CELL
APPLICATIONS, INC.

General Instructions for Culturing Human Mesenchymal Stem Cells (HMSC-AD)

Be sure to wear face protection mask and gloves when retrieving cryovials from the liquid nitrogen storage tank. The dramatic temperature change from the tank to the room could cause any trapped liquid nitrogen in the cryovials to burst and cause injury.

Open all the packages immediately upon arrival and examine each component for shipping damage. Notify Cell Applications, Inc. or your distributor immediately if there is any problem.

I. STORAGE

A. CRYOPRESERVED VIALS (492Ad-05a)

Store the cryovials in a liquid nitrogen storage tank immediately upon arrival.

B. PROLIFERATING FLASKS (493Ad-25, -75)

1. Examine under a microscope to check if all the cells are attached to the bottom of the flask. If not, notify CAl or your distributor immediately.
2. Decontaminate the exterior of the cell culture flask with 70% alcohol.
3. Place the sealed flask in a 37°C, 5% C02 humidified incubator for 2 hours as shipped.
4. In a sterile Biological Safety Cabinet, open the cap of the flask very slowly and carefully.
5. Remove the Transport Medium by aspiration. Add fresh Growth Medium: 5 ml for a T-25 flask and 15 ml for a T-75 flask.
6. Place the flask in a 37°C, 5% C02 humidified incubator with loosened cap to allow gas exchange.
7. Change medium every other day.

C. GROWTH MEDIUM (419-500)

Store at 4°C in the dark immediately upon arrival.

D. SUBCULTURE REAGENT KIT (090K)

Store at -20°C immediately upon arrival. Store at 4°C after thawing.

E. OSTEOBLAST DIFFERENTIATION MEDIUM (417D-250)

Store at 4°C in the dark immediately upon arrival.

F. ADIPOCYTE DIFFERENTIATION MEDIUM (81ID-250)

Store at 4°C in the dark immediately upon arrival.

II. PREPARATION FOR CULTURING

1. Make sure the Class II Biological Safety Cabinet, with HEPA filtered laminar airflow, is in proper working condition.
2. Clean the Biological Safety Cabinet with 70% alcohol to ensure it is sterile.
3. Turn the Biological Safety Cabinet blower on for 10 min. before cell culture work.
4. Make sure all serological pipettes, pipette tips and reagent solutions are sterile.
5. Follow the standard sterilization technique and safety rules:
   a. Do not pipette with mouth.
   b. Always wear gloves and safety glasses when working with human cells even though all the strains have been tested negative for HIV, Hepatitis B and Hepatitis C.
   c. Handle all cell culture work in a sterile hood.

III. CULTURING HMSC-AD

A. PREPARING CELL CULTURE FLASKS FOR CULTURING HMSC-AD

1. Take the Human Mesenchymal Stem Cell Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Pipette 15 ml of Human Marrow Stromal Cell Growth Medium* into a T-75 flask.

***** Use Corning and Grenier flasks for best results.

* Keep the medium to surface area ratio at 1 ml per 5 cm2.
For example,
5-7.5 ml for a T-25 flask or a 60 mm tissue culture dish.
15-20 ml for a T-75 flask or a 100 mm tissue culture dish.

B. THAWING AND PLATING HMSC-AD

1. Remove the cryopreserved vial of HMSC-AD from the liquid nitrogen storage tank using proper protection for your eyes and hands.
2. Turn the vial cap a quarter turn to release any liquid nitrogen that may be trapped in the threads, then re-tighten the cap.
3. Thaw the cells quickly by placing the lower half of the vial in a 37°C water bath for 1 minute.
4. Take the vial out of the water bath and wipe dry.
5. Decontaminate the vial exterior with 70% alcohol in a sterile Biological Safety Cabinet.
6. Remove the vial cap carefully. Do not touch the rim of the rim of the vial.
7. Resuspend the cells in the vial by gently pipetting the cells 5 times with a 2 ml pipette. Be careful not to pipette too vigorously as to cause foaming.
8. Pipette the cell suspension (1ml) from the vial into the T-75 flask containing 15 ml of Human Mesenchymal Stem Cell Growth Medium.
9. Cap the flask and rock gently to evenly distribute the cells.

10. Place the T-75 flask in a 37°C, 5% CO2 humidified incubator.
11. Loosen the cap to allow gas exchange. For best results, do not disturb the culture for 24 hours after inoculation.
12. Change to fresh Human Mesenchymal Stem Cell Growth Medium after 24 hours or overnight to remove all traces of DMSO.
13. Change Human Mesenchymal Stem Cell Growth Medium every other day until the cells reach 60% confluent.
14. Double the Human Marrow Stromal Cell Growth Medium volume when the culture is >60% confluent or for weekend feedings.
15. Subculture the cells when the HMSC-AD culture reaches 85-95% confluent.

IV. SUBCULTURING HMSC-AD

A. PREPARING SUBCULTURE REAGENTS

1. Remove the Subculture Reagent Kit from the -20°C freezer and thaw overnight in a refrigerator.
2. Make sure all the subculture reagents are thawed. Swirl each bottle gently several times to form homogeneous solutions.
3. Store all the subculture reagents at 4°C for future use. The activity of Trypsin/EDTA Solution will be stable for 2 weeks when stored at 4°C.
4. Aliquot Trypsin/EDTA solution and store the unused portion at -20°C if only portion of the Trypsin/EDTA is needed.

B. PREPARING CULTURE FLASK

1. Take the Human Mesenchymal Stem Cell Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Pipette 35 ml of Human Marrow Stromal Cell Growth Medium to a T-75 flask (to be used in Section IV C Step 15)

C. SUBCULTURING HMSC-AD

Trypsinize Cells at Room Temperature. Do Not Warm Any Reagents to 37°C.

1. Remove the medium from culture flasks by aspiration.
2. Wash the monolayer of cells with HBSS and remove the solution by aspiration.
3. Pipette 5 ml of Trypsin/EDTA Solution into the T-75 flask.
4. Rock the flask gently to ensure the solution covers all the cells.
5. Remove 4 ml of the solution immediately.
6. Re-cap the flask tightly and monitor the trypsinization progress at room temperature under an inverted microscope. It usually takes about 2 to 5 minutes for the cells to become rounded. The cells may not be completely round during trypsinization and some cells may maintain some processes even though they are loosened from the culture surface.
7. Release the rounded cells from the culture surface by hitting the side of the flask against your palm until most of the cells are detached.
8. Pipette 5 ml of Trypsin Neutralizing Solution to the flask to inhibit further tryptic activity.
9. Transfer the cell suspension from the flask to a 50 ml sterile conical tube.
10. Rinse the flask with an additional 5 ml of Trypsin Neutralizing
11. Solution and transfer the solution into the same conical tube.
12. Examine the T-75 flask under a microscope. If there are >20% cells left in the flask, repeat Steps 2-9.
13. Centrifuge the conical tube at 220 x g for 5 minutes to pellet the cells.
14. Aspirate the supernatant from the tube without disturbing the cell pellet.
15. Flick the tip of the conical tube with your finger to loosen the cell pellet.
16. Resuspend the cells in 5 ml of Human Mesenchymal Stem Cell Growth Medium by gently pipetting the cells to break up the clumps.
17. Count the cells with a hemocytometer or cell counter.
18. Inoculate at 10,000 cells per cm2 for rapid growth, or at 5,000 cells per cm2 for regular subculturing.

V. DIFFERENTIATING HMSC-AD

A. SEEDING HMSC-AD FOR DIFFERENTIATION INTO OSTEOBLASTS

1. Seed HMSC-AD in the desired format at 10,000 per cm2.
2. Place the cells 37°C. 5% C02 humidified incubator.
3. Change to Osteoblast Differentiation Medium the next day by removing the growth medium from culture tissue ware by aspiration and adding the appropriate volume of Osteoblast Differentiation Medium. Do not allow cells to dry during medium changes.
4. Incubate cell in a 37°C, 5% CO2 humidified incubator.
5. Change to fresh Osteoblast Differentiation Medium every three (3) days.
6. Extracellular matrix will be mineralized in 30 days.

B. SEEDING HMSC-AD FOR DIFFERENTIATION INTO ADIPOCYTES

1. Seed the HMSC-AD at 40,000 cells/cm2 in the desired format.
2. Place the cells in a 37°C, 5% CO2 humidified incubator.
3. Take out the appropriate amount of Human Adipocyte Differentiation Medium and equilibrate in the incubator for 2 hours prior to use in Step 4.
4. Change to Human Adipocyte Differentiation Medium after three days by removing growth medium from culture tissue ware by aspiration and adding the appropriate volume of pre- equilibrated Human Adipocyte Differentiation Medium in Step 3. Do not allow cells to dry during medium changes.
5. Incubate cells in a 37°C, 5% CO2 humidified incubator.
6. Change to fresh Human Adipocyte Differentiation Medium every three (3) days.
7. Cells are differentiated into adipocytes with large lipid droplets in 3-4 weeks.

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C. SEEDING HMSC-AD FOR DIFFERENTIATION INTO CARTILAGE-BUILDING CHONDROCYTES

1. Expand the HMSC-AD until 1-2.5 x 10^7 cells is reached (roughly three T175 flasks).
2. Take out the appropriate amount of Human Chondrocyte Cartilage-Building Differentiation Medium and allow to come to room temperature.
3. Harvest HMSC-AD and count cells.
4. Determine amount of alginate required: Use 32 µL of 1.5% alginate for each 1 x 10^6 cells (e.g. 10 x 10^6 cells = 320 µL alginate).
5. Rinse cells once with PBS and suspend in 1.5% alginate (see directions in CAI Cartilage-Building Kit).
6. Carefully mix cells with alginate until a uniform homogenate is achieved. Take care to avoid bubbles.
7. Load the homogenate into a syringe and attach a 27-gauge needle.
8. Form microbeads by positioning syringe ~10 cm above 100 mL of a rapidly stirring 100 mM $CaCl_2$ solution.
9. Quickly expel homogenate into 100 mM $CaCl_2$ solution.
10. Allow microbeads to settle and remove as much $CaCl_2$ solution as possible.
11. Transfer microbeads to 50 mL conical tube and rinse twice with 150 mM sterile NaCl.
12. Rinse microbeads once with Cartilage-Building Differentiation Medium and transfer to appropriate culture ware.
13. Incubate cells in in a 37°C, 5% CO2 humidified incubator.
14. Change to fresh Human Chondrocyte Cartilage-Building Differentiation Medium every three to four (3-4) days.
15. Cells are differentiated with cartilage in three weeks.