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

Applicable Products: SC00A1, SC00A2, SC00A3, SC00A4 and SC00A5.

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. Mesenchymal stem cells exist in hypoxic environments within the body and we have found accelerated growth rates under reduced oxygen conditions (1 to 5% O_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 MSC culture medium. We provide MSC media for optimal self-renewal of these MSC cells. Our growth media is provided as low serum, humanized and serum-free. (Catalog numbers SC00B1, SC00B2 and SC00B3). Other commercially available MSC may be used.

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! It is essential to ensure that no liquid nitrogen is present in the vial! If liquid nitrogen is present in the vial, allow this to evaporate before proceeding. Please use proper precautions including appropriate gloves to protect skin from exposure to liquid nitrogen, eye protection and other personal protective equipment when transferring vials containing cryopreserved cells from liquid nitrogen to a 37°C water bath.

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. 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 7,500 to 10,000 cells/cm^2 in suitable tissue culture dishes or flasks. 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°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 3 to 4 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.

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°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

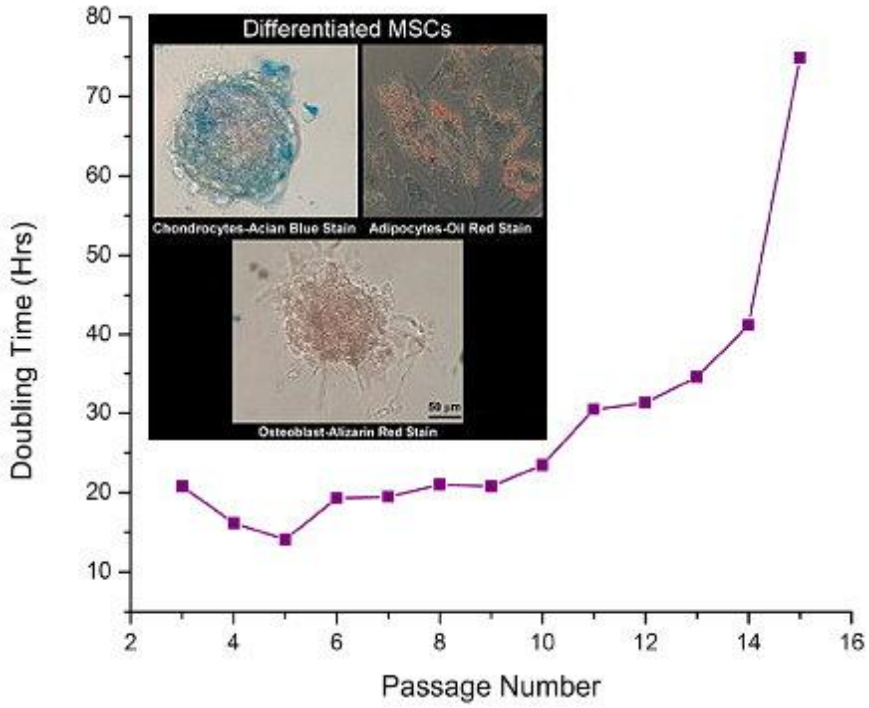
Neuromics Antibodies • 5325 West 74th Street, Suite 8 • Edina, MN 55439
phone 866-350-1500 • fax 612-677-3976 • e-mail pshuster@neuromics.com

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 □m. Inoculate the cells at 4,000 to 5,000 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 4 to 5 days to reach about 90% confluence, although this is dependent on several different factors. We recommend feeding every three days. 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., 1 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.

Images: MSCs (Catalog #: SC00A1) Growth in MSCGro™, Low Serum Medium (Catalog#: SC00B1). Inset: MSCs differentiatiated using MSCGro Differentiation Media.



FOR RESEARCH USE ONLY

Neuromics Antibodies • 5325 West 74th Street, Suite 8 • Edina, MN 55439
phone 866-350-1500 • fax 612-677-3976 • e-mail pshuster@neuromics.com