PREPARATION OF PHA-STIMULATED UNINFECTED DONOR PERIPHERAL BLOOD MONONUCLEAR CELLS

1. PRINCIPLE

1.1 Peripheral blood mononuclear cells (PBMC) are isolated from healthy, uninfected donor blood for use in various assays and to culture HIV.

1.2 The PBMC are stimulated with the mitogen phytohemagglutinin-P (PHA-P), in the presence of human interleukin (IL-2) for 24-72 hours before use to promote blast formation and replication of T-cells.

2. SPECIMEN REQUIREMENTS

2.1 Whole blood anticoagulated with heparin may be used (heparin should be at a concentration of 600 units/mL). The volume drawn is 120-240 mL.

2.2 Leukocyte concentrates (buffy coats) can be obtained from the American Red Cross and are usually anticoagulated with EDTA or CPD. This is a unit of whole blood from which most of the plasma and red blood cells have been removed. The usual volume is 30-50mL.

2.3 For use of either of the sources of blood, the blood should be stored at room temperature and processed within 30 hours of collection.

3. REAGENTS

3.1 Ficoll-hypaque density gradient solution,endotoxin tested and sterile (i.e. Ficoll-Paque, Amersham-Pharmacia, cat# 17-1440-02 or Sigma Histopaque-1077 Hybri-Max, sterile filtered, endotoxin and hybridoma tested, cat# H8889): Store at room temperature. Shelf life = six months after opening. It is best to purchase small volumes of this reagent and replace frequently. (Reagent may be aseptically aliquoted and stored in small volumes.) Label container with date after opening.

3.2 Fetal Bovine Serum (FBS, heat-inactivated at 56^{o} C for 30 minutes.)
Specific lots of heat inactivated and filter-sterilized fetal bovine serum are reserved for ACTG. Contact Operations Center or check ACTG web page for ordering information.

3.3 **Growth medium:** Supplement RPMI with 2mM final L-glutamine, 100units Pen and100ugStrep/500mL (or 50ug/mL gentamicin), 5% human IL-2 and 20% fetal bovine serum. Media may be filter sterilized after addition of supplements. Sterility should be confirmed by incubating test aliquots for 3 days before use. Store medium at 4ºC for up to one month. Warm medium to room temperature before using. For more specific reagent information, see Qualitative PBMC Macrococulture Assay SOP.

3.5 Phosphate Buffered Saline (PBS) without Ca++ and Mg++, 1X: Store at 18 to 25°C. Observe manufacturer's outdate. Label bottle with open date; use opened bottle within three months.

3.6 Hanks Balanced Salt Solution (HBSS) without Ca++ and Mg++: Store at 18 to 25°C. Observe manufacturer's outdate. Label bottle with open date; use opened bottle within three months.

3.4 0.4% Trypan Blue Stain. This stains non-viable cells dark blue and is used to determine the viable cell count of a culture. Store at 18 to 25°C. Observe manufacturer's outdate. If the solution has crystals on inspection, it should be filtered using a 0.2µm filter.

3.7 PHA-P (Such as Sigma, catalog # L9132, 5mg lyophilized powder). Add 5mL of sterile PBS to the vial to resuspend PHA-P to a final concentration of 1mg/mL.

4. Equipment/Supplies/Reagents

4.1 Laminar flow hood (minimum class 2, type A biosafety hood).
4.2 Gloves (latex, vinyl, nitrile).
4.3 Bleach (household bleach diluted 1/100 with tap water)
4.4 Lab coat or protective gown.
4.5 Centrifuge with horizontal rotor, with speeds up to 1800 X g, and equipped with aerosol safe canisters.
4.6 Centrifuge with horizontal rotor, capable of speeds up to 1800xg, and equipped with aerosol safe canisters.
4.7 Microcentrifuge tube for cell counting, 0.5 mL
4.8 Sterile pipettes, graduated and transfer.
4.9 Pipetting device.
4.10 Sterile plugged pipette tips.
4.11 Micropipettors of various volumes.
4.12 Sterile conical centrifuge tubes, 15mL and 50mL.
4.13 Accuspin™ tubes (Sigma Accuspin™ System-Histopaque®-1077, cat#A6929, A7054, or A0561)
4.14 Hemacytometer and microscope, or automated cell counter (i.e. flow cytometer or Coulter Counter)
4.15 Density gradient solution (density = 1.077), sterile and endotoxin tested. Label container with date after opening. The shelf life for Ficoll is 6 months after opening. However, discard if manufacturer's expiration date occurs before this 6-month period. It is best to purchase small volumes of this reagent and replace frequently. Examples: Ficoll-Paque, Amersham-Pharmacia, cat# 17-1440-02; Sigma Histopaque-1077 Hybri-Max, cat# H8889, Sterile phosphate buffered saline (PBS), Ca^{++}-free and Mg^{++}-free or Sterile Hanks Balanced Salt Solution (HBSS). Observe manufacturer's outdate. Label bottle with open date; use opened bottle within three months.
4.16 Fetal bovine serum (FBS), heat-inactivated at 56° C for 30 minutes (mix larger volumes several times while inactivating). Specific lots of heat-inactivated and filter-sterilized fetal bovine serum are reserved for ACTG. Contact Operations Center for ordering information.
4.17 Sterile complete RPMI 1640 medium
Supplement RPMI with L-glutamine (2 mM final concentration), 100 units

Pen/mL, 100ug Strep/mL, and 10% fetal bovine serum. Medium may be filter-sterilized after addition of supplements.

4.18 25 and 75 cm^2 tissue culture flask

4.19 Laminar flow hood (Class 2 biosafety hood)

4.20 CO_2 incubator (37 ± 10°C with humidity)

5. PROCEDURE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

5.1 Twice a week the lab should obtain blood for donor preparation. If a leukocyte concentrate prepared from whole blood unit from the American Red Cross (ARC) is received, it will be tested by the ARC for anti-HIV, as well as hepatitis B and syphilis. Testing may not be complete when the unit is released, in which case the ARC will call or fax those results as soon as they are available. If heparinized whole blood is received it may be treated the same way as the leukocyte concentrate, but the volume will be higher and the number of necessary tubes for white cell separation will be greater.

5.2 Remove the cells from the bag, by pouring the blood into a 50mL conical tube via the coupler or by using 60mL syringe.

5.3 Separate PBMC from the blood as follows:

5.3.1 Accuspin Method: Carefully pour 20-30mL of blood into large Accuspin tubes (as many as needed). Centrifuge the tubes at room temperature at 800 X g for 20 minutes.

5.3.2 Overlay Method: Add one part PBS or HBSS to one part blood. Blood should be carefully and slowly overlaid at a ratio of 4 parts diluted blood to 3 parts Ficoll reagent in 50mL sterile tubes, being careful not to disturb the interface. Centrifuge the tubes at room temperature at 400 X g for 30 minutes. Note: The centrifuge brake must be turned OFF for the separation to be clean and to maximize the retrieval of the PBMCs.

5.3.3 After centrifugation, remove cloudy interface (PBMC layer) into appropriately labeled 50mL conical tubes.

5.3.4 Wash cells by filling tubes with sterile PBS or HBSS and centrifuge at 400 x g for 10 minutes.

5.3.5 Decant supernatant after centrifugation, resuspend cells and fill tubes with sterile PBS or HBSS and wash again.

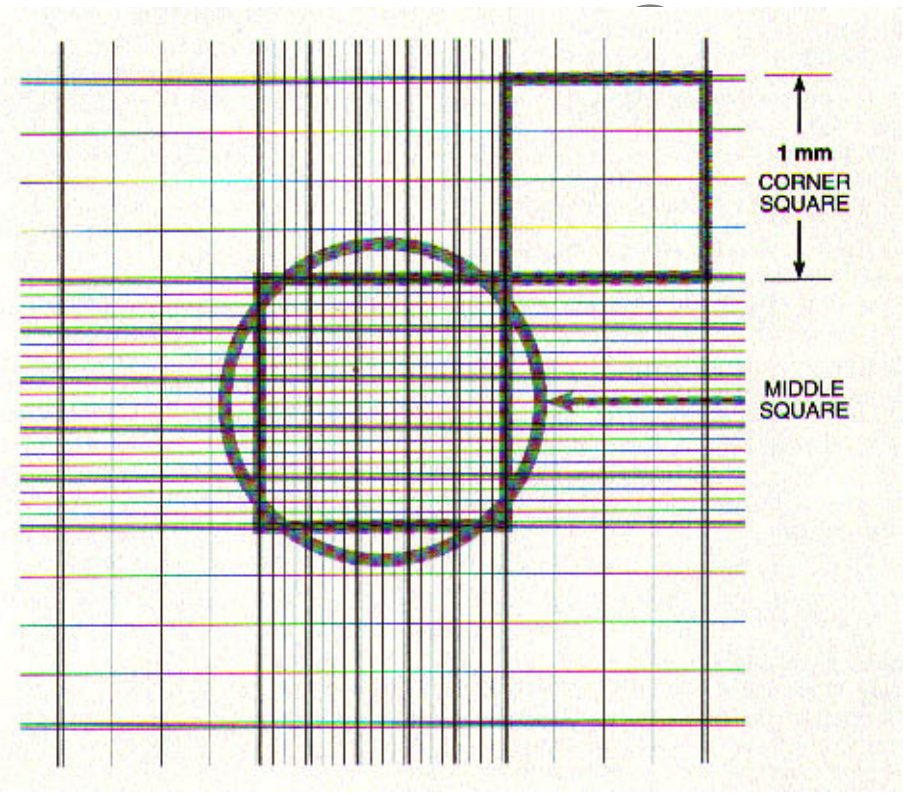
5.3.6 Resuspend each pellet in 10-30mL of Growth Medium, depending on whether whole blood or leukocyte concentrate was used.

5.3.7 Count and record the number of viable PBMC/mL:

5.3.7.1 Pipette 10 μL of PBMC suspension into a 0.5 mL microcentrifuge tube. Add 90 μL of 0.4% Trypan Blue stain, making a 1:10 dilution (final concentration of Trypan Blue is 0.36%). Mix carefully to avoid aerosol formation.

$$\text{Dilution Factor: } \frac{90\mu l \text{ Trypan Blue} + 10\mu l \text{ PBMC}}{10\ \mu l \text{ PBMC}} = 10^1$$

5.3.7.2 Load the hemacytometer with cell mixture (Trypan Blue + PBMC's) until the area under the cover slip is sufficiently filled. Make sure to use a cover slip that is specific for the hemacytometer. Allow the cell suspension to settle in the hemacytometer for at least 10 seconds before counting. Count the 4 large corner squares (see diagram below). Viable PBMCs will be clear; nonviable PBMCs will be blue.



Count cells in the 4 corner 1mm squares. Include cells that touch either the top line or left vertical perimeter line of any corner square. Do NOT count any cells that touch either the bottom line or right vertical perimeter line of any corner square.

5.3.7.3 Calculate the number of PBMC/mL:
10^4 = volume conversion factor to 1 mL
10^1 = specimen dilution factor

$$\text{PBMC/mL} = \frac{\text{PBMC in all four squares}}{4} \times 10^4 \times 10^1$$

example: $\frac{88}{4} \times 10^5 = 2.2 \times 10^6$ PBMC/mL

5.3.7.4 To calculate Cell Viability:

$$\% \text{ Viability} = \frac{\text{Number of Viable Cells Counted}}{\text{Total Number of Cells Counted}} \times 100$$

5.3.7.5 To determine the total number of cells, multiply the number obtained above (PBMC/mL) by the cell suspension volume (mL).

$$\text{Total Cells} = \frac{\text{PBMC}}{\text{mL}} \times \text{Volume (mL) of PBMC suspension}$$

5.3.7.6 Automated counting may also be used. Follow manufacturer's instructions.

5.3.8 Place the cells in 75cm^2 flasks (number of flasks depending upon workload for the week) at a concentration of 2x10^6/mL in Growth Medium. Total volume in each flask may be from 40-120mL.

5.3.9 Add PHA-P at a final concentration of 5µg/mL (e.g. 200µL/40mL medium).

5.3.10 Incubate at 37°C, 5% CO_2 with humidity for 1-3 days before use.

6. QUALITY CONTROL

6.1 Set up a qualitative HIV culture using newly prepared donor PBMC as "patient cells" to verify that the new donor is HIV culture negative. (See the Qualitative PBMC Macroculture Method located elsewhere in this manual).

6.2 Do not use PHA-stimulated donor PBMC older than 3 day post stimulation.

7. REFERENCES

7.1 Levy JA, Shimabukuro J. Recovery of AIDS-associated retroviruses from patients with AIDS or AIDS-related conditions and from clinically healthy individuals. J Infect Dis 1985;152:734-8.

7.2 Gallo RC, Salahuddin SZ, Popovic M, et al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 1985;224:500-3.

7.3 Jackson JB, Coombs RW, Sannerud K, Rhame F, Balfour HH Jr. A rapid and sensitive viral culture method for human immunodeficiency virus HIV-1. J Clin

Microbiol 1988;26:1416-8.

Procedure: ACTG Lab Manual Preparation of PHA-Stimulated HIV Uninfected Donor PBMC

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Supersedes Archived Protocol: DAIDS Virology Manual for HIV Laboratories, Version January 1997