CELL
APPLICATIONS, INC.

General Instructions for Culturing

iPSC-Derived Human Neural Stem Cells (i-HNSC)

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. i-HNSC TOTAL KIT STORAGE

A. CRYOPRESERVED VIALS (i820-10)

Store the cryovials in a liquid nitrogen storage tank immediately upon arrival.
One cryovial of i-HNSC is for seeding one well on the 6 well plate.

B. i-HNSC GROWTH MEDIUM (i813-50)

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

C. HiPSC COATING SOLUTION (126-10)

Store at -20°C immediately upon arrival.

D. i-HNSC DISSOCIATION SOLUTION (076-05)

Store at -20°C immediately upon arrival.

E. ROCK inhibitor (13-0.1):

A 100X free sample included in the kit for initiating i-HNSC culture.
Only add ROCK Inhibitor in Growth Medium for first 24 hours:
1 X: initial culture from cryopreservation
0.5 X: after subculture

II. i-HNSC DIFFERENTIATION REAGENTS

*** <u>Not</u> included in the i-HNSC Total Kit**
*** Store all the reagents and medium at 4°C**

A. DIFFERENTIATION COATING SOLUTION A (034-10**)**

B. DIFFERENTIATION COATING SOLUTION B (035-10)

C. i-HNSC DIFFERENTIATION MEDIUM

- Neuron Lineage (i813D-100N)
- Astrocyte Lineage (i813D-100A)
- Oligodentrocyte Lineage (i813D-100O)

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 standard sterilization technique and safety rules:
   a. Do not pipette with mouth.
   b. Always wear lab coat, gloves, and safety glasses.
   c. Handle all cell culture work in a sterile hood.

III. CULTURING i-HNSC

A. PREPARATION OF CULTURE WARE FOR CULTURING

1. Thaw Coating Solution at room temperature.
2. Add 1 ml of the solution to a well of a 6 well plate and gently distribute to obtain a homogeneous coating surface.
3. Incubate for 1h at 37°C.
4. Take i-HNSC Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood and warm up to room temperature.

B. THAWING AND PLATING i-HNSC

* **Pre-wet the pipette and tips with medium to reduce cells sticking to the pipette and tips and avoid the loss of cells.**
*** Never warm media to 37°C.**

1. Remove the cryopreserved vial of i-HNSC 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. Take the vial out of the water

bath when only small amount of ice remaining in the vial. Do not let cells thaw completely.
4. Decontaminate the vial exterior with 70% alcohol in a sterile Biological Safety Cabinet.
5. Remove the vial cap carefully. Do not touch the rim of the the vial.
6. Resuspend the cells in the vial by gently pipetting the cells 2 times with a pre-wetted, 1 ml aerosol pipette tip set at 950 µl. Be careful not to pipette too vigorously as to cause foaming.
7. Transfer the cell suspension from the cryovial into a 15 ml conical tube. Dropwise add 9 ml of i-HNSC Growth Medium to the cells while swirling the tube to mix.
8. Rinse the cryovial with media to recover all the remaining cells. Collect and transfer to the tube.
9. Centrifuge at 300 x g for 5 minutes to pellet the cells.
10. Aspirate the supernatant from the tube without disturbing the cell pellet.
11. Flick the tip of the conical tube with your finger to loosen the cell pellet.
12. Resuspend the i-HNSC in 3 ml of i-HNSC Growth Medium and gently pipette to mix well. Add 30 µL of 100X ROCK Inhibitor to achieve a final concentration of 1X.
13. Aspirate HiPSC Coating Solution from the well of the 6 well plate.
14. Transfer 3 ml of i-HNSC suspension to the coated well prepared in Section 3A Step 2. Rock gently to evenly distribute the cells.
15. Incubate the i-HNSC culture in a 37ºC, 5% CO_2 humidified incubator.
16. Change the i-HNSC Growth Medium every 3rd day until the cells reach nearly 100% confluency.

IV. SUBCULTURING i-HNSC

1. Take out i-HNSC Dissociation Solution from refrigerator and warm up to room temperature.
2. Coat the cell culture plate(s) as described in Section IIIA.
3. Aspirate the medium from the i-HNSC culture dish.
4. Wash cells once with PBS and aspirate.
5. Add 1 ml i-HNSC Dissociation Solution to one well of the 6 well plate and rock gently to cover the whole dish.
6. Incubate for 3 min at 37ºC. Monitor the detachment during incubation and when clusters of cells start to detach, bring into the culture hood.
7. Triturate gently to break up the clumps and collect all cells with a 1 ml pipette. Add to a fresh 15 ml tube.
8. Add 3 ml of PBS to wash and triturate 3 times with 5 ml pipette. Add the wash to the same tube.
9. Add 6 ml PBS to a final volume of 10 ml. Invert the tube gently for 2-3 times to mix the content. Centrifuge cells at 300 x g for 5 minutes. Aspirate the supernatant carefully.
10. Resuspend the cell pellet in 1 ml of i-HNSC Growth Medium containing 0.5X ROCK inhibitor. Count the cells and plate at a concentration of 1E6 cells/well in coated wells containing 3 ml iHNSC Growth Medium with 0.5X ROCK inhibitor.
11. Incubate i-HNSC culture in a 37ºC, 5% CO_2 humidified incubator.
12. Change i-HNSC with the i-HNSC Growth Medium every 3rd day until the cells reach nearly 100% confluency.

V. DIFFERENTIATING i-HNSC TO NEURONAL CELLS

A. PREPARATION OF CULTURE WARE

1. Dispense Poly-L-Ornithine Coating Solution into tissue culture ware or chamber slide with the ratio of coating solution to surface area at 1 ml per 5 cm^2.
2. If cover slips are to be used for differentiation:
   a. Clean each cover slip by soaking in ethanol overnight, wiping with Kim wipes, and sterilizing in an autoclave.
   b. Coat each cover slip in the well
      12-well plate: 18 mm circle coverslip with 1.5 ml
      24-well plate: 12 mm circle coverslip with 1 ml
3. Coating:
   a. Incubate the tissue culture ware with DIFFERENTIATION COATING SOLUTION A for a minimum of 1 hour, or a minimum of 3 hours for glass surfaces at 37ºC. Incubation overnight at 4ºC is preferred.
   b. Aspirate the coating solution.
   c. Wash the coated surface three times with sterile water, 15 minutes per wash,.
5. Repeat Step 1-3 for coating DIFFERENTIATION COATING SOLUTION B with 6 hours the coating time at 37ºC (incubation overnight at 4ºC is preferred) and wash the coated surface with sterile PBS instead of sterile water.

B. DIFFERENTIATION

1. Seeding density for differentiation is 50,000 cells per cm^2.
2. Resuspend dissociated i-HNSC in i-HNSC Growth Medium as 100,000 cells per ml.
3. Seed the following cell suspension volumes to each well:
   12-well format: 2 ml per well
   24-well format: 1 ml per well
   96-well format: 165 µl per well
4. Incubate i-HNSC in i-HNSC Growth Medium overnight in a 37ºC, 5% CO_2 humidified incubator.
5. Change to respective Differentiation Medium the next day.
6. Check the culture daily and change half of the Neural Stem Cell Differentiation Medium every other day for neurons. **Do not let cells dried up any time during differentiation.** For astrocytes and oligodendrocytes, change medium every other day.
13. Observe the differentiation of i-HNSC into matured neuronal cells of your choice with inverted microscope. Approximately 2 to 3 weeks are needed for differentiation to neurons and oligodendrocytes; approximately 7 to 10 days to differentiate to astrocytes.

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