HIV QUANTITATIVE PBMC MICROCOCULTURE ASSAY

1 PRINCIPLE

The quantitative PBMC micrococulture assay estimates the number of infectious units of HIV per million mononuclear cells (IUPM) in peripheral blood mononuclear cells (PBMC). The greater the number of input patient cells needed to produce a positive result, the lower the virus load in the PBMCs. The assay, as described in detail below, is performed in duplicate in a 24-well tissue culture plate using six 5-fold dilutions, beginning with one million patient PBMCs. Each sample of patient cells is cocultured with PHA-stimulated normal donor PBMCs for 14 days. The supernatant from each individual well is assayed for viral expression of HIV- I p24 antigen by the standard HIV p24 EIA assay.

2 SPECIMEN REQUIREMENTS

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

2.2 Expected volume: 10 to 20 ml from adults or children; minimum volume: 1 to 2 ml 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 on month after opening (whichever is earlier).

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

3.4 Sterile Ficoll-Hypaque or Lymphocyte Separation Medium (LSM). Follow manufacturer's outdate and storage recommendations.

3.5 Penicillin - available in 5 $x10^6$ unit vials. Store at room temperature. Observe manufacturer's outdate. Prepare as follows:

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 0.32 mL aliquots in sterile 1.5mL labeled microfuge tubes. 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. 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 (5000u/mL and 5000ugm/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 H_2O 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 (2mM) - 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 50 mL bottle (freeze the remaining 25 mL).

3.11 Basic Medium - To make 620mL:

3.11.1 Add 120mL FBS to 500 mL 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 620 µL Gentamicin.* (Concentration of Gentamicin used is 50mg/mL or 50µg/µL = 31,000µg. 31,000µg/620mL final volume of medium = 50µg/mL for final concentration).

3.11.4 *Note: 6.5mL of commercial Penicillin-streptomycin solution may be substituted for (3.11.2) and (3.11.3).

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 500 mL:

3.12.1 To 497.5mL sterile Basic Medium, aseptically add 2.5mL IL-2 to make a final concentration of 5%.

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

3.12.3 Growth Medium should be warmed in 37^{o}C incubator or waterbath before use. Store at 4^{o}C for up to two weeks.

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 (0.4gm Trypan Blue to 99 mL saline). After dissolving, filter solution through Whatman filter paper or 0.45 µ filter.*

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

4. EQUIPMENT AND SUPPLIES

Gloves.
Lab coat or gown.
Laminar flow hood (Class 2 biosafety hood).
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 24-well tissue culture plates.
Sterile 500 mL bottles.
Sterile 1.5 and 0.5 mL microcentrifuge tubes.
20µL, 200µL, and 1000µL pipetman.
Sterile 200µL and 1000µL 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.
CO2 incubator (37 ± 1°C).
37°C and 56°C water baths.
Pipette aid.

5. PROCEDURE

5.1 Log patient information into the LDMS 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 STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

5.2 Obtain PBMC 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 Make six 5-fold serial dilutions of patient PBMCs as follows:

5.3.1 Pipet 3.0mL Growth Medium into one sterile dilution tube. Pipet 2.4mL Growth Medium into each of 5 sterile dilution tubes.

5.3.2 For the first tube in the series, start with 3x 10^6 patient cells in 3mL of Growth Medium. A minimum of 2.7xl0^6 patient PBMC (in 2.7mL) are required for the following scheme. See note(*) below if minimum requirement is not met.

5.3.3 Transfer 0.6mL of the cell suspension from step 5.3.2 to the next tube in the series containing 2.4mL of Growth Medium. Mix.

5.3.4 Continue as in step 5.3.3, using a new pipette tip for the removal from each tube, to make 6 dilutions. The resulting dilution scheme is 1: 1, 1:5, 1:25, 1: 125, 1:625, 1:3125. The resulting counts per mL will be 1,000,000, 200,000, 40,000, 8,000, 1600, and 320 patient PBMCs per mL.

**NOTE: If fewer than 2.7x10^6 but more than 2x10^6 patient PBMC are recovered from a sample, the first tube should be adjusted to contain 2.0x10^6 PBMC in 2.0mL of Growth Medium. Proceed with step 5.3.3 above.*

If fewer than 2.0x10^6 PBMC are recovered from a sample, dilute the total number of cells in 3.0 mL of Growth Medium and proceed to make the 5-fold dilutions from this starting concentration. In the computer, it will be necessary to enter the new estimated concentration per mL (total number of PBMC recovered divided by 3) rather than defaulting to 1 x 10^6 for the number of cells in the 1: 1 dilution.

5.3.5 Pipette 1.5mL of sterile water into each of 4 comer wells of an appropriately labeled 24-well plate. In duplicate, pipette 1mL of each of the 6 patient cell dilutions into respective wells. Store remaining patient PBMCs according to eachprotocol (viable PBMC, pellets, etc.).

NOTE: In the case of fewer than 2.7x10^6 but >2.0x10^6 PBMC, the first tube will have just 1.4 mL and must be tested in singleton. The first set of wells will include only one well with a single sample of lx10^6 PBMC.

5.3.6 Prepare donor cells at a concentration of 1 x 10^6 cells/mL. One mL will be needed for each well of the plate (12 wells) plus extra to facilitate pipetting.

5.3.7 Centrifuge 13 x 10^6 PHA-stimulated donor cells at 400 xg for 10 minutes.

5.3.8 Remove supernatant.

5.3.9 Suspend donor cell pellet in 13mL of Growth Medium.

5.3.10 Add 1.0mL of donor cell suspension to each well. Replace plate cover. Incubate at 37^0 C, 5% $C0_2$ with humidity (or dry incubator if plate is enclosed in gas permeable ziplock bag.

5.3.11 Feed and sample for p24 antigen detection as follows: Remove 1mL of supernatant from each well without disturbing the cells.

Day 7 – Feed: *discard supernatant.* (These may be saved if desired, in case of trouble with the assay, but may only be used to troubleshoot the assay and not to calculate an IUPM.)

Day 14 – sample: *save supernatant* from each well, frozen in appropriately labeled tubes for HIV p24 antigen testing.

On day 7 add 1mL of 0.5 x 10^6 PHA-stimulated donor cells to each well.

5.3.12 Prepare donor cells for feeding at a concentration of 0.5x$I0^6$ cells/mL. One mL will be needed for each well plus extra to facilitate pipetting *NOTE: This concentration differs from that used to set up the initial assay.*

5.3.12.1 Centrifuge 7x10^6 donor cells at 400xg for 10 minutes.

5.3.12.2 Decant supernatant.

5.3.12.3 Mix pellet and suspend in 14 mL of Growth Medium.

5.3.13 Culture wells to be sampled are listed in a "sampling list" which is computer -generated each day from the LDMS program. Supernatant aliquots are saved in sterile tubes and stored at -20 ^{0}C or -70 ^{0} C until assayed for HIV p24 antigen level.

5.3.14 Infectious Units per Million PBMCs (IUPMs) values are calculated by the LDMS using the “Method of Maximum Likelihood” from the pattern of positive culture wells in the assay. A well is scored positive if the HIV p24 antigen assay detects at least 30 pg/mL.

5.3.15 At the end of culture, save the appropriate samples according to each protocol. Culture isolates should be stored at -70ºC or colder.

6. REPORTING

6.1 The IUPM calculated by the LDMS is reported.

6.2 A “Goodness of Fit”, also calculated by the LDMS, is similarly reported. A low Goodness of Fit (<0.05) reflects an irregular, problematic pattern of positive and negative wells and thus may invalidate the result.

7 REFERENCES

Ho DD, Moudgil T and Alam M. Quantitation of human immunodeficiency virus type- I in the blood of infected persons. New Engl J Med 321:1621-1625, 1989.

Gupta PA, Enrico A, Armstrong J, Doerr M, Ho M, and Rinaldo C. A semiquantitative microassay for measurement of relative number of blood mononuclear cells infected with human immunodeficiency virus. AIDS Res Hum Retro 6:1193-1196, 1990.

Dimitrov DH, Melnick JL, and Hollinger FB. Microculture assay for isolation of human immunodeficiency virus type I and for titration of infected peripheral blood mononuclear cells. J Clin Microbio 28:734-737, 1990.

Myers LE, McQuay LJ, Hollinger FB. Dilution Assay Statistics. J Clin Microbio 32:732-739, 1994.

Scott WA, Brambilla D, Siwak W, Beatty C, Bremer J, Coombs RW, Homayoon F, Fiscus SA, Hammer SM, Hollinger FB, Khan N, Rasheed S, Reichelderfer PS, and Participating Laboratories of the AIDS Clinical Trials Group. Evaluation of an infectivity standard for realtime quality control of human immunodeficiency virus type 1 quantitative microculture assays. J of Clin Microbio 34.

Procedure: HIV QUANTITATIVE PBMC CULTURE ASSAY

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