HIV QUALITATIVE PBMC MACROCOCULTURE ASSAY

1 PRINCIPLE

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*. Most 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: 10 to 20mL 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) 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 50 mg/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 manufacturer outdate (whichever is earlier).

3.7 Penicillin-streptomycin solution (5000 u/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. Store at 4°C for a maximum of one month after thawing. (If fetal bovine serum is purchased without the heat inactivation, the lab will need to treat it before using in culture media. When needed, 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. Store at 4°C after thawing. Heat-inactivated FBS has a one month outdate.)

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

3.10 IL-2 (interleukin-2) Store at -20°C. Note manufacturer's 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 approximately 20%.

3.11.2 Add 310μL stock penicillin.* (Concentration of penicillin used is 5 million units/25mL or 200,000 units/mL; 0.31mL or 200,000 units/mL = 62,000 units and 62,000 units/620mL final volume of medium = 100 units/mL for final concentration).

3.11.3 Add 620μL Gentamicin.* (Concentration of Gentamicin used is 50mg/mL or 50 μg/μL = 31,000μg and 31,000μg/620mL final volume of medium = 50μg/mL for final concentration).

3.11.4 *6.5 mL of commercial Penicillin-streptomycin solution may be substituted for (b) and (c).

3.11.5 Incubate a 5mL aliquot for 3 days at 37°C 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 (0.4 gm Trypan Blue to 99mL saline). After dissolving, filter solution through Whatman filter paper or a 0.45µ 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

Accuspin tubes with Ficoll. Sigma in 12 mL or 50 mL size (optional).
Sterile 15 and 50 mL conical tubes.
Sterile 2, 5, 10, and 25 mL pipettes.
Hemacytometer.
Sterile 25 cm tissue culture flask.
Sterile 500 mL bottles.
Sterile 1.5 and 0.5 mL microcentrifuge tubes.
20 µL, 200 µL, and 1000 µL pipetman.
Sterile 200 µL and 1000 µL pipette tips.
Bleach (household bleach diluted 1/10 with tap water).
Centrifuge capable of speeds up to 1500 x g and equipped with a horizontal rotor and O-ring sealed safety cups.
Compound microscope.
CO_2 incubator (37 ± 1°C).
37°C and 56°C water baths.
Pipette aid.
Gloves.
Lab coat or gown.
Laminar flow hood (Class 2 biosafety hood).

5 PROCEDURE

5.1 Log patient information into the lab computer and label specimen with the assigned specimen number. 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 BSL3 standards.

5.2 Obtain PBMCs from patient blood according to ACTG Consensus Specimen Processing Guide sections 1.2.7-1.2.11. (http://aactg.s-3.com/labs.htm) Count cells and proceed to section 5.3.

5.3 Set up culture as follows:

5.3.1 Pipette a volume of patient cell suspension equal to 10 million PBMCs into a labeled 25cm^2 sterile cell culture flask.

5.3.2 Add 5 to 10 million PHA-stimulated uninfected donor PBMCs.

5.3.3 Add sufficient Growth Medium to make volume = 10 to 15mLs.

5.3.4 Note: if patient PBMC cell count is low, a culture may be set up using an equal number of patient PBMCs and donor cells, 1 million cells/mL each, in a minimum of 5 mL per flask. For very low patient cell counts, see Qualitative PBMC Micrococulture Assay.

5.4 Incubate at 37^{o}C, 5% CO_2.

5.5 Store remaining PBMCs according to protocol specifications.

5.6 Feed cultures and obtain samples for HIV p24 antigen testing according to the following schedule:

5.6.1 On day 3 or 4, carefully remove 5 to 7mL of supernatant from the flask without disturbing the cells. Freeze an aliquot for HIV p24 antigen testing. Feed with 5 to 7mL of warmed growth medium. (If less than 10mL were used for the culture, remove half of the volume and feed with an equal volume of fresh growth medium.)

5.6.2 On day 7, carefully remove 5mL to 7mL of supernatant from the flask without disturbing the cells. Freeze an aliquot for HIV p24 antigen testing. Replenish with 5 to 7mL of warmed growth medium containing 10 million PHA-stimulated PBMCs.

5.6.3 Continue sampling and feeding in this manner until the end of the culture:
- sampling twice per week,
- adding fresh medium once per week,
- adding fresh medium plus donor PBMCs weekly.

5.7 Print a computer-generated sampling list every feeding day to identify cultures that need an aliquot of supernatant saved for p24 antigen testing. Save the aliquots in tube or sample trays, depending on the type of p24 antigen assay used. Store at -20^{o}C or colder until p24 antigen assay is performed.

5.8 Maintain cultures for 28 days or until culture meets the criteria for positivity. Avoid potential false negative results: do not terminate negative cultures early.

5.9 The recommended harvest from positive cultures is 4 x 1mL supernatant and 2 dry cell pellets. Store all culture isolates 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 Qualitative cultures with supernatant p24 antigen results remain consistently below the cutoff (30 pg/mL) until at least day 28 are reported as "negative".

7.2 Qualitative cultures with supernatant p24 antigen results meeting one of the following criteria are considered "positive".

7.2.1 Two consecutive HIV p24 antigen VQA adjusted values that are "out of range (O.D. >/= 2.0 or 3.0, depending on the assay and reader used.)

or

7.2.2 Two consecutive HIV p24 antigen VQA adjusted values of >30 pg/mL, of which the second value is at least four times greater than the first value or "out of range" (O.D. >/= 2.0 or 3.0, depending on the assay and reader used.)

or

7.2.3 Three consecutive increasing HIV p24 antigen VQA adjusted values of >30 pg/mL, where neither consecutive value is **>/=** 4 times the previous sample, but the third value is at least four times greater than the first.

7.3 Qualitative cultures that do not meet the criteria for a positive or negative culture are considered “indeterminate” (e.g. p24 values >/= 30pg/mL, followed by p24 values < 30 pg/mL, or p24 values that are >/= 30pg/mL but never meet the criteria defined in section 7.2).

7.4 Qualitative cultures that are not maintained according to this protocol (e.g. terminated before the criteria of a negative or positive culture have been achieved) are considered invalid.

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Procedure: HIV Qualitative PBMC Macrococulture

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