# StemXVivo™

# Human/Mouse Dopaminergic Neuron Differentiation Kit

Catalog Number SC001B

Reagents for the differentiation of human and mouse pluripotent stem cells to dopaminergic neurons.

This package insert must be read in its entirety before using this product.
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.

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## INTRODUCTION

In the mammalian central nervous system, Dopamine is primarily supplied by neurons located in two adjacent structures in the midbrain; the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc) (1). Dopaminergic neurons from the VTA innervate the ventral striatum and the prefrontal cortex, forming the mesolimbic and mesocortical pathways which regulate cognition, motivation, and emotional/rewarding behaviors. In contrast, dopaminergic neurons in the substantia nigra innervate the dorsolateral striatum and caudate putamen, forming the nigrostriatal pathway, which regulates posture and voluntary movement. Dysfunction of dopaminergic neurons has been associated with several pathological conditions including addiction, schizophrenia, and Parkinson's disease (2-4).

Parkinson's disease is a neurodegenerative movement disorder caused by the loss of dopaminergic neurons in the substantia nigra (2). Data from animal models and clinical trials have suggested that cell replacement therapies may be an effective treatment for Parkinson's disease (5-7). However, cell replacement therapy requires a rich and effective source of neural precursor cells for the production of dopaminergic neurons. Pluripotent stem cells, which can be differentiated into dopaminergic neurons under appropriate culture conditions, may prove useful in the treatment of Parkinson's disease and other dopaminergic disorders (8, 9).

## PRINCIPAL OF THE ASSAY

The StemXVivo™ Human/Mouse Dopaminergic Neuron Differentiation Kit is a system designed for in vitro neuronal differentiation of human and mouse pluripotent stem cells (PSCs) in a serum-free environment. This kit contains specially formulated ITS and N-2 MAX Media Supplements, which are used to select and enrich for neural stem cell populations. Bovine Fibronectin is included to provide 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 differentiation. The quantity of each component provided in the kit is estimated to be sufficient for the induction of $3 \times 10^7$ PSCs cells. This kit has been shown to generate an average of $15 \pm 5\%$ dopaminergic neurons as estimated from double labeling various mouse pluripotent stem cell lines with antibodies specific to Tyrosine Hydroxylase and Neuron-specific β-III Tubulin.

## ACKNOWLEDGEMENTS

R&D Systems would like to thank Dr. Jong-Hoon Kim and Dr. Ron McKay from the National Institute of Neurological Disorders and Stroke (NINDS) at NIH for their assistance in developing this kit. The instructions in this kit pertain to mouse PSCs. The kit has also been tested and shown to generate dopaminergic neurons from human PSCs (personal communication with Dr. Ron McKay, NINDS).

## LIMITATIONS OF THE PROCEDURE

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix or substitute reagents with those from other lots or sources.
- 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 pluripotent stem cells and any modification of 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 MAX 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.

## MATERIALS PROVIDED

Store unopened kit at ≤ -20 °C in a manual defrost freezer. Do not use past kit expiration date.

| PART | PART # | DESCRIPTION | STORAGE OF OPENED/ RECONSTITUTED MATERIAL |
|---|---|---|---|
| **ITS Supplement** | 390154 | 5 mL of a 100X concentrated solution containing bovine insulin, human transferrin, and sodium selenite. | Store at 2-8 °C for up to 1 month or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles. |
| **N-2 MAX Supplement** | 390524 | 5 mL of a 100X concentrated solution containing recombinant human insulin, human transferrin, sodium selenite, putrescine, and progesterone. | |
| **Bovine Fibronectin Stock** | 390213 | 1 vial containing 2 mL of a 1000X (1 mg/mL) solution of purified bovine fibronectin. | |
| **Human FGF basic** | 390348 | 1 vial of lyophilized recombinant human FGF basic; enough to make 250 µL of a 1000X stock. | |
| **Mouse Shh-N** | 390349 | 1 vial of lyophilized recombinant mouse Shh-N; enough to make 250 µL of a 1000X stock. | |
| **Mouse FGF-8b** | 390350 | 1 vial of lyophilized recombinant mouse FGF-8b; enough to make 250 µL of a 1000X stock. | |

*Provided this is within the expiration of the kit.

## OTHER SUPPLIES REQUIRED

### Materials

- Mouse Pluripotent Stem Cells (refer to reference 10 or use D3 cells from ATCC, www.atcc.org)
- Irradiated Mouse Embryonic Fibroblast (iMEF) (R&D Systems, Catalog # PSC001)
- 10 cm tissue culture plates
- 10 cm bacterial culture plates
- 12 mm coverslips
- 24-well culture plates
- 15 mL centrifuge tubes
- 0.2 µm syringe filter
- 0.2 µm, 500 mL filter units
- Cryotubes
- Serological pipettes
- Pipettes and pipette tips
- 10 mL syringes

### Equipment

- 37 °C and 5% $CO_2$ incubator
- 37 °C water bath
- 60 °C hot plate
- Centrifuge
- Hemocytometer
- Microscope

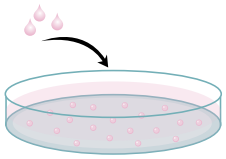
### Reagents

- Dulbecco's Modified Eagle Medium (DMEM)
- DMEM/F-12, no HEPES
- Fetal Bovine Serum, ES Cell Qualified
- Phosphate Buffered Saline (PBS)
- 0.05% Trypsin/EDTA
- Gelatin
- ESGRO® (recombinant mouse LIF) (Millipore, Catalog # ESG1106 or equivalent)
- Knock-out DMEM
- MEM Non-essential AA Solution
- Penicillin-Streptomycin-Glutamine, 100X
- Penicillin-Streptomycin, 100X
- 2-Mercaptoethanol, 1000X
- Glucose
- L-Glutamine
- Sodium Bicarbonate ($NaHCO_3$)
- Poly-L-ornithine
- Ascorbic Acid (Tocris, Catalog # 4055)
- Sterile, deionized water
- BSA, very low endotoxin (Millipore, Catalog # 81-068-3 or equivalent)
- Anti-Nestin Antibody (R&D Systems, Catalog # AF2736)
- Anti-Tyrosine Hydroxylase Antibody (R&D Systems, Catalog # MAB7566 or AF7566)
- Anti-Neuron-Specific β-III Tubulin Antibody (R&D Systems, Catalog # MAB1195)
- NorthernLights™ 493 (NL493)-Conjugated Mouse Anti-Neuron-Specific β-III Tubulin Antibody (R&D Systems, Catalog # NL1195G)

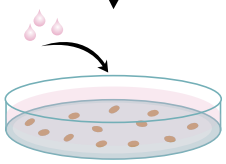
# PROCEDURE OUTLINE

**Selection of Nestin-positive Cells (10-12 Days)**

**Generate** embryoid bodies (EB) from pluripotent stem cells.
**Transfer** EB to a 10 cm culture plate containing KO-ES Media.
**Culture** the cells for 24 h at 37 °C and 5% $CO_2$.

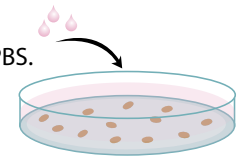


**Replace** the KO-ES Media with ITS/Fibronectin Media.
**Culture** the cells for 6-8 days at 37 °C and 5% $CO_2$.
**Replace** the media every 2 days.
**Verify** successful differentiation by staining cells for Nestin.

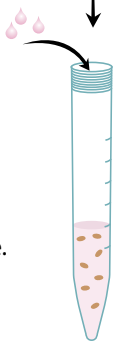


**Expansion of Nestin-Positive Cells (4-6 Days)**

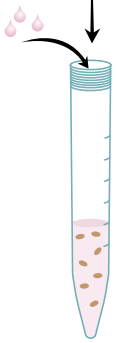
**Wash** the cells twice with sterile PBS.
**Dissociate** the cells with 0.05% Trypsin/EDTA.
**Add** 5 mL of KO-ES Media to neutralize the trypsin.



**Transfer** the cells to a 15 mL tube.
**Remove** the cell clumps by allowing the tube to stand for approximately 5 minutes and then transferring the suspended cells to a new 15 mL tube.



**Centrifuge** the samples at 220 x g for 5 minutes.
**Resuspend** the cell pellet in N-2 MAX/FGF basic/FGF-8b/ Shh-N/Ascorbic Acid Media.



**Perform** a cell count.

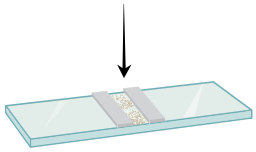
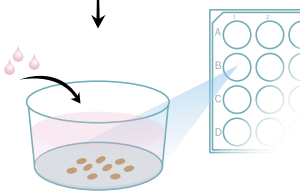
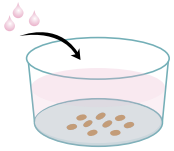


**Plate** the cells on Poly-L-ornithine/ Fibronectin-coated plates at 3-5 x $10^5$ cells/well in 500 µL of media.
**Replace** the media daily for 4-6 days with N-2 MAX/FGF basic/FGF-8b/ Shh-N/Ascorbic Acid Media.

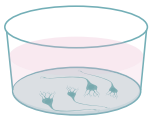


**Differentiation of Nestin-positive Cells to Dopaminergic Neurons (10-15 Days)**

**Culture** the cells in N-2 MAX/Ascorbic Acid Media without growth factors for 10-15 days.
**Replace** the media every 2 days.



**After** 10-15 days, dopaminergic neurons can be identified by staining for expression of Tyrosine Hydroxylase and Neuron-specific β-III Tubulin.



## PREPARATION OF REAGENTS FOR PLURIPOTENT STEM CELL PLATING & EXPANSION

**Use serological pipettes to transfer and remove solutions.**

**Note:** *The procedures described here are for mouse pluripotent stem cells. If using a different starting population, these procedures may need to be modified.*

**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.

**MEF Media** - Mix the following sterile ingredients to make 50.5 mL of media. Store at 2-8 °C for up to 1 month.

| Item | Amount | Final Concentration |
|---|---|---|
| DMEM | 45 mL | 90% |
| Fetal Bovine Serum | 5 mL | 10% |
| 100X Penicillin-Streptomycin-Glutamine | 0.5 mL | 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine |

**KO-ES Media** - Mix the following sterile ingredients to make 510.5 mL of media. 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 Media** - Dilute the ESGRO in KO-ES Media to make a 1400 U/mL solution. Prepare fresh as needed.

## PREPARATION OF MOUSE PLURIPOTENT STEM CELLS

Use serological pipettes to transfer and remove solutions.

### THAWING AND PLATING OF THE iMEF FEEDER CELLS

1. Gelatin coat one 100 mm tissue culture plate by covering the surface of the plate with 0.1% sterile gelatin for 15 minutes. One vial of 6 x $10^6$ iMEF is enough for two 100 mm plates.
2. Warm the MEF Media to 37 °C.
3. Thaw the vial of iMEF cells by quickly warming the cryotube in a 37 °C water bath until cells are just thawed and then immediately transferring the contents of one vial to a 15 mL conical tube containing at least 5 mL of pre-warmed MEF Media. Rinse the vial with an additional 1 mL of media to ensure the removal of all the cells.
4. Centrifuge at 200 x g in a clinical centrifuge for 5 minutes.
5. Remove the supernatant and flick the pellet.
6. Aspirate the 0.1% gelatin from the plate(s).
7. Resuspend the iMEF cells from step 5 (above) in 10 mL of MEF Media and transfer to the gelatin-coated plate at a density of approximately 3 x $10^6$ cells/100 mm plate. Incubate for 24 hours at 37 °C and 5% $CO_2$.

### PLURIPOTENT STEM CELL CULTURE

1. Twenty-four hours after the iMEF feeder cells are plated, warm the KO-ES/ESGRO Media in a 37 °C water bath for 20 minutes. In a 15 mL tube, resuspend 3 x $10^6$ pluripotent stem cells from a frozen stock vial with 10 mL of warm KO-ES/ESGRO Media.
2. Remove the MEF Media from feeder cells and wash once with 5 mL of KO-ES Media.
3. Add the PSC suspension on top of the feeder cells and incubate the culture at 37 °C and 5% $CO_2$. Twenty-four hours after the PSCs are plated, the cells should become attached to the feeder cell layer.
4. Feed the cells daily by replacing with fresh KO-ES/ESGRO Media. PSCs should start proliferating as indicated by the increasing size of colonies on top of the feeder cell layer.
5. Incubate the cells for 2 days (or until just before individual colonies contact each other) at 37 °C and 5% $CO_2$. Harvest the cells as follows:
    a. Remove the media. 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% $CO_2$.
    c. Tap the plate gently to dislodge the cells. Add 5 mL of KO-ES Media to neutralize the Trypsin.
    d. Transfer the cells to a 15 mL tube by gentle pipetting and centrifuge for 5 minutes at 220 x g.
    e. Remove the supernatant and gently resuspend the pluripotent stem cells with 5 mL of KO-ES/ESGRO Media. Count the cells. At this point, cells can be used in Stage I of this protocol.

## STAGE I: EXPANSION OF UNDIFFERENTIATED PLURIPOTENT STEM CELLS (3-4 DAYS)

1. Warm the KO-ES/ESGRO Media in a 37 °C water bath for 20 minutes.
2. Seed 3 x $10^6$ PSCs cells in 10 mL of warm KO-ES/ESGRO Media on a gelatin-coated plate.

   **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% $CO_2$, or until just before the cell colonies contact each other. Replace the KO-ES/ESGRO Media **daily.**

## STAGE II: FORMATION OF EMBRYOID BODIES (4 DAYS)

1. Discard the media. 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% $CO_2$.
3. Gently tap the plate to dislodge the cells. Add 5 mL of KO-ES Media 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 supernatant and resuspend the cell pellet in 5 mL of KO-ES Media. Count the cells. Approximately 1.5 x $10^7$ cells/plate can be expected from the harvest.
6. Seed 2 x $10^6$ cells on a 10 cm bacterial culture plate containing 10 mL of KO-ES Media.
7. Culture the cells for 4 days at 37 °C and 5% $CO_2$. Change the media on day 2 in the following manner:
   a. Transfer the embryoid bodies (EB) to a 15 mL tube by gently pipetting. **Do not centrifuge.**
   b. Allow the tube to stand until the EB settle to the bottom (about 5 minutes).
   c. Remove the media. Add 10 mL of fresh KO-ES Media.
   d. Transfer the EB back into the original plate by gently pipetting.
8. On day 4, proceed to Stage III.

# STAGE III: SELECTION OF NESTIN-POSITIVE CELLS (6-8 DAYS)

## REAGENT PREPARATION FOR THE SELECTION OF NESTIN-POSITIVE CELLS

**ITS Media** - Mix the following ingredients with deionized water to make 500 mL of media. Adjust the pH to 7.5 ± 0.2. Sterile filter the solution using a 500 mL, 0.2 µm 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 |
| $NaHCO_3$ | 1.2 g |
| ITS Supplement (100X) | 5 mL |

*Liquid DMEM/F-12 (no HEPES) can be used as an alternative to the powder form.

**Bovine Fibronectin Stock (1000X)** - Allow the Bovine Fibronectin Stock to stand for 30 minutes at room temperature **without agitation.** Swirl very gently. Aliquot and store at ≤-20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

**ITS/Fibronectin Media** - Dilute the Bovine Fibronectin Stock 200-fold in ITS Media to make a 5 µg/mL solution (e.g. 50 µL of Bovine Fibronectin Stock in 10 mL of ITS Media). Mix by gently swirling without vortexing. Prepare fresh as needed.

## PROCEDURE FOR THE SELECTION OF NESTIN-POSITIVE CELLS

1. At the end of Stage II, transfer the EB to a 15 mL tube by gently pipetting.
2. Allow the tube to stand until the EB settle to the bottom. Remove half of the media.
3. Rinse the original plate with 5 mL of fresh KO-ES Media and add to the 15 mL tube by gently pipetting.
4. Transfer the EB to a 10 cm tissue culture plate by gentle pipetting. Culture for 24 hours at 37 °C and 5% $CO_2$. At this time, the EB should become attached.
5. Change the media from KO-ES Media to 10 mL of ITS/Fibronectin Media.
6. Culture the cells for 6-8 days at 37 °C and 5% $CO_2$. Change the ITS/Fibronectin Media every 2 days. During this period, a monolayer will grow from the attached EB.

## STAGE IV: EXPANSION OF NESTIN-POSITIVE CELLS (4-6 DAYS)

### REAGENT PREPARATION FOR THE EXPANSION OF NESTIN-POSITIVE CELLS

**N-2 MAX Media** - Mix the following ingredients with deionized water to make 500 mL of media. Adjust the pH to 7.2 ± 0.2. Sterile filter the solution using a 500 mL, 0.2 µm 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 |
| $NaHCO_3$ | 845 mg |
| N-2 MAX Media Supplement (100X) | 5 mL |

*Liquid DMEM/F-12 (no HEPES) can be used as an alternative to the powder form.

**0.1% BSA in PBS** - Dissolve 10 mg of BSA in 10 mL of PBS. Sterile filter the solution using a 0.2 µm syringe filter and store at 2-8 °C for up to 3 months.

**FGF basic Stock (1000X)** - Add 250 µL of sterile 0.1% BSA in PBS to the hHman FGF basic vial. Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

**FGF-8b Stock (1000X)** - Add 250 µL of sterile 0.1% BSA in PBS to the Mouse FGF-8b vial. Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

**Shh-N Stock (1000X)** - Add 250 µL of sterile 0.1% BSA in PBS to the Mouse Shh-N vial. Aliquot and store at ≤ -20 °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 µm syringe filter. Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

**N-2 MAX/FGF basic/FGF-8b/Shh-N/Ascorbic Acid Media** - Dilute the FGF basic, FGF-8b, Shh-N and Ascorbic Acid stocks 1000-fold in N-2 MAX Media. Prepare fresh as needed.

**Poly-L-ornithine Stock (1000X)** - Dissolve the Poly-L-ornithine in sterile PBS to make a 15 mg/mL stock. Aliquot and store at ≤ -20 °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 µg/mL solution. Prepare fresh as needed.

**Fibronectin Solution (1X)** - Dilute the Fibronectin Stock 1000-fold in sterile PBS to make a 1 µg/mL solution. Mix by gently 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 °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 sterile PBS to each well. Incubate overnight at 37 °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 °C for 3 to 30 hours.
7. Discard the Fibronectin Solution. Wash each well once with 1 mL of sterile PBS before use.

## PROCEDURE FOR THE EXPANSION OF NESTIN-POSITIVE CELLS

1. Remove the ITS/Fibronectin Media 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 at 37 °C and 5% $CO_2$ for 5 minutes. Gently tap the plate to dislodge the cells. Add 5 mL of KO-ES Media to neutralize the Trypsin.
3. Transfer the cells to a 15 mL tube by gently 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 new 15 mL tube by gently pipetting.
5. Centrifuge the suspension for 5 minutes at 220 x g to pellet the cells.
6. Discard the supernatant and resuspend the cell pellet in N-2 MAX/FGF basic/FGF-8b/Shh-N/Ascorbic Acid Media.
7. Count the cells and seed on a Poly-L-ornithine/Fibronectin-coated 24-well plate.

   **Note:** *Seeding density: 3-5 x $10^5$ cells/well in 500 µL of media.*
8. Feed the cells with N-2 MAX/FGF basic/FGF-8b/Shh-N/Ascorbic Acid Media every day for 4-6 days. Cell confluency should reach close to 100% at this time.
9. **Optional:** Remove and fix one coverslip for staining with Anti-Nestin Antibody.

# STAGE V: DIFFERENTIATION TO DOPAMINERGIC NEURONS (10-15 DAYS)

## REAGENT PREPARATION FOR DIFFERENTIATION TO DOPAMINERGIC NEURONS

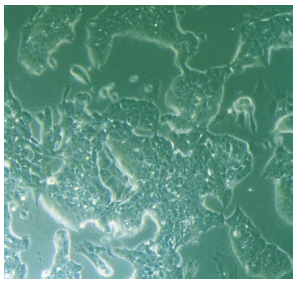
**N-2 MAX/Ascorbic Acid Media** - Dilute the Ascorbic Acid Stock 1000-fold in N-2 MAX Media to make a 200 µM solution. Prepare fresh as needed.

## PROCEDURE FOR DIFFERENTIATION TO DOPAMINERGIC NEURONS

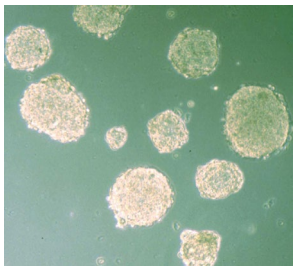
1. Induce differentiation of the expanded Nestin-positive cells from Stage IV by culturing the cells in N-2 MAX/Ascorbic Acid Media (without growth factors) for 10-15 days. Change the N-2 MAX/Ascorbic Acid Media every 2 days.
2. After 10-15 days, dopaminergic neurons can be identified by staining with Tyrosine Hydroxylase and Neuron-specific β-III Tubulin antibodies (refer to reference 8).

## CELL MORPHOLOGY BY STAGE

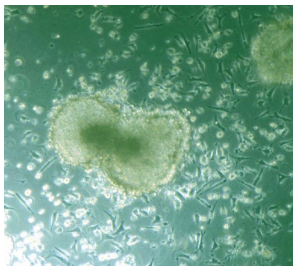
**Stage I: Expansion of Undifferentiated ES Cells**



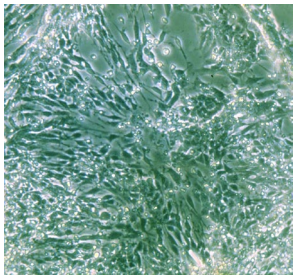
**Stage II: Formation of Embryoid Bodies**



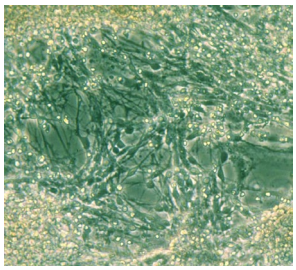
**Stage III: Selection of Nestin-positive Cells**



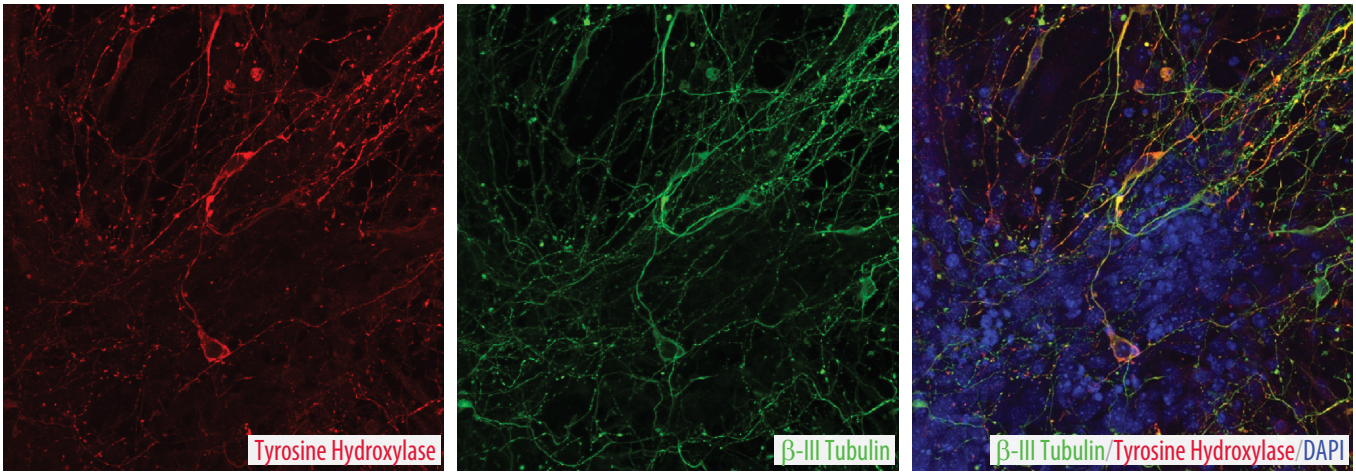
**Stage IV: Expansion of Nestin-positive Cells**



**Stage V: Differentiation of Dopaminergic Neurons**



## DATA EXAMPLES




**Characterization of Dopaminergic Neurons Generated from Human Pluripotent Stem Cells.** Dopaminergic neurons were generated from human pluripotent stem cells using the StemXVivo Dopaminergic Neuron Differentiation Kit. Tyrosine Hydroxylase was detected using Mouse Anti-Human Tyrosine Hydroxylase Monoclonal Antibody (R&D Systems, Catalog # MAB7566). The cells were stained with NorthernLights™ (NL)557-conjugated Donkey Anti-Mouse Antigen Affinity Purified Secondary Antibody (R&D Systems, Catalog # NL007; red). Neuron-specific β-III Tubulin was detected using NL493-conjugated Mouse Anti-Neuron-specific β-III Tubulin (Clone TuJ-1) Monoclonal Antibody (R&D Systems, Catalog # NL1195G; green).

## REFERENCES


1. Alves dos Santos, M.T. and M.P. Smidt (2011) Neural Development **6**:23.
2. Volkow, N.D. and J.S. Fowler (2000) Cereb. Cortex **10**:318.
3. Eyles, D. *et al.* (2012) Transl. Psychiatry (2012) **2**:e81.
4. Zigmond, M.J. *et al.* (1999) Fundamental Neuroscience, Academic Press, p. 965.
5. Kriks, S. *et al.* (2011) Nature **480**: 547.
6. Bjorklund, A. and O. Lindvall (2000) Nature Neuroscience **3**:537.
7. Hayashi, T. *et al.* (2013) J. Clinical Investigation **123**:272.
8. Lee, S.H. *et al.* (2000) Nature Biotechnology **18**:675.
9. Kawasaki, H. *et al.* (2000) Neuron **28**:31.
10. Nagy, A. (1993) Proc. Natl. Acad. Sci. USA **90**:8424.