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 KIT (i813K-50)

Store Basal Medium (BM) at 4°C and Growth Supplement (GS) at -20°C in the dark immediately upon arrival. When ready to use, thaw GS and mix it completely with BM to make i-HNSC Growth Medium.

C. HiPSC ECM COATING SOLUTION (126-05)

Store at 4°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 each subculture

i-HNSC DIFFERENTIATION REAGENTS

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

i-HNSC DIFFERENTIATION MEDIUM
- Neuron Lineage (i813D-100N)
- Astrocyte Lineage (i813D-100A)

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 SEEDING

1. Dispense 2 mL of HiPSC ECM Coating Solution into one well of 6 well plate
2. Incubate the tissue culture ware with HiPSC ECM Coating Solution for 1 hour or a minimum of 3 hours for glass surfaces at 37ºC.
3. Aspirate the coating solution, Coated well is ready for use

B. THAWING AND PLATING i-HNSC

*** Pre-wet the pipette and tips with medium to reduce cells sticking to the pipette and tips that minimize the loss of cells.**
*** Do not 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 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 the remaining cells and transfer the rinse to the same 15 ml conical 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 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 the last wash from the well prepared in Section IIIA Step 4.
14. Transfer 3 ml of i-HNSC suspension to the coated well to seed at cell density of 1E6 cells/well of 6 well plate. Rock gently to evenly distribute the cells.
15. Incubate i-HNSC culture in a 37ºC, 5% CO_2 humidified incubator.
16. Change i-HNSC Growth Medium every 3rd day until the cells reach nearly 100% confluency.

IV. SUBCULTURING i-HNSC

1. Coat 6 wells of a new 6 well plate described in Section IIIA aspirate Coating Solution and add 3 ml of i-HNSC Growth Medium with 0.5X ROCK inhibitor to each well.
2. Take out i-HNSC Dissociation Solution from refrigerator and warm up to room temperature.
3. Aspirate the medium from the i-HNSC well.
4. Wash cells once with PBS and aspirate.
5. Add 1 ml i-HNSC Dissociation Solution to the well and rock gently to cover the whole well.
6. Incubate for 3 min at 37ºC. Monitor the detachment during incubation and when clusters of cells start to detach, bring plate to the Biological Cabinet.
7. Triturate gently to break up the clumps and collect all cells with a pre-wetted 1 ml pipette tip set at 950 ul and transfer to a 15 ml conical tube.
8. Add 3 ml of PBS into the i-HNSC well to wash and triturate 3 times with 5 ml pipette and transfer the wash to the same 15 ml conical tube as in section IV step 7.
9. Add 6 ml more PBS to the same 15 ml conical tube for a final volume of 10 ml.
10. Invert the 15 ml conical tube gently for 2-3 times to mix the content. Centrifuge cells at 300 x g for 5 minutes. Aspirate the supernatant carefully. Flick the tip of the conical tube with your finger to loosen the cell pellet.
11. Resuspend the cell pellet in 1 ml of i-HNSC Growth Medium containing 0.5X ROCK inhibitor. Count the cells and plate at a seeding density of 1E6 cells/well in coated wells containing 3 ml i-HNSC Growth Medium.
12. Incubate i-HNSC culture in a 37ºC, 5% CO_2 humidified incubator.
13. 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 i-HNSC Coating Solution A 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 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 for overnight at 4ºC is preferred.
   b. Aspirate the coating solution.
   c. Wash the coated surface two times with sterile water.
3. Repeat Step 1-3 for coating with Differentiation Coating Solution B for 2 hours at 37ºC (incubation overnight at 4ºC is preferred)
4. Wash the coated surface three times with sterile PBS, 15 minutes per wash,

B. DIFFERENTIATION

1. Seeding density for differentiation is 10,000 cells per cm^2.
2. Resuspend dissociated i-HNSC in i-HNSC Growth Medium as 20,000 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 third day for neurons. **Do not let cells dried up any time during differentiation.** For astrocytes and oligodendrocytes, change medium every other day.
7. Observe the differentiation of i-HNSC into matured neuronal cells of your choice with inverted microscope. Approximately 3 weeks are needed for differentiation to neurons and oligodendrocytes; approximately 10 days to differentiate to astrocytes.

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