CELL
APPLICATIONS, INC.

General Instructions for Culturing

Rat Brain Microvascular Endothelial Cells (RBMVEC)

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 (R840-05a)

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

B. PROLIFERATING FLASKS (R841-25a, -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% CO_2 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% CO_2 humidified incubator with loosened cap to allow gas exchange.
7. Change medium every other day.

C. GROWTH MEDIUM (R819-500)

Store the Growth Medium 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. ATTACHMENT FACTOR SOLUTION (123-100)

Store at 4°C temperature.

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 protective lab gear (lab coat, gloves, safety glasses, etc.) when working with cell cultures.
   c. Handle all cell culture work in a sterile hood.

III. CULTURING RBMVEC

A. PREPARING CELL CULTURE FLASK

1. Swirl the Attachment Factor Solution bottle a few times to form a homogenous solution.
2. Decontaminate the bottle with 70% alcohol in a sterile hood.
3. Coat 1 T-75 flasks by adding 7.5 ml of Attachment Factor Solution* and rock the flasks gently to distribute the solution evenly to cover the whole culture surface.
4. Coat the culture ware for 30 minutes at 37°C or 2 hours (overnight is OK) at room temperature.
5. Remove the Attachment Factor Solution by aspiration in a sterile hood.
6. The coated flask can be used immediately or stored at 4°C for up to 2 weeks.
7. Prepare the coated flask for culturing RBMVEC by pipetting 15 ml of Growth Medium** into this coated T-75 flask and wait for seeding.

*The coating concentration is 1 ml per 10cm^2 surface area of culture ware.
2.5 ml for coating a T-25 flask or a 60mm culture dish
7.5 ml for coating a T-75 flask or a 100mm culture dish

**Keep the medium to surface area ration at 1-1.5ml per 5 cm^2. For example,
5 ml for a T-25 flask or a 60 mm tissue culture dish.
15 ml for a T-75 flask or a 100 mm tissue culture dish

B. THAWING AND PLATING RBMVEC

1. Remove the cryopreserved vial of RBMVEC 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 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 coated T-75 flask containing 15 ml of Rat Brain Microvascular Endothelial Cell Growth Medium.
9. Cap the flask and rock gently to evenly distribute the cells.
10. Place the T-75 flask in a 37ºC, 5% CO_2 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 Brain Microvascular Endothelial Cell Growth Medium after 24 hours or overnight to remove all traces of DMSO.
12. Change Rat Brain Microvascular Endothelial Cell Growth Medium every other day until the cells reach 60% confluent.
13. Double the Rat Brain Microvascular Endothelial Cell Growth Medium volume when the culture is >60% confluent or for weekend feedings.
14. Subculture the cells when the RBMVEC reach 80% confluent.

IV. SUBCULTURING RBMVEC

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.

B. PREPARING CULTURE FLASK

Prepare as in Step III A.

C. SUBCULTURING RBMVEC

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 4.5 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 1 minute for the cells to become rounded but still attached to the flask. (When rounded cells detach by itself without hitting, it means the cells are over trypsinized.)
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 Brain Microvascular Endothelial 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 cm^2 for regular subculturing.

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