

General Instructions for Culturing

Rat Mesenchymal Stem Cells (RMSC)

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 (R492-05a)

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

B. PROLIFERATING FLASKS (R493-25a, -75a)

1. Examine under a microscope to check if all the cells are attached to the bottom of the flask. If not, notify CAI 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% CO₂ 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% CO₂ humidified incubator with loosened cap to allow gas exchange.
7. Change medium every other day.

C. GROWTH MEDIUM (R419-500)

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 (R417D-250)

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

F. ADIPOCYTE DIFFERENTIATION MEDIUM (R811D-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 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 RMSC

A. PREPARING CELL CULTURE FLASKS FOR CULTURING RMSC

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

* 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 RMSC

1. Remove the cryopreserved vial of RMSC 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 and watch the vial closely during the thawing process.
4. Take the vial out of the water bath when only small amount of ice left in the vial. Do not let cells thaw completely.
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 cap or 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 Rat 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. Loosen the cap to allow gas exchange. For best results, do not disturb the culture for 24 hours after inoculation.
11. Change to fresh Rat Mesenchymal Stem Cell Growth Medium after 24 hours or overnight to remove all traces of DMSO.

12. Change Rat Mesenchymal Stem Cell Growth Medium every other day until the cells reach 60% confluent.
13. Double the Rat Mesenchymal Stem Cell Growth Medium volume when the culture is >60% confluent or for weekend feedings.
14. Subculture the cells when the RMSC culture reaches 85-95% confluent.

IV. SUBCULTURING RMSC

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 Rat Mesenchymal Stem Cell Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Pipette 35 ml of Rat Mesenchymal Stem Cell Growth Medium to a T-175 flask (to be used in Section IV C Step 15).

C. SUBCULTURING RMSC

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. Rock the flask gently to ensure the solution covers all the cells.
4. Remove 4 ml of the solution immediately.
5. 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.
6. 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.
7. Pipette 5 ml of Trypsin Neutralizing Solution to the flask to inhibit further tryptic activity.
8. Transfer the cell suspension from the flask to a 50 ml sterile conical tube.
9. Rinse the flask with an additional 5 ml of Trypsin Neutralizing Solution and transfer the solution into the same conical tube.
10. Examine the T-75 flask under a microscope. If there are >20% cells left in the flask, repeat Steps 2-9.
11. Centrifuge the conical tube at 220 x g for 5 minutes to pellet the cells.
12. Aspirate the supernatant from the tube without disturbing the cell pellet.
13. Flick the tip of the conical tube with your finger to loosen the cell pellet.
14. Resuspend the cells in 5 ml of Rat Mesenchymal Stem Cell Growth Medium by gently pipetting the cells to break up the

clumps.

15. Count the cells with a hemocytometer or cell counter. Inoculate at 10,000 cells per cm² for rapid growth, or at 5,000 cells per cm² for regular subculturing.

V. DIFFERENTIATING RMSC

A. SEEDING RMSC FOR DIFFERENTIATING INTO OSTEOBLAST

1. Seed RMSC in the desired format at 10,000 per cm².
2. Place the cells 37°C, 5% CO₂ humidified incubator.
3. Change to Rat Osteoblast Differentiation Medium the next day by removing the growth medium from culture tissue ware by aspiration and adding the appropriate volume of Rat 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 Rat Osteoblast Differentiation Medium every three (3) days.
6. Extracellular matrix will be mineralized in 30 days.

B. SEEDING RMSC FOR DIFFERENTIATING INTO ADIPOCYTE

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