

## **Human/Mouse Dopaminergic Neuron Differentiation Kit**

**Reagents for the differentiation of  
human and mouse embryonic stem cells**

**Product Number: SC15000**

*The product insert must be read in its entirety before using this product*

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## INTRODUCTION

Dopamine cell bodies exist in the midbrain, where they are mainly located in the substantia nigra, the ventral tegmental area, and the retrorubral field. These dopaminergic (DA) neurons give rise to extensive forebrain dopamine innervation ( e.g. projections to the caudate nucleus, putamen nucleus accumbens, olfactory tubercle, and several cortical regions and limbic regions, including the amygdala, lateral septum and ventral hippocampus) (1). The basic organization of midbrain dopamine neurons and their projections is consistent across most mammals. The midbrain dopamine projections to the striatum have been among the most extensively studied catecholamine neurons, in part because the degeneration of DA neurons in the substantia nigra results in Parkinson s disease (2). Both animal models and clinical trials have suggested that cell replacement therapies may be effective in treating Parkinson s disease (3). This approach is limited, however, by the availability of a rich, effective source of neural precursors for DA neuron generation.

Embryonic stem (ES) cells, derived from the inner cell mass of pre-implantation embryos, have been recognized as the most pluripotent stem cell population (4). ES cells give rise to a collection of differentiated cells and tissues, including neurons. Thus, they may serve as a potential source of replacement DA neurons in Parkinson s disease. Two groups have reported methods for generating DA neurons from ES cells, thus demonstrating the feasibility of identifying conditions required for functional DA neuron differentiation of ES cells (5, 6). Experimental models established from these studies might facilitate the *in vitro* examination of various aspects of neural development, in particular the underlying mechanisms controlling cell lineage and fate commitment.

## DESIGN OF THE KIT

The Mouse Dopaminergic Neuron Differentiation Kit is a system designed for *in vitro* neural differentiation of mouse ES cells in a serum-free environment. This kit contains specially formulated ITS and N-2 Plus Media Supplements, which are used to select and enrich neural stem cell populations. Bovine Fibronectin is included to provide proper support for cell attachment and spreading. A growth factor panel, consisting of human fibroblast growth factor (FGF) basic, mouse FGF-8b, and mouse sonic hedgehog amino-terminal peptide (Shh-N), is included for effective DA differentiation. The quantity of each component provided in the kit is estimated to be sufficient for the induction of  $3 \times 10^7$  ES cells. This kit has been shown to generate an average of  $15 \pm 5\%$  DA neurons as estimated from double labeling various mouse ES cell lines with antibodies specific to tyrosine hydroxylase and neuron-specific  $\beta$ -III tubulin.

## LIMITATIONS OF THE PROCEDURE

**FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.**

The safety and efficacy of this product in diagnostic or other clinical uses has not been established. The kit should not be used beyond the expiration date on the kit label.

**The quality of the embryonic stem cells and any variation in this procedure can cause variation in the efficiency of dopaminergic neuron generation.**

## PRECAUTIONS

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed and protective clothing should be worn when handling kit reagents.

The ITS and N-2 Plus Media Supplements contain human transferrin. This transferrin was tested at the donor level using an FDA licensed method and found to be non-reactive for anti-HIV-1/2 and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, these reagents should be handled as if capable of transmitting infection.

## REAGENTS

ITS Media Supplement (Part 390154) - 5 mL of a 100X concentrated solution, containing bovine insulin, human transferrin, and sodium selenite.

N-2 Plus Media Supplement (Part 390155) - 5 mL of a 100X concentrated solution, containing bovine insulin, human transferrin, sodium selenite, putrescine, and progesterone.

Bovine Fibronectin Stock (Part 390213) - 1 vial containing 2 mL of a 1000X (1 mg/mL) solution of purified bovine fibronectin.

Human FGF basic (Part 390348) - 1 vial of lyophilized recombinant human FGF basic; enough to make 250  $\mu$ L of a 1000X stock.

Mouse FGF-8b (Part 390350) - 1 vial of lyophilized recombinant mouse FGF-8b; enough to make 250  $\mu$ L of a 1000X stock.

Mouse Shh-N (Part 390349) - 1 vial of lyophilized recombinant mouse Shh-N; enough to make 250  $\mu$ L of a 1000X stock.

## STORAGE

Unopened Kit	Store at $\leq -20^{\circ}$ C in a manual defrost freezer. Do not use past kit expiration date.	
Opened Reagents	ITS Media Supplement	Store at 2 - 8 $^{\circ}$ C for up to 1 month or aliquot and store at $\leq -20^{\circ}$ C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
	N-2 Plus Media Supplement	
	Human FGF basic	
	Mouse FGF-8b	
	Mouse Shh-N	
	Bovine Fibronectin Stock	

\*Provided this is within the expiration date of the kit.

## OTHER SUPPLIES REQUIRED

### Materials

Mouse Embryonic Stem (ES) cells (refer to reference 7 or use D3 cells from ATCC, [www.atcc.org](http://www.atcc.org) )  
Mouse Embryonic Fibroblasts (MEF) feeder cells (prepare according to reference 8 or purchase From [www.specialtymedia.com](http://www.specialtymedia.com) )  
10 cm tissue culture dishes (Falcon Catalog # 353003 or equivalent)  
10 cm bacterial culture dishes (Falcon Catalog # 351029 or equivalent)  
12 mm cover slips (Assistant 1001/0012 Catalog # BA-63-3009 from [www.carolina.com](http://www.carolina.com) or equivalent)  
24-well culture plates (Corning Costar Catalog # 3526 or equivalent)  
15 mL centrifuge tubes (Corning Costar Catalog # 430052 or equivalent)  
T-75 flasks (Corning Costar Catalog # 3375 or equivalent)  
0.2  $\mu$ m syringe filter (PALL Gelman Laboratories Part # 4187)  
0.2  $\mu$ m, 500 mL filter units (Nalgene Catalog # 161-0020 or equivalent)  
Cryotubes (Nalgene Catalog # 5000-0012 or equivalent)  
Serological pipettes  
Pipettes and pipette tips  
10 mL syringes (BD Catalog # 309604)

### Reagents

Dulbecco s Modified Eagle Medium (DMEM) (GIBCO Catalog # 11965-092 or equivalent)  
DMEM/F-12, no HEPES (GIBCO Catalog # 12500-062, 11320-033, or equivalent)  
Fetal Bovine Serum, ES Cell Qualified (GIBCO Catalog # 16141-061 or equivalent)  
Mitomycin C (Sigma Catalog # M0503 or equivalent)  
Phosphate Buffered Saline (PBS) (GIBCO Catalog # 10010-023 or equivalent)  
0.05% Trypsin/EDTA (GIBCO Catalog # 25300-054 or equivalent)  
Gelatin (Sigma Catalog # G2500 or equivalent)  
ESGRO<sup>®</sup> (recombinant mouse LIF) (Chemicon Catalog # ESG1106 or equivalent)  
Knock-out DMEM (GIBCO Catalog # 10829-018 or equivalent)  
MEM Non-essential AA Solution (GIBCO Catalog # 11140-050 or equivalent)  
Penicillin-Streptomycin-Glutamine, 100X (GIBCO Catalog # 10378-016 or equivalent)  
Penicillin-Streptomycin, 100X (GIBCO Catalog # 15140-148 or equivalent)  
2-Mercaptoethanol, 1000X (GIBCO Catalog # 21985-023 or equivalent)  
Glucose (Sigma Catalog # G6152 or equivalent)  
L-Glutamine (Sigma Catalog # G5763 or equivalent)  
Sodium Bicarbonate (NaHCO<sub>3</sub>) (Sigma Catalog # S5761 or equivalent)  
Poly-L-ornithine (Sigma Catalog # P3655 or equivalent)  
Ascorbic Acid (Sigma Catalog # A4403 or equivalent)  
Sterile, deionized water  
BSA, very low endotoxin (Serologicals Proteins Inc. Catalog # 81-068-3 or equivalent)  
Acetic acid (J.T. Baker Catalog # 9508-03 or equivalent)  
Anti-nestin antibody (Dr. Ronald McKay, NINDS, NIH or equivalent)  
Anti-tyrosine hydroxylase antibody (Sigma Catalog # T1299, Pel-freez Catalog # P40101-0 or equivalent)  
Anti-neuron-specific  $\beta$ -III tubulin antibody (R&D Systems Catalog # MAB1195 or equivalent)

### Equipment

37° C and 5% CO<sub>2</sub> incubator  
37° C water bath  
60° C hot plate  
Centrifuge  
Hemocytometer  
Microscope

## PROCEDURE OUTLINE

Stage I: Expansion of Undifferentiated ES Cells (3 - 4 days)



Stage II: Formation of Embryoid Bodies (4 days)



Stage III: Selection of Nestin-positive Cells (6 - 8 days)

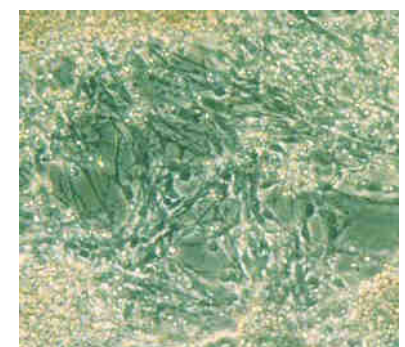
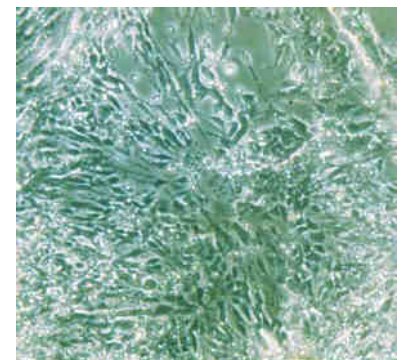
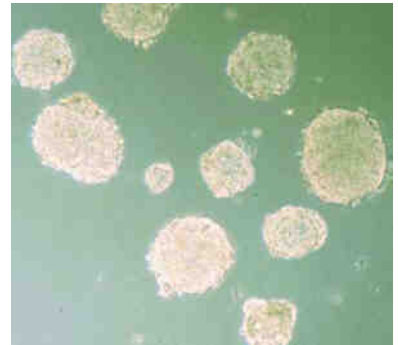
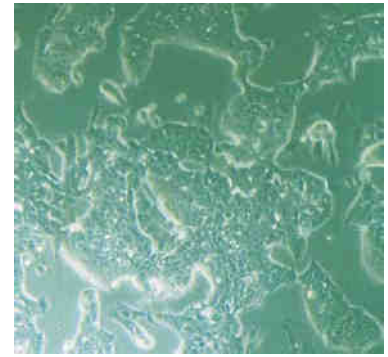


Stage IV: Expansion of Nestin-positive Cells (4 - 6 days)



Stage V: Differentiation of Dopaminergic Neurons (10 - 15 days)

Total: 27 - 37 days





## REAGENT AND MATERIAL PREPARATION

Use serological pipettes to transfer and remove solutions.

### Preparation of ES Cells

Mitomycin C Stock (100X) - Add 2 mL of sterile, deionized water to a 2 mg vial of Mitomycin C to make a 1 mg/mL stock solution. Protect from the light. Store at 2 - 8° C for up to 6 weeks.

0.1% Gelatin Solution - Add 0.25 g of gelatin to 250 mL of deionized water to make a 0.1% solution. Dissolve by heating to 60° C. Water may evaporate during the heating process. Additional water should be added to bring the final volume up to 250 mL. Sterile filter the solution using a 500 mL, 0.2 µm filter unit and store at room temperature for up to 6 months.

Gelatin-coated Dishes - Prepare immediately before use.

1. Add 10 mL of 0.1% Gelatin Solution to a 10 cm tissue culture dish (0.127 mL/cm<sup>2</sup>).
2. Incubate at room temperature for 15 minutes.
3. Aspirate the 0.1% Gelatin Solution.
4. Air dry for 30 minutes by leaving the lid half-open in a hood at room temperature.

MEF Medium - Mix the following sterile ingredients to make 505 mL of medium. Store at 2 - 8° C for up to 1 month.

Item	Amount	Final Concentration
DMEM	450 mL	90%
Fetal Bovine Serum	50 mL	10%
100X Penicillin-Streptomycin-Glutamine	5 mL	100 U/mL Penicillin 100 µg/mL Streptomycin 2 mM L-Glutamine

KO-ES Medium - Mix the following sterile ingredients to make 510.5 mL of medium. Store at 2 - 8° C for up to 1 month.

Item	Amount	Final Concentration
Knock-out DMEM	425 mL	85%
Fetal Bovine Serum	75 mL	15%
MEM Non-Essential AA Solution	5 mL	100 µM
100X Penicillin-Streptomycin-Glutamine	5 mL	100 U/mL Penicillin 100 µg/mL Streptomycin 2 mM L-Glutamine
2-Mercaptoethanol	0.5 mL	55 µM

KO-ES/ESGRO Medium - Dilute the ESGRO in KO-ES Medium to make a 1400 U/mL solution. Prepare fresh as need.



## Stage I: Expansion of Undifferentiated ES Cells

0.1% Gelatin Solution - See previous recipe.

Gelatin-coated Dishes - See previous recipe.

KO-ES Medium - See previous recipe.

KO-ES/ESGRO Medium - See previous recipe.

## Stage II: Formation of Embryoid Bodies

KO-ES Medium - See previous recipe.

## Stage III: Selection of Nestin-positive Cells

ITS Medium - Mix the following ingredients with deionized water to make 500 mL of medium. Adjust the pH to  $7.5 \pm 0.2$ . Sterile filter the solution using a 500 mL, 0.2  $\mu\text{m}$  filter unit, and add 5 mL of 100X Penicillin-Streptomycin. Store at 2 - 8° C for up to 2 weeks.

Item	Amount
DMEM/F-12*	6 g (495 mL*)
Glucose	775 mg
L-Glutamine	36.5 mg
NaHCO <sub>3</sub>	1.2 g
ITS Media Supplement (100X)	5 mL

\*Liquid DMEM/F-12 (GIBCO Catalog # 11320-033; no HEPES) can be used as an alternative to the powder form (GIBCO Catalog # 12500-062).

Bovine Fibronectin Stock - Allow the Bovine Fibronectin Stock to stand for 30 minutes at room temperature, **without agitation**. Swirl very gently. Aliquot and store at  $\leq -20^{\circ}\text{C}$  in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

ITS/Fibronectin Medium - Dilute the Bovine Fibronectin Stock 200-fold in ITS Medium to make a 5  $\mu\text{g/mL}$  solution (e.g. 50  $\mu\text{L}$  of Fibronectin Stock in 10 mL of ITS Medium). Mix by gently swirling without vortexing. Prepare fresh as needed.

## Stage IV: Expansion of Nestin-positive Cells

N-2 Plus Medium - Mix the following ingredients with deionized water to make 500 mL of medium. Adjust the pH to  $7.2 \pm 0.2$ . Sterile filter the solution using a 500 mL, 0.2  $\mu\text{m}$  filter unit, and add 5 mL of 100X Penicillin-Streptomycin. Store in the **dark** at 2 - 8° C for up to 2 weeks.

Item	Amount
DMEM/F-12*	6 g (495 mL*)
Glucose	775 mg
L-Glutamine	36.5 mg
NaHCO <sub>3</sub>	845 mg
N-2 Plus Media Supplement (100X)	5 mL

\*Liquid DMEM/F-12 (GIBCO Catalog # 11320-033; no HEPES) can be used as an alternative to the powder form (GIBCO Catalog # 12500-062).

0.1% BSA in PBS - Dissolve 10 mg of BSA into 10 mL of PBS. Sterile filter the solution using a 0.2  $\mu\text{m}$  syringe filter and store at 2 - 8° C for up to 3 months.

FGF basic Stock (1000X) - Add 250  $\mu\text{L}$  of sterile 0.1% BSA in PBS to the human FGF basic vial. Aliquot and store at  $\leq -20^\circ\text{C}$  in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

FGF-8b Stock (1000X) - Add 250  $\mu\text{L}$  of sterile 0.1% BSA in PBS to the mouse FGF-8b vial. Aliquot and store at  $\leq -20^\circ\text{C}$  in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

Shh-N Stock (1000X) - Add 250  $\mu\text{L}$  of sterile 0.1% BSA in PBS to the mouse Shh-N vial. Aliquot and store at  $\leq -20^\circ\text{C}$  in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

Ascorbic Acid Stock (1000X) - Add 0.176 g of ascorbic acid into 5 mL of sterile, deionized water to make a 200 mM solution. Sterile filter the solution using a 0.2  $\mu\text{m}$  syringe filter. Aliquot and store at  $\leq -20^\circ\text{C}$  in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

N-2 Plus/FGF basic/FGF-8b/Shh-N/Ascorbic Acid Medium - Dilute the FGF basic, FGF-8b, Shh-N and Ascorbic Acid Stocks 1000-fold in N-2 Plus Medium. Prepare fresh as needed.

Poly-L-ornithine Stock (1000X) - Dissolve the Poly-L-ornithine in sterile PBS to make a 15  $\mu\text{g/mL}$  stock. Aliquot and store at  $\leq 20^{\circ}\text{C}$  in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

Poly-L-ornithine Solution (1X) - Dilute the Poly-L-ornithine Stock 1000-fold in sterile PBS to make a 15  $\mu\text{g/mL}$  solution. Prepare fresh as needed.

Fibronectin Solution (1X) - Dilute the Fibronectin Stock 1000-fold in sterile PBS to make a 1  $\mu\text{g/mL}$  solution. Mix by gentle swirling, without vortexing. Prepare fresh as needed.

Poly-L-ornithine/Fibronectin Coated Plates -

1. Insert a sterile cover slip (sterilized with 95% EtOH and flamed) into each well of a 24-well plate.
2. Add 0.5 mL of Poly-L-ornithine Solution (1X) to each well. Incubate overnight at  $37^{\circ}\text{C}$ .
3. Discard the Poly-L-ornithine Solution. Wash each well 3 times with 1 mL of sterile PBS.
4. Add 0.5 mL of PBS to each well. Incubate overnight at  $37^{\circ}\text{C}$ .
5. Discard the PBS. Wash each well once with 1 mL of sterile PBS.
6. Add 0.5 mL of Fibronectin Solution (1X) to each well. Incubate at  $37^{\circ}\text{C}$  for 3 to 30 hours.
7. Discard the Fibronectin Solution. Wash each well once with 1 mL of sterile PBS before use.

Stage V: Differentiation to Dopaminergic Neurons

N-2 Plus/Ascorbic Acid Medium - Dilute the Ascorbic Acid Stock 1000-fold in N-2 Plus Medium to make a 200  $\mu\text{M}$  solution. Prepare fresh as needed.

## PREPARATION OF ES CELLS

Use serological pipettes to transfer and remove solutions.

### MEF Feeder Cells

1. Plate the MEF feeder cells from the frozen stock ( $1 - 2 \times 10^6$  cells) into a T-75 flask containing 25 mL of MEF Medium. Incubate at 37° C and 5% CO<sub>2</sub>. Cells should become adherent after 4 hours.
2. Feed the cell culture daily by replacing with fresh medium until subconfluent.
3. Remove the medium. Add 10 mL of MEF Medium supplemented with 0.1 mL of Mitomycin C Stock (final concentration of 10 µg/mL). Incubate for 3 hours at 37° C and 5% CO<sub>2</sub>.
4. Remove the MEF Medium containing Mitomycin C. Wash the feeder cells 3 times with 10 mL of sterile PBS.
5. Add 2 mL of 0.05% Trypsin/EDTA. Incubate for 5 minutes at 37° C and 5% CO<sub>2</sub>.
6. Tap the flask to dislodge the feeder cells. Add 10 mL of MEF Medium to neutralize the trypsin.
7. Transfer the cells to a 15 mL tube by gentle pipetting. Centrifuge for 5 minutes at 220 x g.
8. Remove the supernate and resuspend the feeder cells with 10 mL of MEF Medium. Count the cells. At this point, extra cells can be frozen for future use, according to the procedure described in reference 8.
9. Seed  $3 \times 10^6$  Mitomycin C-treated feeder cells in 10 mL of MEF Medium in a Gelatin-coated Dish. Incubate for 24 hours at 37° C and 5% CO<sub>2</sub>.

## ES Cell Culture

1. Twenty-four hours after the Mitomycin C-treated feeder cells are plated, warm the KO-ES/ESGRO Medium in a 37° C water bath for 20 minutes. In a 15 mL tube, resuspend  $3 \times 10^6$  ES cells from a frozen stock vial with 10 mL of warm KO-ES/ESGRO Medium.
2. Remove the MEF Medium from feeder cells and wash once with 5 mL of KO-ES Medium.
3. Add the ES cell suspension on top of the feeder cells and incubate the culture at 37° C and 5% CO<sub>2</sub>. Twenty-four hours after the ES cells are plated, the ES cells should become attached to the feeder cell layer.
4. Feed the cells daily by replacing with fresh KO-ES/ESGRO Medium. ES cells should start proliferating as indicated by increasing size of colonies on top of the feeder cell layer. Refer to reference 8 for images of the cell colonies.
5. Incubate the cells for 2 days (or until just before individual colonies contact each other) at 37° C and 5% CO<sub>2</sub>. Harvest the cells as follows:
  - a. Remove the medium. Wash the feeder cells 3 times with 10 mL of sterile PBS.
  - b. Add 1 mL of 0.05% Trypsin/EDTA. Incubate for 5 minutes at 37° C and 5% CO<sub>2</sub>.
  - c. Tap the dish gently to dislodge the cells. Add 5 mL of KO-ES Medium to neutralize the trypsin.
  - d. Transfer the cells by pipetting to a 15 mL tube and centrifuge for 5 minutes at 220 x g.
  - e. Remove the supernate and gently resuspend the ES cells with 5 mL of KO-ES/ESGRO Medium. Count the cells. At this point, cells can either be frozen by following the protocol described in *Disaggregation, expansion and freezing of transfected ES clones* (<http://medicine.wustl.edu/~escore/>) or used in Stage I of this protocol.

## PROCEDURE

### Stage I: Expansion of Undifferentiated ES Cells

1. Warm the KO-ES/ESGRO Medium in a 37° C water bath for 20 minutes.
2. Seed  $3 \times 10^6$  ES cells in 10 mL of warm KO-ES/ESGRO Medium on a Gelatin-coated Dish.

**Note:** *Some MEF cells may be carried over to this stage. However, they will adhere to the Plate at Stage II and will be separated from the floating cells after the embryoid bodies are formed.*

3. Culture the cells for 3 - 4 days at 37° C and 5% CO<sub>2</sub>, or until just before the cell colonies contact each other. Replace the KO-ES/ESGRO Medium **daily**.

### Stage II: Formation of Embryoid Bodies

1. Discard the medium. Wash the cells from Stage I twice with 10 mL of sterile PBS.
2. Add 1 mL of 0.05% Trypsin/EDTA solution. Incubate for 5 minutes at 37° C and 5% CO<sub>2</sub>.
3. Gently tap the dish to dislodge the cells. Add 5 mL of KO-ES Medium to neutralize the trypsin.
4. Transfer the cells to a 15 mL tube by gentle pipetting. Centrifuge for 5 minutes at 220 x g.
5. Remove the supernate and resuspend the cell pellet in 5 mL of KO-ES Medium. Count the cells. Approximately  $1.5 \times 10^7$  cells/dish can be expected from the harvest.
6. Seed  $2 \times 10^6$  cells on a 10 cm bacterial culture dish containing 10 mL of KO-ES Medium.
7. Culture the cells for 4 days at 37° C and 5% CO<sub>2</sub>. Change the medium on day 2 in the following manner:
  - a. Transfer the embryoid bodies (EB) to a 15 mL tube by gentle pipetting. Do not centrifuge.
  - b. Allow the tube to stand until the EB settle to the bottom (about 5 minutes).
  - c. Remove the medium. Add 10 mL of fresh KO-ES Medium.
  - d. Transfer the EB back into the original dish by gentle pipetting.
8. On day 4, proceed to Stage III.

### Stage III: Selection of Nestin-positive Cells

1. At the end of Stage II, transfer the EB to a 15 mL tube by gentle pipetting.
2. Allow the tube to stand until the EB settle to the bottom. Remove half of the medium.
3. Rinse the original dish with 5 mL of fresh KO-ES Medium and add to the 15 mL tube by gentle pipetting.
4. Transfer the EB to a 10 cm tissue culture dish by gentle pipetting. Culture for 24 hours at 37° C and 5% CO<sub>2</sub>. At this time, the EB should become attached.
5. Change the medium from KO-ES Medium to 10 mL of ITS/Fibronectin Medium.
6. Culture the cells for 6 - 8 days at 37° C and 5% CO<sub>2</sub>. Change the ITS/Fibronectin Medium every 2 days. During this period, a monolayer will grow from the attached EB.

### Stage IV: Expansion of Nestin-positive Cells

1. Remove the ITS/Fibronectin Medium from the cell culture in Stage III. Wash the attached cells twice with 10 mL of sterile PBS. Remove the PBS.
2. Add 1 mL of 0.05% Trypsin/EDTA solution. Incubate for 5 minutes at 37° C and 5% CO<sub>2</sub>. Gently tap the dish to dislodge the cells. Add 5 mL of KO-ES Medium to neutralize the trypsin.
3. Transfer the cells to a 15 mL tube by gentle pipetting. Remove the cell clumps (remnants of EB) by allowing the tube to stand just long enough to allow the cell clumps to settle to the bottom (about 5 minutes).
4. Transfer the suspended cells to a 15 mL tube by gentle pipetting.
5. Centrifuge the suspension for 5 minutes at 220 x g to pellet the cells.
6. Discard the supernate and resuspend the cell pellet in N-2 Plus/FGF basic/FGF-8b/Shh-N/Ascorbic Acid Medium.
7. Count the cells and seed on a Poly-L-ornithine/Fibronectin Coated Plate.

**Note:** Seeding density:  $3-5 \times 10^5$  cells/well in 500  $\mu$ L of medium.

8. Feed the cells with N-2 Plus/FGF basic/FGF-8b/Shh-N/Ascorbic Acid Medium every day for 4 - 6 days. Cell confluence should reach close to 100% at this time.

### Stage V: Differentiation of Dopaminergic Neurons

1. Induce differentiation of the expanded Nestin-positive cells from Stage IV by feeding the cells in N-2 Plus/Ascorbic Acid Medium (without growth factors) for 10 - 15 days. Change the N-2 Plus/Ascorbic Acid Medium every 2 days.
2. After 10 - 15 days, dopaminergic neurons can be identified by staining with tyrosine hydroxylase and neuron-specific  $\beta$ -III tubulin antibodies (refer to reference 5).



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