Procedure for Maintenance of A3R5 Cells in Culture

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I. Introduction

The performance of neutralizing antibody assays under properly standardized and optimized conditions requires that the cells are at their log phase of growth and at least 80-90% viable when performing neutralization assays using A3R5 (A3.01/R5.7) cells. This is a derivative of the human lymphoblastoid cell line CEM, that naturally expressed CD4 and CXCR4 [1] and was engineered by Dr. Robert McLinden in Colonel Kim's laboratory of the US Medical HIV Research Program (USMHRP) to express the CCR5 receptor. The cells are maintained in growth medium containing 1.0 mg/ml geneticin (G418) to select for cells containing this receptor.

II. Definitions

GM: Growth Medium

RPMI-1640: Roswell Park Memorial Institute Growth Medium

FBS: Fetal Bovine Serum

HEPES: N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid

DMSO: Dimethyl Sulfoxide

CO_2: Carbon Dioxide

III. Reagents and Materials

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

A3R5.7 Cells
Vendor: NIH AIDS Reagent Program

Complete Growth Medium for A3R5 Assay (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in A3R5 Cells)

Geneticin (G418), 250 mg/ml
Vendor: Gibco BRL Life Technologies
For every 10 ml of Complete GM, add 40 µl of G418

Microliter pipettor tips, sterile
Vendor: Biohit

Trypan Blue (0.4%)
Vendor: Sigma

Disposable serological pipettes, sterile, individually wrapped
Vendor: Fisher

Culture flasks with vented caps, sterile
Vendor: Fisher
T-25 cm^2
T-75 cm^2

70% Ethanol
Vendor: VWR

Microtubes
Vendor: Sarstedt Brand Products

IV. Instrumentation

Biological Safety Cabinet
Manufacturer: Baker Co.

CO_2 Incubator
Manufacturer: Forma Scientific

Water bath
Manufacturer: VWR

Hemacytometer
Manufacturer: INCYTO

> ***NOTE 1****:* An automated cell counting device (e.g., Countess, Manufacturer: Invitrogen) may be used in lieu of a light microscope / hemacytometer for cell counting and viability calculation.

Pipettor
Manufacturer: Biohit
Single channel 5-50 µl
Single channel 40-200 µl

Light Microscope
Manufacturer: Olympus

Liquid Nitrogen Freezer
Manufacturer: MVE, Inc.

4°C Refrigerator
Manufacturer: Sci-Cool

−20°C Freezer
Manufacturer: Sci-Cool

Countess Automatic Cell Counter
Manufacturer: Invitrogen

V. Protocol

1. Thawing and Recovering A3R5 Cells

NOTE 2: All personnel must wear a full-face shield during the handling of frozen specimens.

1.1. Transfer microtube containing frozen cells from liquid nitrogen to a room temperature water bath in the biological safety cabinet.

1.2. If liquid nitrogen has seeped into the microtube, loosen the cap slightly to allow the nitrogen to escape during thawing.

1.3. Hold the microtube on the surface of the water bath with an occasional gentle "flick" during thawing. Thawing only takes a few seconds.

NOTE 3: Cells should be thawed quickly to prevent formation of ice crystals that can cause cell lysis.

1.4. Dry off the outside of the microtube and wipe it with 70% ethanol before opening to prevent contamination.

1.5. Transfer the thawed cells to a T-75 cm^2 culture flask containing 30 ml of Complete GM.

1.6. Record the information from the label of the vial to the T-75 cm^2 flask and add the thaw date.

NOTE 4: It is important to dilute the cryo-protective DMSO present in the microtube at least 30-fold at this point to avoid cell toxicity.

1.7. Incubate the cells at 37°C/5% CO_2 overnight.

1.8. The next day, remove the medium from the T-75 cm^2 flask without disturbing the cells, and add 20-30 ml of fresh, pre-warmed to 37°C, Complete GM containing 1.0 mg/ml of G418.

1.9. Examine the cells via light microscopy, observing the morphology and density to ensure they are healthy and growing as expected (see NOTE 5). If the cells are allowed to become too dense, they will go into stationary phase where their metabolic action decreases.

NOTE 5: The cells should look round and refracting light around their membrane. The medium should have a pink-orange hue. Discard the culture if the cells do not appear to be growing at all.

1.10. After 3 to 4 days, remove the Complete GM and replace with fresh Complete GM. Add G418 to the flask(s) at a final concentration of 1 mg/ml.

1.11. If the cells appear to be growing well, increase the volume of GM or split the cells into two or more flasks at a density of 2×10^5 cells/ml. Examine the culture(s) in 3 days.

NOTE 6: Cells will initially go through a lag phase. Then they will go into an exponential growth phase where they have the highest metabolic activity (log phase).

2. Splitting, Cutting and Maintaining A3R5 Cells in Culture

2.1. Warm the Complete GM to 37°C.

2.2. Label the T-75 cm^2 flask(s) appropriately with the cell line name and date of thawing.

2.3. Carefully remove GM from the flask using a pipette, leaving approximately 1-2 ml of cell suspension.

2.4. Add 10-15 ml of fresh Complete GM, gently mix the cells and pipette about 100 µl into a microtube.

2.5. In a separate vial, pipette 20-50 µl of Trypan Blue (0.4%).

2.6. Gently mix the 100 µl of cell suspension and pipette an equal volume of cell suspension into the vial containing the Trypan Blue (to create a 1:2 dilution). Mix and pipette 10-12 µl of this mixture into a hemacytometer or cell counting chamber and count the viable cells using the appropriate site-specific protocol for cell counting. Dead cells will stain blue and live cells are bright. As an alternative, an automated cell counter with viability check capabilities can be used. Viability should be at least 80%.

2.7. Based on the cell count, seed the cells into a T-75 cm^2 flask(s), as needed (see ***NOTE 7***).

NOTE 7: Split ratios can be used to ensure cells should be ready for an experiment on a particular day or just to keep the cell culture running for future use. Recommended split ratio should be about 2 x 10^5 cells/ml or 1:10 in a total volume of 50 ml of GM if the cells are needed in 3 days. If the cells are needed in less than 3 days, consider the split ratio at about 4 x 10^5 cells per ml, or a 1:5 in a total volume of 50 ml of GM.

NOTE 8: Cells seeded at too low density may be inhibited or delayed in entry into log phase growth. Overcrowded cells will enter stationary phase and start to die off. Split cells when they reach 1.5 x 10^6 cells per ml. Do not allow cells to grow at a density higher than 2.0 x 10^6 cells per ml.

2.8. After the cell count is complete, based on the viable cell count, seed the flask(s) as needed at a ratio of about 2 x 10^5 cells per ml in a total volume of 50 ml of warmed fresh Complete GM. Add 1.0 mg/ml of G418 to each flask. Return the flask(s) to the incubator.

2.9. Observe the cells after three days. Count and split the cells (if needed) to replenish the nutrients and keep the correct pH.

2.10. Thaw a new batch of cells every 3 months. Cells in culture will undergo changes in growth, morphology, and genetic characteristics over time. Such changes can adversely affect reproducibility of laboratory results.

2.11. Cells should be tested for the detection of *Mycoplasma* species at the end of their 3 months in culture (see Protocol for Preparation of Cells for Detection of *Mycoplasma* Species). A3R5 cells should only be cultured for 3 months total.

2.12. A Master Archive Lot and a Master Working Lot should be established to ensure that enough cell supply is available to complete studies (see Protocol for Preparation of Cells for Detection of *Mycoplasma* Species).

VI. References

1. Folks, T., Benn, S., Rabson, A., Theodore T., Hoggan, M.D., Martin, M., Lightfoote, M., and Sell, K. (1985) Characterization of a continuous T-cell susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. Proc. Natl. Acad. Sci. (USA) 82: 4539-4543.

2. Sarzotti-Kelsoe M, Daniell X, Todd CA, Bilska M, Martelli A, LaBranche C, Perez LG, Ochsenbauer C, Kappes JC, Rountree W, Denny TN, Montefiori DC. Optimization and validation of a neutralizing antibody assay for HIV-1 in A3R5 cells. J Immunol Methods. 2014 Jul; 409: 147-60. doi: 0.1016/j.jim.2014.02.013. Epub 2014 Mar 6.

3. McLinden RJ, LaBranche C, Chenine AL, Polonis VR, Eller MA, Wieczorek L, Ochsenbauer C, Kappes JC, Perfetto S, Montefiori DC, Michael NL, Kim JH. Detection of HIV-1 neutralizing antibodies in a human $CD4^{+}/CXCR4^{+}/CCR5^{+}$ T-lymphoblastoid cell assay system. PLos One. 2013 Nov 28; 8(11):e77756. doi: 10.1371/journal.pone.0077756. eCollection 2013.