Protocols for Neural Progenitor Cell Expansion and Dopaminergic Neuron Differentiation

In vitro neurological research presents many challenges due to the difficulty in establishing high-yield neuronal cultures as well as batch-to-batch consistency. Human induced pluripotent stem cells (iPSCs) have a high expansion capacity and can differentiate into neurological cells types; thus, these cells hold great promise for both regenerative medicine and drug discovery. This protocol describes a method for expanding large quantities of iPSC-derived neural progenitor cells 6. iPSC-derived Neural Progenitor Cells (ATCC® ACS-5003™, ACS-5004™, ACS-5005™, ACS-5006™, ACS-5007™, and ACS-5001™) using the Growth Kit for Neural Progenitor Cell Expansion (ATCC® ACS-3003™). In addition, a protocol for differentiating neural progenitor cells into dopaminergic neurons using Dopaminergic Neuron Differentiation Kit (ATCC® ACS-3004™) is detailed herein.

I. MATERIALS, SUPPLIES, AND REAGENTS

A. Materials and Supplies

1. 12-well cell culture plates (Costar-Corning 3513 or equivalent)
2. Sterile Centrifuge tube 15, 50 mL
3. Serological pipettes, various sizes
4. Micropipette 10, 200 and 1000 µl
5. Pasteur pipettes
6. iPSC-derived Neural Progenitor Cells (ATCC® ACS-5003™, ACS-5004™, ACS-5005™, ACS-5006™, ACS-5007™, and ACS-5001™)

B. Reagents

1. Growth Kit for Neural Progenitor Cell Expansion (ATCC® ACS-3003™)
2. Dopaminergic Neuron Differentiation Kit (ATCC® ACS-3004™)
3. DMEM: F-12 (ATCC® 30-2006™)
4. Cell Basement Membrane Gel (ATCC® ACS-3035™)
5. Accutase (StemCell Technologies 07920)
6. Stem Cell Freezing Media (ATCC® ACS-3020™)
7. 4% Paraformaldehyde (PFA, Diluted from 20%; Electron Microscopy Sciences 157-4 or equivalent)
8. Water, Cell Culture Grade (ATCC® 30-2205 or equivalent)
9. Dulbecco's Phosphate Buffered Saline (DPBS; ATCC® 30-2200™ or equivalent)
10. 70% alcohol
11. Tween-20 (Sigma 9005-64-5 or equivalent)
12. Triton X-100 (Sigma 9002-93-1 or equivalent)
13. Normal goat serum (Thermo Fisher 10000C or equivalent)

14. Hoechst nuclear stain (Thermo Fisher H3570 or equivalent)
15. Mouse anti-human Tuj1 antibody (BioLegend 801201 or equivalent)
16. Rabbit anti-human tyrosine hydroxylase (TH) antibody (EMD-Millipore AB152 or equivalent)
17. Secondary antibodies [Thermo Fisher Alexa Flour 488 (mouse) and 594 (rabbit) or equivalent].

II. EQUIPMENT

1. Biological safety cabinet (Forma Scientific or equivalent)
2. Cell counter (Vi-Cell Analyzer or hemocytometer or equivalent)
3. Incubator (Thermo Fisher or equivalent)
4. Microscope (EVOS or equivalent)
5. Table centrifuge (Sorvall or equivalent)
6. Pipette aid
7. Fluorescent microscope (Nikon Ti or equivalent)
8. Water bath (Boekel or equivalent)

III. PREREQUISITES

A. Prepare complete NPC Growth Media as the following:

1. Obtain one Growth Kit for Neural Progenitor Cell Expansion (ATCC® ACS-3003) from the freezer and make sure that the caps of all components are tight.
2. Thaw the components of the Growth Kit for Neural Progenitor Cell Expansion at 4°C overnight.
3. Obtain one bottle of DMEM: F-12 (500 mL) from cold storage
4. Decontaminate the external surfaces of all growth kit component vials and the basal medium bottle by spraying them with 70% ethanol.
5. Using aseptic technique and working in a biosafety cabinet, remove 36 mL of DMEM: F-12 and then transfer the indicated volume of each Growth Kit for Neural Progenitor Cell Expansion component, as indicated in **Table 1**, to the basal medium bottle using a separate sterile pipette for each transfer.
6. Tightly cap the bottle of complete NPC Growth Medium and swirl the contents gently to assure a homogeneous solution. Do not shake forcefully to avoid foaming. Label and date the bottle.
7. Complete NPC Growth Media should be stored in the dark at 2°C to 8°C. When stored under these conditions, the complete NPC Growth Media is stable for two weeks.

B. If dopaminergic neurons are the desired differentiated product, prepare complete NPC Dopaminergic Neuronal Differentiation Media as the following:

1. Obtain one Dopaminergic Neuron Differentiation Kit (ATCC® ACS-3004) from the freezer and make sure that the caps of all components are tight.
2. Thaw the components of the Dopaminergic Neuron Differentiation Kit at 4°C overnight.
3. Obtain one bottle of DMEM: F-12 (500 mL) from cold storage.

4. Decontaminate the external surfaces of all Dopaminergic Neuron Differentiation Kit component vials and the basal medium bottle by spraying them with 70% ethanol.
5. Using aseptic technique and working in a laminar flow hood or biosafety cabinet, Take 237 mL of DMEM: F-12 and add it to a new sterile bottle.
6. Transfer the indicated volume of each Dopaminergic Neuron Differentiation Kit component, as indicated in **Table 2**, to the basal medium bottle using a separate sterile pipette for each transfer.
7. Tightly cap the bottle of complete Dopaminergic (Dopa) Differentiation Media and swirl the contents gently to assure a homogeneous solution. Do not shake forcefully to avoid foaming. Label and date the bottle.
8. Complete Dopa Differentiation Media should be stored in the dark at 2°C to 8°C. When stored under these conditions, complete NPC Dopa Differentiation Media is stable for four weeks.

C. Pre-warm complete NPC Growth Medium and Dopa Differentiation Media to room temperature prior to use.

D. Dilute Accutase with equal volume of DPBS for dissociation of ACS-5003, ACS-5005, ACS-5006, and ACS-5007 NPCs while using undiluted Accutase for passaging ACS-5004.

IV. PROCEDURE

This protocol for expansion and dopaminergic neuron differentiation of NPCs is based on a 12-well plate. Adjust volumes for Cell coating solution, culture media, and Accutase solution based on the surface area of the cell culture vessel as described in **Table 3**.

1. **Preparation of Cell Basement Membrane gel-coated plates**
   This protocol is designed for coating a 12-well plate. Half mL of diluted Cell Basement Membrane gel is required per well of a 12-well plate. Volumes can be directly scaled according to the size and numbers of tissue culture vessels used (**Table 3**).
   a. Thaw Cell Basement Membrane gel in a refrigerator overnight.
   b. Aliquot thawed Cell Basement Membrane gel, place one aliquot at 4°C for immediate use, and store the remaining aliquots at -20°C.
   c. Place 6 mL cold DMEM: F-12 Medium in a 15 mL conical tube on ice.
   d. Place thawed Cell Basement Membrane gel in an ice box and add cold Cell Basement Membrane gel to the 6 mL cold DMEM: F-12 Medium on ice. The final Cell Basement Membrane gel concentration should be 150 µg/mL (e.g. Add 43 µL of concentrated Cell Basement Membrane gel at 14 mg/mL into 4 mL cold DMEM: F-12).
   e. Mix the diluted Cell Basement Membrane gel well and add 0.5 mL diluted Cell Basement Membrane gel per well of a 12-well-plate.
   f. Leave a coated plate for 1 ± 0.5 hour at 37°C.
   g. Aspirate coating solution and immediately plate the cells. **Note**: Keep Cell Basement Membrane gel cold at all times and it is critical that the coating doesn't dry out.
2. **Thawing of cryopreserved NPCs**
   a. Pre-warm NPC Growth Media to room temperature.
   b. Prepare a Cell Basement Membrane gel-coated 12-well plate described above.
   c. Remove a cryovial of NPCs from liquid nitrogen storage.
   d. Thaw the cells in a 37°C water bath. To reduce the possibility of contamination, keep the cap out of the water. Thawing should be rapid (approximately 1 to 2 minutes). Remove the cryovial from water bath when only a few ice crystals are remaining.

e. Sterilize the cryovial with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.

f. Remove cells from the vial using a P1000 micropipette and transfer cells drop-wise into the 15 mL conical tube containing 9 mL DMEM: F-12 Medium.

g. Centrifuge cells at 270 x g for 5 minutes at room temperature.

h. Aspirate the supernatant and discard. Gently tap the bottom of the tube to loosen the cell pellet.

i. Add 4 mL of the complete NPC growth medium to the tube. Gently resuspend the pellet by pipetting up and down for 4-6 times to make a single-cell suspension.

j. Perform cell count by a Vi-Cell Analyzer or hemocytometer. **Note**: Do not perform cell count by a Vi-Cell Analyzer without removal of serum-free freezing medium.

k. Seed NPCs at a seeding density of 80,000 viable cells/cm^2 (e.g. 0.30 $x10^6$/well of a 12-well plate) onto a Cell Basement Membrane-coated plate containing 1.5 mL complete NPC Growth Media/well.

l. Incubate the plate at 37°C with 5% CO_2 overnight.

m. Change medium at a 100% media change rate (1.5 mL media/well) the next day and every other day thereafter.

n. Monitor cell growth daily and passage cells when they reach ~95% confluence. **Note**: Do not passage NPCs when the cells are <85% confluence.

3. Maintenance and passaging of NPCs

a. Monitor NPC growth daily and change the media every other day thereafter until cells reach ~95% confluence.

b. Passage NPCs with Accutase when cells are about 95% confluence as the following:

1). Dilute Accutase with equal volume of DPBS for passaging ACS-5003, ACS-5005, ACS-5006, and ACS-5007 NPCs while passaging ACS-5004 and ACS-5001 NPCs by using undiluted Accutase. Warm Accutase to room temperature before using.

2). Prepare a Cell Basement Membrane gel-coated 12-well plate described above.

3). Aspirate the media and add 1 mL diluted or undiluted Accutase per well based on cell types of NPCs.

4). Incubate the plate at a 37°C incubator until majority of cells are detached (It may take 3-10 minutes).

5). Add 1 mL DMEM: F-12 Medium per well and transfer cells into a 15 mL conical tube.

6). Gently pipette the cells up and down 3-4 times to mix thoroughly.

7). Perform cell count by Vi-Cell Cell Analyzer or a hemocytometer.

8). Centrifuge cells at 270 x g for 5 minutes at room temperature.

9). Aspirate the supernatant and discard. Gently tap the bottom of the tube to loosen the cell pellet.

10). Add 5 mL of the complete NPC Growth Medium to the tube. Gently resuspend the pellet by pipetting up and down 3-4 times to make a single-cell suspension.

11). Aspirate Cell Basement Membrane solution; add 1.5 mL complete NPC Growth Medium per well.

12). Seed passaged NPCs at 40,000 viable cells/cm^2 (i.e. Seed 0.15$x10^6$ cells/well of a 12-well plate).

13). Incubate the plate at 37°C with 5% CO_2 overnight.

14). Change the media at 100% media change rate the following day and change the media every other day thereafter.

15). Monitor NPC growth daily and subculture NPCs with Accutase when cells are about 95% confluence as described above.

16). Harvest NPCs for cryopreservation of NPCs with Stem Cell Freezing Media (~2 x10^6 cells/vial) or seed NPCs for neural differentiation studies.

4. **Dopaminergic neuron differentiation of NPCs**
   a. Passage NPCs 1-2 times with complete NPC Growth Media to have sufficient NPCs for dopaminergic neuron differentiation.
   b. Prepare a Cell Basement Membrane gel-coated 12-well plate.
   c. Pre-warm NPC Growth Media to room temperature.
   d. Passage and seed NPCs at seeding densities of 5,000, 10,000, and 15,000 viable cells/cm^2 in a 12-well plate coated with Cell Basement Membrane.
   e. Culture NPCs in the complete NPC Growth Media overnight
   f. Pre-warm complete Dopaminergic Neuron Differentiation Media to room temperature.
   g. Aspirate the NPC Growth Media and add 1.5 mL of complete Dopaminergic Neuron Differentiation Media per well next day
   h. Monitor neuronal differentiation of NPCs daily and change the dopaminergic differentiation media every other day for 3 weeks as the following (**Figure 1**):
      1). Gently remove ~85% of spent media by using a 5 ml serological pipette.
      2). Slowly add 1.5 mL of fresh dopaminergic differentiation media to each well through the wall by using a 5 ml serological pipette in the first week of dopaminergic neuron differentiation.
      3). During the second week of dopaminergic neuron differentiation, gently remove ~85% of spent media and slowly add 2 mL of fresh dopaminergic differentiation media to each well through the wall.
      4). During the third week of dopaminergic neuron differentiation, gently remove ~85% of spent media and slowly add 2.5 mL of fresh dopaminergic differentiation media to each well through the wall.
   i. **Note**: Handle the culture carefully and avoid detachment of differentiated NPCs. Some floating cells are visible when the cells reach 100% confluence. However, this will not affect dopaminergic differentiation.
5. **Cell Fixation**
   a. Differentiate NPCs with Dopaminergic Neuron Differentiation Media for 3 weeks.
   b. Carefully remove all culture media using a 5 mL serological pipette.
   c. Slowing add 1 ml of DPBS per well through the wall of a well using a 5 mL serological pipette.
   d. Carefully remove all DPBS using a 5 mL serological pipette.
   e. Slowly add 1 ml of 4% PFA per well through the wall of a well.
   f. Incubate at room temperature (RT) for 15 minutes.
   g. Rinse cells 3 times using DPBS by using a 5 mL serological pipette.
   h. Do not use aspirator to remove solution.
   i. Proceed for ICC or store at 4C in DPBS.
6. **Immunocytochemistry with TH and Tuj1 antibodies**
   a. Prepare Wash Buffer (0.05% Tween 20 in DPBS) and store at RT.
   b. Prepare Permeabilization Buffer (0.2% Triton-100 + 0.01% Tween 20 in DPBS) and store at RT.
   c. Freshly prepare Blocking Buffer (5% normal goat serum in Wash buffer).
   d. Remove DPBS from the wells by using a 5 mL serological pipette.
   e. Add 1 mL Permeabilization Buffer and incubate at RT for 40 minutes.

f. Remove Permeabilization Buffer and add 1 mL Blocking buffer.
g. Incubate at RT for 30 minutes.
h. Remove Blocking Buffer and wash 3 times with Wash Buffer using a 5 mL serological pipette.
i. Mix primary TH antibodies (1:75 dilution) and Tuj1 (1:200 dilution) in Wash Buffer.
j. Remove the Wash Buffer and add 250 µL of TH and Tuj1 antibody mix per well.
k. Incubate at 4°C overnight.
l. Remove the antibody mix next day and wash 3 times with Wash Buffer using a 5 mL serological pipette.
m. Add 250 µL of goat anti-rabbit fluorescence conjugated secondary antibodies (1:100 dilution) and goat anti-mouse fluorescence conjugated secondary antibodies (1:200 dilution) per well.
n. Incubate at RT for 1 hour in a slow shaker.
o. Wash 3 times with Wash Buffer.
p. Incubate with Hoechst stain (1:5000 dilution in Wash buffer) for 10 minutes at RT.
q. Wash 2 times with Wash Buffer.
r. Add 1 mL of Wash Buffer per well.
s. Visualize the immune-stained cells under a fluorescence microscope (**Figure 2**).

Table 1. NPC Growth Media Components

Component	ATCC No.	Volume Added
DMEM/F12	30-2006	464 mL
L-Alanyl-L-Glutamine	PCS-999-034	5 mL
Non-Essential Amino Acids Mix	PCS-999-052	5 mL
Growth Kit for Neural Progenitor Cell Expansion Component A	PCS-999-050	10 mL
Growth Kit for Neural Progenitor Cell Expansion Component B	PCS-999-056	5 mL
Growth Kit for Neural Progenitor Cell Expansion Component C	PCS-999-055	1 mL
Growth Kit for Neural Progenitor Cell Expansion Component D	PCS-999-054	10 mL

Table 2. Dopaminergic Neuron Differentiation Media Components

Component	ATCC No.	Volume added
DMEM/F12	30-2006	237 mL
L-Alanyl-L-Glutamine	PCS-999-034	2.5 mL
Non-Essential Amino Acids Mix	PCS-999-052	2.5 mL
Ascorbic Acid	PCS-999-006	0.5 mL
Dopaminergic Neuron Differentiation Kit Component A	PCS-999-051	5 mL
Dopaminergic Neuron Differentiation Kit Component B	PCS-999-057	2.5 mL
Dopaminergic Neuron Differentiation Kit Component C	PCS-999-053	0.5 mL

Table 3. Recommended Volumes for Cell Basement Membrane solution, Accutase solution, and NPC Growth Media

Culture vessel	12-well plate	6-well plate	6-cm dish	10-cm dish
Surface Area (cm^2)	3.8	9.6	21.5	59
Volume Cell Basement Membrane (mL)	0.5	1	2.5	5
Volume of Accutase (mL)	1	2	3	6
Volume of Complete Growth Media (mL)	1.5	3	6	12

Figure 1. Time-course of dopaminergic neuron differentiation of ACS-5003 NPCs (10x).

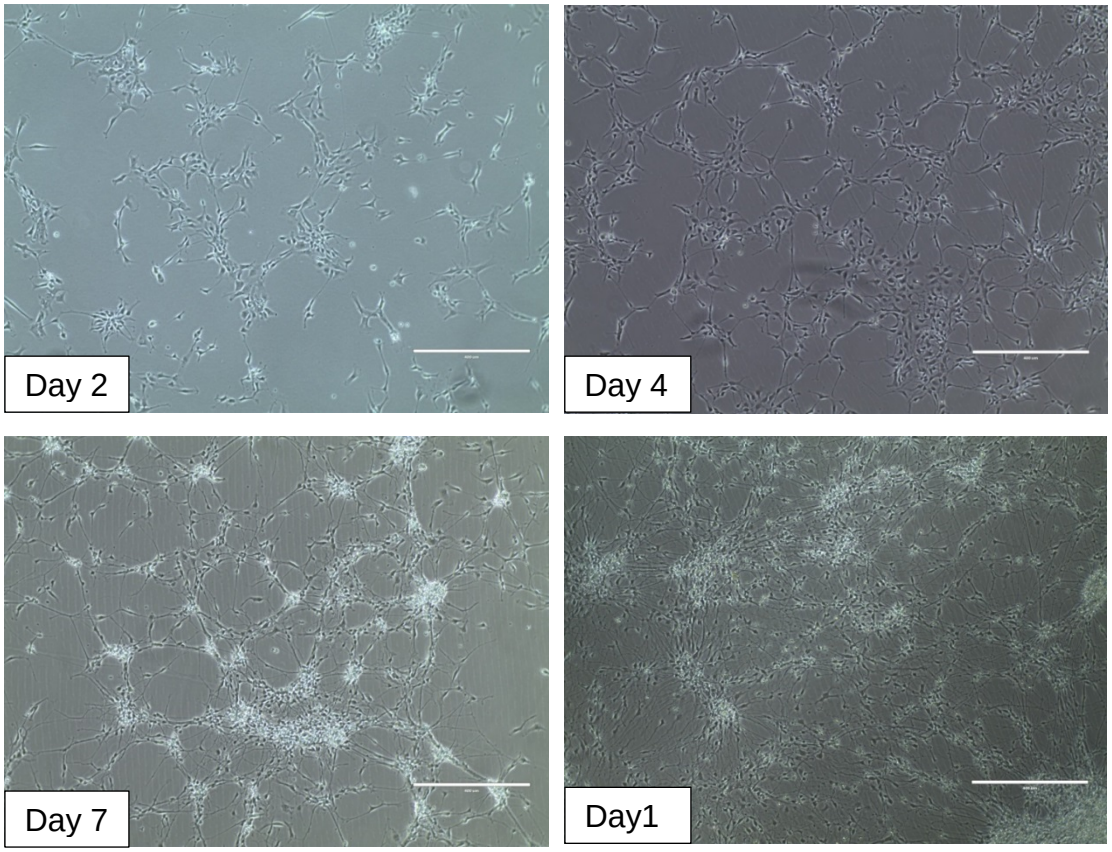

Figure 2. ICC of ACS-5003 NPC-derived dopaminergic neurons with TH and Tuj1 antibodies (20x).