CELL
APPLICATIONS, INC.

General Instructions for Culturing

HiPSC-Derived Human Alveolar Epithelial Cells, Type 2 (AEC2/i-HAEpC2)

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 (i536-05)

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

B. GROWTH MEDIUM (519-100)

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

C. COATING MEDIUM (1020-10)

Store the Coating medium at -20°C in the dark immediately upon arrival and should be kept at 4°C after thawing and used within 4 weeks.

D. DISSOCIATION SOLUTION (077-10)

Store at 4°C

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 AEC2/i-HAEpC2

A. PREPARING CELL CULTURE PLATES FOR CULTURING AEC2/ i-HAEpC2

1. Take the AEC2/i-HAEpC2 Coating Medium and the Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Add recommended volume of the Coating Medium to the respective wells of the culture ware **as per the table**.
3. Incubate coated plate at 37°C incubator for 1h.
4. After 1h, take off the Coating Medium completely and add the Growth medium **as per the table (day 1)**.

B. THAWING AND PLATING AEC2/i-HAEpC2
**** Starting seeding density: 25000 cells/cm^2**

1. Remove the cryopreserved vial of AEC2/i-HAEpC2 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 2 times with a 1 ml pipette tip. Be careful not to pipette too vigorously as to cause foaming.
8. Pipette the cell suspension from the vial into the wells of the plates containing the Growth Medium **as per the table**.
9. Rock the plate gently to evenly distribute the cells.
10. Place the plate in a 37ºC, 5% CO_2 humidified incubator. For best results, do not disturb the culture for 24 hours after inoculation.
11. Change to fresh Growth Medium after 24 hours to remove all traces of DMSO **as per the table (day 2)**.
12. Change Growth Medium every other day until the cells become 80% confluent.
13. Subculture the cells when the AEC2/i-HAEpC2 reach 80% confluent.

IV. SUBCULTURING AEC2/i-HAEpC2

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 Dissociation Solution will be stable for 2 months when stored at 4°C.

B. PREPARING CULTURE PLATES

1. Take the Coating Medium and the Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Prepare the culture plate of choice as described in Section III and as per the table.

C. SUBCULTURING AEC2/i-HAEpC2

Dissociate Cells at Room Temperature. Do Not Warm Any Reagent to 37°C.

1. Remove the medium from culture plates by aspiration.
2. Wash the monolayer of cells with PBS without Ca++, Mg++ and remove the solution by aspiration.
3. Pipette recommended volume of Dissociation Solution into the wells **as per the table**. Rock the plate gently to ensure the solution covers all the cells.
4. Monitor the dissociation progress at room temperature under an inverted microscope. Do this for 60-90 seconds.
5. Aspirate Dissociation solution.
6. Place the plate in a 37ºC incubator for 90 seconds.
7. Release the rounded cells from the culture surface by hitting the side of the plate against your palm until most of the cells are detached.
8. If the cells are not visually detached place the plate into the incubator for an additional thirty seconds to 1 minute, then repeat step 7.
9. Pipette recommended volume of AEC2/i-HAEpC2 Growth Medium **as per the table** to the plate to inhibit further dissociation activity.
10. Transfer the cell suspension from the plate to a 50 ml sterile conical tube.
11. Rinse the plate with PBS and transfer the solution into the same conical tube.
12. Examine the plate under a microscope. If there are >20% cells left in the plate, 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 minimum volume of Growth Medium by gently pipetting the cells to break up the clumps.
17. Count the cells with a hemocytometer or cell counter. Inoculate at 25000 cells per cm^2.

Table for cell seeding

TC Well Plate	Volume per Well			
	Coating Medium	Cell suspension from the original vial	Growth Medium (day 1)	Growth Medium (day 2)
6	1 ml	500 ul	5 ml	3 ml
12	0.5 ml	200 ul	2 ml	1.5 ml
24	0.25 ml	100 ul	1 ml	1 ml

Table for subculture

TC Well Plate	Volume per Well			
	Coating Medium	Growth Medium	Dissociation Solution	Neutralizing Solution
6	1 ml	3 ml	1 ml	1 ml
12	0.5 ml	1.5 ml	0.5 ml	0.5 ml
24	0.25 ml	1 ml	0.25 ml	0.25 ml

5820 Oberlin Drive, Suite 101
San Diego, CA 92121
Tel: (858) 453-0848 Toll-Free: (800) 645-0848 Fax: (858) 453-2862
Email: info@cellapplications.com Web: www.cellapplications.com