HIV QUALITATIVE PBMC MICROCOCULTURE ASSAY

1. PRINCIPLE:

   1.1 A co-culture of patient peripheral blood mononuclear cells (PBMC) and uninfected PHA-stimulated PBMCs is maintained under ideal conditions to allow viral replication in vitro. PBMC cultures from HIV-1 seropositive patients will yield detectable HIV-1 antigen by this method. Culture positivity rates will vary with patient treatment regimen, viral load, and condition of specimen (e.g."fresh" or frozen) at time of culture inoculation.

2. SPECIMEN REQUIREMENTS

   2.1 ACD, CPD, heparin or EDTA anticoagulated peripheral blood.

   2.2 Expected volume: 3-10mL from adults or children; minimum volume: 1 to 2mL from infants.

   2.3 The blood must be kept at room temperature until processing and should be processed within 30 hours of collection. Blood older than 50 hours will have a significantly decreased yield for culture and is not recommended.

3. REAGENTS:

   3.1 All reagents are prepared using deionized, type I water.

   3.2 Sterile Phosphate Buffered Saline (PBS), without calcium or magnesium; store at room temperature. Note manufacturer's outdate or discard one month after opening (whichever is earlier).

   3.3 Sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store at room temperature. Note manufacturer's outdate or discard one month after opening (whichever is earlier).

   3.4 Sterile Ficoll-Hypaque or Lymphocyte Separation Medium (LSM) with a density of 1.077. Note manufacturer's outdate and storage recommendations.

   3.5 Penicillin - available in 5×10^6 unit vials. Store at room temperature. Observe manufacturer's outdate.

      3.5.1 Add 25mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

3.5.2 Divide into 0.32mL aliquots in sterile 1.5mL microcentrifuge tubes and freeze at -20°C in a labeled box. Label with a 1 year outdate or manufacturer outdate (whichever is earlier).

3.6 Gentamicin - available in 50mg/mL bottles. Open bottles under laminar flow hood only. Divide into 0.640mL aliquots in sterile 1.5mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4°C. Outdate one month after opening or manufacturers outdate (whichever is earlier).

3.7 Penicillin-streptomycin solution (5000 μ/mL and 5000 μgm/mL) may be substituted for the penicillin and gentamicin listed above.

3.8 Fetal Bovine Serum (FBS), heat-inactivated - available in 500mL sterile bottles from various manufacturers; store frozen at -20°C; observe manufacturer's outdate. Once thawed, store opened FBS at 4°C for a maximum of one month. (NOTE: If fetal bovine serum is purchased without the heat inactivation, the lab will need to treat it before using in culture media. Thaw a bottle in a 37°C water bath, then heat-inactivate in a 56°C water bath for 30 minutes with occasional mixing with a gentle swirl- do not shake the FBS bottle because the liquid will froth. The level of H2O in the water bath should be as high as the level of the serum in the bottle.)

3.9 RPMI 1640 medium with L-glutamine (2mM) - Store at 4°C and observe manufacturers outdate.

3.10 IL-2 (interleukin-2) - Store at -20°C. Note manufacturers outdate. As needed, thaw a 50mL bottle (freeze the remaining 25mL).

3.11 Basic Medium. To make 620mL:

3.11.1 Add 120mL FBS to 500mL of RPMI 1640 medium with L-glutamine. Final concentration is 20%.

3.11.2 Add 310μL stock penicillin.* (Concentration of penicillin used is 5 million units/25mL or 200,000 units/mL; final concentration = 100 units/mL).

3.11.3 Add 620μL Gentamicin.* (Concentration of stock Gentamicin used is 50 mg/mL; final concentration = 50 μg/mL).

3.11.4 6.5mL of commercial Penicillin-streptomycin solution may be substituted for .2 and .3.

3.11.5 Incubate a 5mL aliquot for 3 days at 37oC for a sterility check.

3.11.6 Store Basic Medium at 4°C for up to 1 month.

3.12 Growth Medium - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium. To make 500mL:

3.12.1 475mL Basic Medium

3.12.2 Aseptically add sufficient IL-2 to make a final concentration of 5%.

3.12.3 Incubate a 5mL aliquot for 3 days at 37°C for a sterility check.

3.12.4. Store Growth Medium at 4°C for up to 1 month.

3.12.5 Growth Medium should be warmed in 37°C incubator or waterbath before use.

3.13 Trypan Blue Stain Solution (available from Sigma and Gibco) - stains non-viable cells blue, and is used to determine the viable cell count. If powdered Trypan Blue is purchased, prepare a 0.4% solution (add sufficient volume of normal saline to 0.4 gm Trypan Blue to equal 100mL). After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

3.14 PHA-stimulated uninfected donor PBMCs - see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC).

4. EQUIPMENT AND SUPPLIES

4.1 Laminar flow hood (biosafety cabinet class II)

4.2 Gloves

4.3 Lab coat

4.4 Nalgene bottle, 500 ml

4.5 Sterile 50 ml conical graduated polypropylene centrifuge tubes

4.6 Sterile 15 ml conical graduated polystyrene centrifuge tubes

4.7 Sterile 75 x 100 mm tube

4.8 Serological pipettes, 10 and 2ml

4.9 Polypropylene transfer pipettes

4.10 Disposable polystyrene blood dilution vials

4.11 Isoton II (Coulter)

4.12 Isoterge II (Coulter)

4.13 Bleach

4.14 Millipore filter, 0.22µm

4.15 Tissue culture plate, 24-well

4.16 Pipette aid filter/controller (e.g., Drummond)

4.17 Tabletop centrifuge

4.18 Automated cell counter (e.g., Coulter Cell Counter) or manual cell count by hemacytometer

4.19 Phase Bright or Light microscope with 10x ocular

4.20 Cell diluter (e.g., Dade dilutor)

4.21 Tissue culture incubator - 5% CO2, 37°C and 98% humidity
56°C water bath

5. PROCEDURE

5.1 Log patient information into the LDMS (see LDMS Manual; also posted on the FSTRF website at http://www.fstrf.org/ldms/manual/3.9/virology_3.9.pdf). Carefully label all tubes and flasks for each sample of blood being processed.

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

5.2 Obtain PBMC from patient blood according to the ACTG consensus methods (http://aactg.s-3.com/specshipproc.htm). The PBMCs can the be resuspended in culture media, counted and used for culture; washed in PBS and stored as dry cell pellets; or stored as viable cell suspensions (see ACTG consensus methods for cryopreservation, http://aactg.s-3.com/cryo.htm).

5.3 Set up qualitative microculture as follows:

5.3.1 In two wells of a 24-well tissue culture plate, add 1x 10^6 PHA-stimulated donor cells (see “Preparation of PHA-Stimulated Uninfected Donor Peripheral Blood Mononuclear Cells”) and 1 x10^6 patient PBMC’s. Adjust final volume to 2ml with growth media.

5.3.2. Put 2ml of sterile water in corner wells to help maintain humidity.

5.3.3 Incubate at 37°C with 5% CO2.

5.3.4. On day 7 remove 1.0ml of medium without disturbing cells. Replace with 1 ml fresh growth media containing 5 x 10^5 PHA-stimulated donor cells.

5.3.5 On day 14 remove 1.0ml of medium without disturbing cells. Replace with 1mL fresh growth media containing 5 x 10^5 PHA-stimulated donor cells.

5.3.6 Save day 14 supernatant fractions from duplicate wells separately and store at -20°C until analyzed for HIV p24 antigen by EIA.

5.3.7. Assay day 14 supernatant fractions for HIV p24 antigen and if both wells are positive, terminate culture; otherwise, continue culture until day 21.

5.3.8 Terminate culture on day 21, save supernatant fractions from duplicate wells separately, and store at -70°C until analyzed for HIV p24 antigen by EIA.

5.3.9 Assay day 21 supernatant fractions for HIV p24 antigen.

5.3.10 NOTE: Supernatant should be saved from positive culture wells as defined in the protocol. Culture supernatants should be aliquotted into cryovials with a minimum volume of 0.5ml per tube and stored at -70ºC or colder.

6. QUALITY CONTROL

6.1 The input number of patient cells used for a culture must be entered into the computer. Similarly, it is important to document whether the culture was set up using "fresh" or cryopreserved PBMCs. A positive culture is interpretable and provides an isolate no matter how many (or few) patient PBMCs were set up for culture. However, a negative result may not be accurate if too few patient cells were used or if the condition of the patient cells was compromised due to freezing or age of the culture.

6.2 All laboratories performing ACTG cultures must participate successfully in the ACTG Viral Quality Assurance program.

7. INTERPRETATION AND REPORTING OF RESULTS

7.1 Coculture Well Results: Criteria

7.1.1 The criteria for a positive or negative microculture well is based on VQA standardized HIV p24 antigen results obtained from the day 14 and/or day 21 culture supernatants. A culture well is considered positive if the HIV p24 antigen level is $\geq$ 30 pg/mL. A culture well is considered negative if the HIV p24 antigen level is < 30 pg/mL.

7.2 Culture Results: Criteria (NOTE: The criteria for determining a positive, negative or indeterminate result for a qualitative microculture depends upon the HIV status of the patient.

7.2.1 For confirmed HIV-positive patients, a microculture is considered positive if either well (or both wells) of a qualitative microculture is positive. The culture is considered negative if both wells are negative for p24 antigen on day 14 and day 21.

7.2.2 For HIV-negative or HIV-undetermined patients, a microculture is considered positive if both wells are positive at day 14 and/or day 21. If either well of a qualitative microculture is positive at day 14 and day 21 and the other negative, the culture is considered indeterminate and a subsequent specimen should be requested for testing. The culture is considered negative if both wells are negative for p24 antigen on day 14 and day 21.

7.3 PROCEDURE NOTES

7.3.1 To reduce the chances of cross contamination and/or specimen mix-up, cells from only one patient should be set-up per 24-well tissue culture plate.

7.3.2 Laboratories performing this assay for ACTG or other DAIDS sponsored protocols, should be participating in and certified by the Virology Quality Assurance Quantitative Microculture certification program.

7.3.3 Cultures contain large quantities of HIV and are potentially infective to the technician handling the cultures. Gowns are required when working with any potential HIV containing specimens (i.e., peripheral blood, CSF, tissue specimens, etc.) and are changed weekly (daily if work is being done in BSL-3 facility). Gloves must be worn whenever working with any potentially HIV containing specimens. Gloves should be changed often, especially if punctured or contaminated. All work must be performed in a certified biological safety laminar flow hood. All work areas in the laboratory must be wiped down with 10% sodium hypochlorite (bleach) at the beginning and end of the working day. The laminar flow hood must also be decontaminated with 10% sodium hypochlorite daily.

7.3.4 The level of CO2 should be checked weekly with fyrite to determine % CO2.

7.3.5 GLP(Good Lab Practices) and excellent sterile technique are very important because the cultures are maintained for up to 3 weeks and must remain free of contamination to give accurate results.

8. REFERENCES

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Procedure: ACTG Lab Man HIV QUALITATIVE PBMC MICROCOCULTURE ASSAY

Prepared by: ACTG Laboratory Technologist Committee

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