QUALITATIVE CSF/PBMC MICROCULTURE ASSAY

1. PRINCIPLE

1.1 Human immunodeficiency virus (HIV) has been shown to be the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). Isolation of HIV-1 from the Cerebral Spinal Fluid (CSF) specimens of AIDS patients is often associated with neurological complications of this disease.

1.2 Isolation of HIV from CSF is accomplished by the modification of the qualitative microculture method describe elsewhere in this manual.

1.3 The main differences in the methods are as follows:

1.3.1 Requires 1.0mL of freshly collected CSF (optimal).

1.3.2 An initial input of 2 million donor cells is used in each culture well.

1.3.3 An additional supernatant harvest is made at 24 hours to replenish culture media.

2. SPECIMEN REQUIREMENTS

Specimens should be stored at room temperature until processed. The assay utilizes freshly collected CSF specimens from lumbar puncture. Tube #3 from the lumbar puncture series should be used in this assay. Residual CSF should be stored at -70°C with appropriate patient identifiers and logged into the LDMS for protocol related studies. If there are visible red cells present in the CSF this should be noted in the LDMS because an HIV positive CSF culture that contains RBCs may be the result of the PBMC contamination rather than HIV being present in the CSF itself.

3. REAGENTS

3.1 Sterile PBS or HBSS, or Sterile 1X Dulbecco's PBS without Ca^{2+} or Mg^{2+}.

3.1.1 Place 450mL of distilled water into a 500mL Nalgene bottle.

3.1.2 Add 50mL of 10X Dulbecco's PBS without Ca^{2+} or Mg^{2+} to the bottle and mix well by inversion.

3.1.3 Open the bottle slightly and sterilize using the liquid setting on the sterilizer or by filtration through 0.22µm Millipore filter.

3.1.4 Allow the Dulbecco's PBS to cool and label with a 6 month expiration date or expiration of the Dulbecco's PBS, whichever is sooner, and preparer's initials.

3.1.5 Alternate: purchase sterile 1X PBS or HBSS.

3.2 Sterile water.

3.3 Lymphocyte separation media (LSM or Ficoll-Hypaque)

3.4 Fetal bovine serum (FBS, sterile)

3.4.1 Thaw the 500mL bottle of FBS completely.

3.4.2 If the FBS is not already heat inactivated, inactivate it by immersing the bottle up to the FBS level in a 56°C water bath for 30 minutes.

3.4.3 Label the bottle with the day of thawing/inactivation and store at -20°C after use. Keep for up to 18 months for inactivation, or until expiration date of FBS, whichever is sooner.

3.5 Antibiotics (100X Penicillin/Streptomycin, Gentamicin, 10 or 50 mg/mL

3.6 L-Glutamine 200mM.

3.7 Interleukin-2 (IL-2)

3.8 RPMI 1640 w/o L-Glutamine, 500mL bottle.

3.8.1 Add 138mL of heat inactivated FBS to a 500mL bottle of RPMI 1640 w/o L-Glutamine.

3.8.2 Mix well by inversion.

3.8.3 Add 34.5mL of Interleukin-2 to the mixture.

3.8.4 Add antibiotics to the mixture (e.g. 3.5mL of Gentamicin).

3.8.5 Mix well by inversion.

3.8.6 Add 14.0mL of 200mM L-Glutamine to the mixture.

3.8.7 The media is finally filtered through a Millipore 0.22 micron filter in a disposable filter/storage unit.

3.8.8 Label the bottle with the date of preparation, a one month expiration date, preparer's initials, and perform a sterility culture prior to use.

4. EQUIPMENT AND SUPPLIES

Laminar Flow hood (biosafety cabinet class II)
Gloves
Lab coat
Nalgene bottle, 500mL
Sterile Millipore 1000ml. filter unit with 0.22 micron filter
Sterile 50mL conical graduated Polypropylene centrifuge tubes

Sterile 15mL conical graduated polystyrene centrifuge tubes
Sterile 75 x 100 mm tube
Serological pipettes, 10 and 2mL
Polypropylene transfer pipettes
Disposable polystyrene blood dilution vials
Bleach
Tissue culture plate, 24 well
Pipette aid filler/controller (e.g., Drummond)
Tabletop centrifuge
Automated cell counter (e.g., Coulter Cell Counter) or hemacytometer
Light microscope with 10x ocular
Tissue culture incubator – 5% CO_2, 37˚C and 98% humidity
56˚C water bath

5. PROCEDURE

5.1 Patient specimen Preparation

No special patient preparation is required. CSF is delivered to the lab ASAP (usually within 30 minutes post collection). Do not refrigerate after collection. Deliver immediately to the laboratory and keep at room temperature prior to culture setup. Log all specimens into the computer and obtain a LDMS specimen number. Perform all subsequent procedures under a biological safety hood.

5.2 Coculture Procedure

5.2.1 In two wells of a 24-well tissue culture plate, add $2x10^6$ PHA-stimulated donor cells (see "Preparation of PHA-Stimulated Uninfected Donor Peripheral Blood Mononuclear Cells" located elsewhere in this manual) in 1.6mL of culture media. Add 0.4mL freshly collected CSF to each well.

5.2.2 Put 2mL of sterile water in corner wells to maintain humidity.

5.2.3 Incubate at 37°C with 5% CO_2.

5.2.4 The next day, remove 1.0mL of culture supernatant and discard into appropriate waste container. Add 1.0mL of fresh coculture medium.

5.2.5 At day 7 remove 1.0mL of medium without disturbing cells. Save supernatant at -20°C until analyzed for HIV p24 antigen by EIA. Replace with 1mL of fresh coculture medium containing $5x10^5$ PHA-stimulated donor cells.

5.2.6 At day 14 remove 1.0mL of medium without disturbing cells. Save supernatant at -20°C until analyzed for HIV p24 antigen by EIA. Replace with 1mL of fresh coculture medium containing $5x10^5$ PHA-stimulated donor cells.

5.2.7 At day 21 remove 1.0mL of medium without disturbing cells. Save supernatant at -20°C until analyzed for HIV p24 antigen by EIA. Replace

with 1mL of fresh coculture medium containing $5x10^5$ PHA-stimulated donor cells.

5.2.8 At day 28 remove 0.5mL of medium without disturbing cells. Save supernatant at -20°C until analyzed for HIV p24 antigen by EIA. Terminate cultures and store remaining supernatants at -70°C.

6. RESULTS / INTERPRETATIONS

6.1 Interpretive Criteria

The criteria for a positive or negative well is based on VQA standardized HIV p24 antigen results obtained from harvested culture samples. A culture well is considered positive if the HIV p24 antigen level is ≥30 pg/mL. Cultures that turn positive at harvest days of 7, 14 or 21, should have successive p24 values that are higher than the first supernatant harvest. A culture well is considered negative if the HIV p24 antigen level is <30 pg/mL at all harvest timepoints.

6.2 Reporting Positive CSF Cultures

If either well (or both wells) of a qualitative micrococulture is positive, the culture is considered positive. Cultures are reported as positive at the first day that the supernatant p24 antigen value equals or exceeds 30 pg/mL. For example, if one or more wells of the culture are positive at day 14 then the culture is reported as “CSF HIV-1 Positive at Day 14”.

6.3 Reporting Negative CSF Cultures

The culture is considered negative if both wells continue to have p24 values <30 pg/mL through 4 weeks of supernatant harvests. These cultures are reported as “CSF Negative for HIV-1 at 28 day”.

7. PROCEDURE NOTES

7.1 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..

7.2 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 Cultures containing large quantities of HIV 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. 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.

7.4 Lymphocyte separation media, coculture medium, and diluents used (Dulbecco's PBS or saline) must be at room temperature to prevent clumping of cells.

7.5 Inspect all 15mL polystyrene and 50mL polypropylene conical centrifuge tubes for cracks prior to use. Loss of cell suspensions will occur when cracked tubes are centrifuged.

7.6 Collection flask on the aspiration system (or waste container if not using an aspiration system) must contain some bleach (i.e. enough to make solution 10% if full).

7.7 Always resuspend the cell pellet in the small quantity of liquid left after aspirating off the supernatant. Trying to resuspend cells in larger volume of liquid, such as that added for washing will result in a suspension of clumped cells. Recovery should be >50% (normally 90%) of retrievable cells. If the Technologist is unfamiliar with lymphocyte isolation procedures, recovery should be not assessed until he or she is satisfactorily recovering >50% of the mononuclear cells. Inadequate recoveries are associated with failure to dilute the blood 1:2 with a buffer prior to layering over lymphocyte separation media, inadequate removal of cells from the plasma/lymphocyte separation media interface, failure to dilute the cells from the interface in Dulbecco's PBS, and inadequate centrifugation during the wash steps.

7.8 Viability of the cells isolated by this method is usually 98%. Viability of cells should be assessed on all specimens by trypan blue exclusion.

7.9 The level of CO_2 should be checked weekly with fyrite to determine % CO_2.

7.10 GLP and excellent sterile technique are very important because the cultures are maintained for up to 4 weeks and must remain free of contamination to give accurate results.

8. QUALITY ASSURANCE

8.1 For each test plate a PBMC control culture will be set up that consists of 2 million PBMCs from the same donor lot as that used for the patient CSF culture. This "negative control culture" will be refed with the same lot and number of cells as the CSF culture at each time point. Supernatant will be tested from the control wells at day 28 only. If a CSF culture tests positive the corresponding control wells should test negative. If the PBMC control wells are positive at final harvest then the CSF results are suspect and should be reported as indeterminate.

8.2 Each donor lot of cells should be held in culture for 28 days and tested for p24 antigen before discarding. If evidence of viral expression by elevated p24 antigen levels is found all cocultures previously performed with that lot of donor cells is suspect and should not be reported. All potentially contaminated cultures should be terminated, new patient samples will need to be resubmitted, and the cell provider must be notified of the finding.

9. REFERENCES

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Procedure: ACTG Qualitative CSF/PBMC Microculture Assay

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