CELL APPLICATIONS, INC.

General Instructions for Culturing Human Brain Neurons (HBN)

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 (8800-10f, 8800-20f)

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

B. PRE-PLATED MULTIWELL PLATES (8801-6w, -24w, -96w)

1. Examine under a microscope to check if all the cells are attached to the bottom of the multiwell plates. If not, notify CAI or your distributor immediately.
2. Decontaminate the exterior of the multiwell with 70% alcohol.
3. Place the multiwell in a 37°C, 5% CO_2 humidified incubator for 2 hours as shipped.
4. In a sterile Biological Safety Cabinet, remove the Transport Medium by aspiration. Add fresh Culture Medium.
5. Place the multiwell in a 37°C, 5% CO_2 humidified incubator.
6. Change medium every other day.

C. NEURON COATING SOLUTION II (029-05)

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

D. NEURON PLATING MEDIUM (817P-10)

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

E. NEURON CULTURE MEDIUM (817-100)

Store the Basal Medium (816-98) at 4°C in the dark immediately upon arrival.
Store Culture Supplements (817-CS1, 817-CS2, 817-CS3, 817-CS4) at -20°C immediately upon arrival.

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 HBN

A. COATING CELL CULTURE WARE FOR HBN

1. Take the Neuron Coating Solution II from refrigerator. . Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Pipette enough amount of Neuron Coating Solution II to the culture ware (for example, 0.5 mL in each well of a 24-well plate) to cover the whole culture surface. Coat 5 wells for each cryovial of HBN
3. Incubate the culture ware at 37°C for overnight.
4. Aspirate Neuron Coating Solution II from the culture ware.
5. Rinse the culture surface twice with sterile PBS prior to use to remove unbound Neuron Coating Solution II.

B. PREPARING FOR SEEDING HBN

1. Take the Neuron Plating Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
2. Equilibrate the 10 mL Neuron Plating Medium in a 37°C, 5% CO_2 humidified incubator for 1 hr.

C. THAWING AND PLATING HBN

1. Remove the cryopreserved vial of HBN 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 retighten 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 pre-wetted aerosol tip set at 950 mL. Be careful not to pipette too vigorously as to cause foaming.
8. Transfer the cell suspension from the vial into a 50 mL tube. Dropwise add 10 mL equilibrated Neuron Plating Medium prepared in Section IIIB Step 2 to 1E6 HBN.while swirling the tube to mix. Rinse the cryovial to recover all the content. Collect the medium to the tube
9. Gently mix the cell suspension in the 50 mL tube by pipetting and aliquot 2 mL into each well of the precoated 24-well plate. A seeding density of 100,000 cells per cm^2 or above is recommended. One cryovial of HBN will seed 5 wells of 24 well plate.
10. Put the lid back to the 24-well plate and rock gently to evenly distribute the cells.
11. Place the 24-well plate in a 37ºC, 5% CO_2 humidified incubator. For best results, do not disturb the culture for 24 hours after inoculation.
12. Equilibrate 10 mL of Neuron Culture Medium for 1 hour in 37ºC, 5% CO_2 humidified incubator to be used in Step 13.
13. Change to pre-equilibrated Neuron Culture Medium after 24 hours or overnight to remove all traces of DMSO. Two milliliters of medium is recommended for 24-well plate culture to minimize the detrimental effect of evaporation on the culture.
14. Change half of the Neuron Culture Medium every three days. Refreshing whole volume is not recommended as neurons are sensitive to changes in culture conditions

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