked electronically to the data analysis computer in the general laboratory.
11. 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.
12. Save the raw data electronically to the secure access file server.
13. 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.
14. 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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