PROTOCOL FOR CELL CULTURE, SUB-CULTURE AND CRYOPRESERVATION OF HUMAN NEURAL LINEAGES DERIVED FROM MESENCHYMAL STEM CELLS MANUFACTURED BY VITRO BIOPHARMA

Establishing cultures from cryopreserved cells: Use of these cell lines requires prior experience in standard methods of mammalian cell culture. In particular, sterile technique is required in a dedicated cell culture facility that is free from contamination.

Cryopreserved cells may be used to establish cultures immediately upon receipt or they may be stored for use at a later time. If stored, it is preferable to store in the vapor phase of liquid N_2. Storage in a –80°C freezer may be used but is likely to result in diminished cell viability proportional to storage time.

To establish cultures from cryopreserved cells, first ensure that adequate equipment and reagents are available to perform the necessary procedures in a timely manner. These cells require culture in a 37°C, CO_2 cell culture incubator, calibrated to 5% CO_2. However, these cells can be cultured at ambient oxygen levels (~20% O_2) which results in reduced growth rates. Please contact technical services for information about various equipment and instrumentation options available to establish reduced oxygen cultures. Also, a water bath equilibrated to 37°C is needed. Required reagents include 1 x PBS, Fisher Catalog number BP665-1 or equivalent at room temperature or 37°C and Neural culture medium. We provide **MSC-Gro™ media** for optimal proliferation of these neural cells. Our growth media is provided as Alpha Motor Neuron Maintaining media, Dopaminergic Neuron Maintaining media and Neural Stem Cell growth media. (Catalog numbers PC00B7, PC00B8 and NSCB1). Data that is presented on our website (www.vitrobiopharma.com) shows positivity ICC staining of neural lineages.

To establish cultures from frozen cell stock, it is first necessary to rapidly thaw cells at 37°C. Remove the desired number of vials containing cells from liquid nitrogen.

Exposure of closed vials containing liquid nitrogen to a 37°C water bath is an **explosion hazard**. Please follow your institutions guidelines for safe handling of cryogenically preserved cells. Provide continuous agitation, e.g., swirling, to the vial while it is submerged in the 37°C water bath. Continue with agitation until the cells are completely thawed and no ice remains within the cell suspension, usually about 1 to 2 minutes. **Maximum cell viability is dependent on rapid and complete thawing of frozen cells.**

Count cells by a suitable method including a hemacytometer or automated cell counting device and determine the concentration of cells within the cell suspension. Our products are provided in 0.5 ml (500 µl) of cryopreservation medium at a nominal concentration of 1 x 10^6 cells/ml. We

recommend direct inoculation of cultures from the cell-cryopreservation media suspension. (Washout of the cryopreservative has been shown to decrease viability.)

We suggest establishing the initial passage culture at a plating density of about 15,000 to 20,000 cells/cm^2 in suitable tissue culture dishes or flasks. Please note that extracellular matrices are important in culture of all cells, especially attachment dependent cells. Neural cells including neural stem cells and differentiated neural lineages require laminin or fibronectin coated flasks. We recommended using Corning, Biocoat Laminin Cellware Catalog Number 354533. Add the appropriate volume of MSC culture medium to the plate or flask to be used for culture. We typically add 10 ml of medium per T-25. Use these guidelines to determine the appropriate volume of medium for your application. Following inoculation with the appropriate volume of cell suspension, gently agitate the flask or plate to ensure homogeneous distribution of the MSCs with the cell culture medium. Allow cultures to incubate in 5% CO_2 in ambient or reduced O_2 as noted above at 37^{o}C in a humidified environment. Monitor cell growth by visual inspection. When these cultures are 80% to 90% confluent, split and subculture the cells as described in the next section. This should require about 7 to 10 days of continuous culture, but this time depends on several factors. Thus the cultures should be monitored by inspection with an inverted microscope with appropriate magnification e.g., 100x. Once cells become attached, within 3 days, change the media every other day until desired confluency is achieved.

Subculture Procedures: Wash each flask 2-3 times with PBS (e.g., 5 ml per T-25) and then add Accutase™ (Innovative Cell Technologies, Catalog number AT104 (4 mls/T-25 or 8 mls/T-75) and incubate at 37^{o}C with gentle agitation for 15 minutes. (Alternatively, trypsin may be used instead of Accutase.) Visualize the culture. If necessary, assure complete detachment of cells as by rapping the flask or plate firmly on a solid surface. Transfer the dissociated cells to a centrifuge tube and combine this with a PBS wash of the flask (5ml/T-25; 10 ml/T75) followed by a second smaller volume wash. Centrifuge for 5-7 minutes at 450 x g and pour off or aspirate the supernatant. Resuspend the pelleted cells in 1 ml PBS by repetitive elutriation. Count the cells using an automated cell counter or hemacytometer. For automated counters, count in the size range 10 to 30 μm. Inoculate the cells at 15,000 to 20,000 cells/cm^2 for routine passage. For optimal viability, complete the subculture process within 2 hours or less of dissociation. Fully adapted MSCs typically require about 7 to 10 days to reach about 90% confluence, although this is dependent on several different factors. We recommend feeding every other day. For longer or shorter periods between subculture, cultures may be inoculated at lower or higher densities. Subculture at lower or higher plating densities may also be used depending on the application. Our suggested procedures are provided as guidelines and may require adjustments within different laboratory environments.

Freeze-down Procedures: Obtain an accurate count of the number of cells to be cryopreserved. Centrifuge these cells and aspirate the supernatant. Suspend the cells at the desired concentration, e.g., 2 million/ml, in cryopreservative medium. (10% DMSO is a suitable cryopreservative for these cells.) Transfer into appropriate cryopreservation vials that are rated for use in liquid nitrogen. Incubate the cells at room temperature for 30 minutes prior to freezing.

Freeze the cell suspension at a slow rate, approximately 1°C/minute to ~ -80°C. After complete freezing, transfer vials of cells to liquid N_2-containing Dewar flask preferably in the vapor phase for long-term storage at maximum viability.

Technical Service: Please contact Vitro Biopharma technical services at (303) 999-2130 x 4 for additional technical assistance.