CELL
APPLICATIONS, INC.

General Instructions for Culturing

Human Preadipocytes (HPAd)

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 (802s-05a, 802h-05a, S802s-05a)

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

B. PROLIFERATING CELLS (803s-25, -75, 803h-25, -75, 803s-6w,-12w,-24w,-48w,-96w, 803h-6w,-12w,-24w,-48w,-96w)

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 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 Medium by aspiration. Add fresh Growth Medium; 5ml for a T-25 flask and 15ml 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. PREADIPOCYTE GROWTH MEDIUM (811-500)

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

D. ADIPOCYTE DIFFERENTIATION MEDIUM (811D-250)

E. SUBCULTURE REAGENT KIT (090K)

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

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 HPAd

1. Remove the cryopreserved vial of HPAd 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.

IV. PLATING PREADIPOCYTES

A. PREPARING CELL CULTURE FLASKS FOR CULTURING HSkMC

1. Take the Preadipocyte Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Pipette 15 ml of Preadipocyte 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 HPAd

1. Remove the cryopreserved vial of HPAd 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 Preadipocyte Growth Medium.
9. Cap the flask and rock gently to evenly distribute the cells.
10. Place the T-75 flask in a 37ºC, 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 Preadipocyte Growth Medium after 24 hours or overnight to remove all traces of DMSO.
12. Change Preadipocyte Growth Medium every other day until the cells reach 60% confluent.
13. Double the Preadipocyte Growth Medium volume when the culture is >60% confluent or for weekend feedings.
14. Subculture the cells when the HPAd culture reaches 85-95% confluent.

V. SUBCULTURING HPAd

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 Preadipocyte Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Pipette 35ml of Preadipocyte Growth Medium to a T-175 flask (to be used in Section IV C Step 14.)

C. SUBCULTURING HPAd

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 6 ml of Trypsin/EDTA Solution into the T-75 flask. Rock the flask gently to ensure the solution covers all the cells.
4. Remove 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 to 3 minutes to loosen the cells. The cells may not become completely round during the 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. Resuspend the cells in 5 ml of Preadipocyte Growth Medium by gently pipetting the cells to break up the clumps.
14. Count the cells with a hemocytometer or cell counter. Inoculate at 15,000 cells per cm^2 for rapid growth, or at 10,000 cells per cm^2 for regular subculturing.

VI. DIFFERENTIATING HPAd

A. SEEDING HPAd FOR DIFFERENTIATION

1. Adjust the volume of Preadipocyte Growth Medium to bring the cell/ml to the desired cell density according to the following chart:

Culture Format	Surface Area/Well	Cell Density cell/ml	Volume per well	# Cells per well	Volume per Format
6 well plate	9.40 cm^2	110,000	4 ml	440,000	24 ml
12 well plate	3.83 cm^2	90,000	2 ml	180,000	24 ml
24 well plate	1.88 cm^2	90,000	1 ml	90,000	24 ml
48 well plate	0.86 cm^2	84,000	500 µl	42,000	24 ml
96 well plate	0.32 cm^2	110,000	150 µl	16,500	14.4 ml
T-25 flask	25 cm^2	240,000	5 ml	1,200,000	5 ml
T-75 flask	75 cm^2	240,000	15 ml	3,600,000	15 ml

2. Seed the Preadipocytes at 44,000 cells/cm^2 in the desired format for differentiation.
3. Place the cells in a 37°C, 5% CO2 humidified incubator.
4. Start Differentiation the when culture is 100% confluent and cells are packed. Usually it takes 1 to 2 days to reach total concluency.

B. PREPARING DIFFERENTIATION MEDIUM

1. Remove Adipocyte Differentiation Medium from the refrigerator, and decontaminate the bottle with 70% alcohol in a sterile hood.
2. Aliquot the needed amount of Adipocyte Differentiation Medium, loosen the cap and equilibrate in 37°C, 5% CO2 humidified incubator for 2 hours to allow gas exchange and medium warm up to 37°C.

C. DIFFERENTIATING HPAd TO HAd

Do Not Let Cells Dry During The Medium Changes.
HPAd must be 100% confluent and cells are packed prior to starting differentiation

1. Remove growth medium from culture flask by aspiration.
2. Add the appropriate volume of Adipocyte Differentiation Medium according to the chart in Section VI A Step 1.
3. Incubate cells in a 37°C, 5% CO2 humidified incubator in the Adipocyte Differentiation Medium.
4. Change to fresh Adipocyte Differentiation Medium every 3 days for 15 days.
5. At the end of 15 days, cells are differentiated into HAd with lipid droplets in the cells.
6. Remove Adipocyte Differentiation Medium and starve the cells in Adipocyte Starving Medium for 1 day prior to assay.

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