Protocol for Neutralizing Antibody Assay in PBMC
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I. INTRODUCTION

The following protocol uses a 96-well microdilution plate format that is designed for optimal sensitivity while maximizing the number of samples evaluated in a single plate (5 samples tested at 8 dilutions per plate, plus a negative control). The principles of the assay as described below may be applied to a variety of formats depending on the needs of the investigator. The assay measures neutralization as a function of a reduction in viral p24 Gag antigen synthesis in PBMC blasts in the presence of a test sample relative to a negative control sample. The assay may be used with either human or rhesus PBMC. It is recommended that all primary isolates be of a low passage number (1 or 2 passages) in PBMC exclusively.

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

ID: Identification

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), 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

Human Interleukin 2 (IL-2), Delectinized. Store at -80°C 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 20°C - 37°C prior to use.

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

Prepare PHA-P 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 vials. 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.

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

Centrifuge, maximum rotational speed = 14,000 rpm
Eppendorf
18 place standard rotor for F-45-18-11 for 1.5 ml microcentrifuge tubes

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

Hemacytometer
Hausser Scientific

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

PipetteAid XP
Drummond Scientific Co.

Specimens:

Samples should be heat-inactivated at 56°C for 1 hour prior to assay as described in "Protocol for Heat-Inactivation of Plasma and Serum Samples." Samples may be serum or plasma although serum is preferred. Anticoagulants in plasma are problematic in the assay, especially when heparin is used. For

example, some forms of heparin have potent and strain-specific antiviral activity. Also, all anticoagulants are toxic to the cells at plasma dilutions lower than 1:60.

IV. PROTOCOL

Day 1: Thaw and stimulate PBMC

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

1. Transfer cryovials containing frozen PBMC (2.5×10^7 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 contained 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 5×10^6 cells/ml and are ready for use. The cells should be used for assay within 3 days. Two vials of cells are sufficient for one 96-well plate.

Day 2: Assay set-up

1. Using the suggested assay format in a 96-well U-bottom plate shown below in Appendix A, place 100 μl of IL-2 GM in all wells of columns 1-12 and additionally 40 μl to row H columns 3-12 and 50 μl to column 1 (column 1 to be used later for p24 standards and blank). This format will permit the titration of 5 serum samples tested at 8 dilutions in duplicate plus a negative control sample – virus control.

2. Place 11 μl of serum sample into the first 2 wells of a set, mix, do serial 3-fold dilutions upward (i.e. 50 μl into 100 μl) for a total of 8 serum dilutions when complete. Discard 50 μl from the last set of wells in a dilution series after sample transfer.

3. Add 50 μl of appropriate dilution of virus (500 - 2500 TCID50) to all wells used. Incubate at 37°C for 1 hr.

4. Add 50 μl of PBMC (500,000 cells, you will have 200 μl total volume at this point). Between each step, either rinse the pipettor tips in a reagent reservoir containing sterile PBS or change the pipettor tips between each plate to minimize carry-over.

5. 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).

Day 3: Washing cells

1. Remove 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. (Centrifugation is not necessary- this will not focus the cells into a tighter button or guarantee that they will remain in place while removing the medium. Centrifugation does not, however, have any negative effects and may be used to complete the washing steps in a short period of time).

2. Replace with 190 µl of fresh IL-2-GM.

> *<u>NOTE 3:</u> It is important to wash the cells by changing the medium nearly completely 4x to remove the virus inoculum and, most importantly, to remove all residual anti-Gag antibodies in samples from infected individuals or animals. Removing the virus inoculum assures that any p24 detected at the time of harvest is due to new virus production. Also, removing anti-Gag antibodies is essential, since very small amounts will interfere with the detection of Gag antigen (serum dilutions as high as 1:10,000 may block Gag antigen detection completely!).*

Days 4 (and Day 5, if necessary): Harvest culture supernatants for p24 measurements

1. Transfer 25 µl of culture supernatant to the corresponding wells of a fresh 96-well flat-bottom plate.

2. Replace with 25 µl of fresh IL-2-GM medium and return the assay plate to the incubator.

3. 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.

4. Between each step, either rinse the pipettor tips in a reagent reservoir containing sterile PBS or change the pipettor tips between each plate to minimize carry-over.

Day 7: P24 ELISA and Data Analysis

1. Read plates at A_{450} on Vmax® Kinetic or Thermomax Microplate Reader.
2. Assign each raw data file an experiment number and file identification number (ID) corresponding to the date and assay number.

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

4. Analyze and print the data using Softmax Pro software. The data print-out must include: i) experiment number, ii) network and protocol number, iii) cells used in the assay, iv) length of incubation in days, v) name, lot number and dilution of the virus stock used, vi) ID, visit number and bleed date of each sample and vii) signature of technician who performed the assay.

5. Check if p24 production in the virus control wells was greater than 2 ng/ml.

6. Continue to harvest the original assay plates for additional days if virus replication remains low on day

7. Perform p24 immunoassays on the entire plate for the chosen harvest day. Values lower than 2 ng/ml (depending on the sensitivity of the p24 assay) might not provide an adequate range of detectable p24 values to determine >90% reductions in cases where neutralization occurred. This depends on the sensitivity of the p24 assay. With a very sensitive assay, values <2 ng/ml may also work. Measuring neutralization when virus production in control wells has peaked may increase the likelihood that non-neutralized virus will overshadow the detection of neutralization. Unless all virus is neutralized, which is rarely the case, the remaining non-neutralized virus will replicate and soon become a significant component of the p24 content of the wells.

8. Calculate the reduction in p24 synthesis relative to the amount of p24 synthesized in the presence of either a negative control serum sample or the corresponding pre-immune sample. These values may be plotted on a linear scale and the titer determined by the reciprocal dilution where the slope intersects a desired cut-off (e.g., 50%, 80%, 90% reductions).

V. REFERENCES

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3. Montefiori, D.C., R.G. Collman, T.R. Fouts, J.Y. Zhou, M. Bilska, J.A. Hoxie, J.P. Moore, and D.P. Bolognesi. (1998) Evidence that antibody-mediated neutralization of human immunodeficiency virus type 1 is independent of coreceptor usage. J. Virol., 72:1886-1893.

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*Best details of the assay are presented here.

VI. APPENDICES

Appendix A: Suggested Assay Format

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	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	VC	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8
B	ST1	VC	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7
C	ST2	VC	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6
D	ST3	VC	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5
E	ST4	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
F	ST5	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
G	ST6	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
H	NEG	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
			Sample 1		*Sample 2*		*Sample 3*		*Sample 4*		*Sample 5*	

Wells in column 1 are left empty when performing the neutralization assays. The corresponding wells of the p24 immunoassay plate are used for the blank (BLK) and p24 standard curve (0 – 10 ng p24/ml). VC, virus control wells (virus and cells but no serum sample are added here).