CELL
APPLICATIONS, INC.

General Instructions for Culturing

HiPSC-Derived Human Hepatic Stellate Cells (i-HHSC)

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 (i782-05)

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

B. PROLIFERATING FLASKS (i783)

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% 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 (i7117-500)

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

D. QUIESCENT MEDIUM (i7121K-100)

Store the Basal Medium at 4°C in the dark immediately upon arrival.
Store i7121-S1, i7121-S2, and i7121-S3 at -20°C immediately upon arrival.
Store i7121-S4 at -80°C immediately upon arrival.

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

III. CULTURING i-HHSC

A. THAWING AND PLATING i-HHSC

1. Take the Hepatic Stellate Cell Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile biological safety cabinet.
2. Pipette 9 ml of Hepatic Stellate Cell Growth Medium to a 15 mL conical tube.
3. Remove the cryopreserved vial of i-HHSC from the liquid nitrogen storage tank using proper protection for your eyes and hands.
4. Turn the vial cap a quarter turn to release any liquid nitrogen that may be trapped in the threads, then re-tighten the cap.
5. Thaw the cells quickly by placing the lower half of the vial in a 37°C water bath for approximately 90 seconds.
6. Take the vial out of the water bath when only small amount of ice left in the vial. Do not let cells thaw completely.
7. Decontaminate the vial exterior with 70% alcohol in a sterile biological safety cabinet.
8. Remove the vial cap carefully. Do not touch the rim of the cap or the vial.
9. Resuspend the cells in the vial by gently pipetting the cells two times with pre-wetted 1 mL aerosol tip set at 950 uL. Do not pipette vigorously and cause foaming.

10. Pipette the cell suspension (1ml) from the vial to the 15 ml sterile conical tube containing 9 ml of Hepatic Stellate Cell Growth Medium.
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 Hepatic Stellate Cell Growth Medium by gently pipetting the cells to break up the pellet.
15. Transfer the cell suspension from the tube into a T-25 flask to seed the cells at 20,000 cells/cm2.
16. Cap the flask and rock gently to evenly distribute the cells.
17. Place the T-25 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.
18. Change Hepatic Stellate Cell Growth Medium every other day.
19. Subculture the cells when the i-HHSC reach 90% confluent.

IV. SUBCULTURING i-HHSC

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 Hepatic Stellate Cell Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile biological safety cabinet.
2. Pipette 30ml of Hepatic Stellate Cell Growth Medium to a T-175 flask (to be used in Section IV C Step 15.)

C. SUBCULTURING i-HHSC

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 PBS and remove the solution by aspiration.
3. Pipette 2 ml of Trypsin/EDTA Solution into the T-25 flask. Rock the flask gently to ensure the solution covers all the cells.
4. Remove 1.5 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 2 to 4 minutes for the cells to become rounded.
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 1.5 ml of Trypsin Neutralizing Solution to the flask to inhibit further tryptic activity.
8. Transfer the cell suspension from the flask to a 15 ml sterile conical tube.
9. Rinse the flask with an additional 3 ml of Trypsin Neutralizing Solution and transfer the solution into the same conical tube.
10. Examine the T-25 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 Hepatic Stellate Cell Growth Medium by gently pipetting the cells to break up the pellet.
15. Count the cells with a hemocytometer or cell counter. Inoculate at 20,000 cells per cm^2 for normal growth.

V. i-HHSC QUIESCENT STATE

A. SUBCULTURING i-HHSC

1. Passage i-HHSC as described in section IV.
2. Plate in a 6-well format for quiescent assays at a seeding density of 20,000 cells per cm^2 in i-HHSC Growth Medium.
3. While i-HHSC can flip between myoblast and quiescent states, treatment with quiescent reagents will affect later ability to proliferate after two flips.
4. It is recommended to reserve a portion of i-HHSC for continued propagation.

B. PREPARING QUIESCENT MEDIUM FOR CULTURING i-HHSC

1. Thaw i-HHSC Quiescent Supplements i7121-S1, i7121-S2, i7121-S3 and i7121-S4. Add Supplements to the Basal Medium and mix gently.
2. Store the Complete Medium at 4°C in the dark. It should be used within one month following preparation.

C. INDUCING THE QUIESCENT STATE

1. Change medium to Quiescent Medium the following day.
2. Continue to change Quiescent Medium every other day. Cells should become quiescent within three days with a maximum signal at seven days. If the researcher wishes to flip the cells back into a proliferative myoblast state, change medium back to Growth Medium

6455 Weathers Place
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

D. ASSAYING i-HHSC IN THE QUIESCENT STATE

1. Lipid droplets should reach a maximum accumulation by seven days.
2. The cells are assayed alive for autofluorescence. Autofluorescence is extremely brief and is exhausted in less than one (1) second. Be prepared to photograph immediately upon exposure to fluorescent laser in the visible light channel. 40x magnification works best for detailed droplet resolution.

6455 Weathers Place
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