# Human Dopaminergic Neurogenesis Kit
# &
# Dopaminergic Differentiation Growth Factor Sampler

Catalog No. SCR135 & SCR128

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

USA & Canada Phone: +1(800) 645-5476
In Europe, please contact Customer Service:
France: 0825.045.645;
Spain: 901.516.645 Option 1
Germany: 01805.045.645
Italy: 848.845.645
United Kingdom: 0870.900.46.45
For other locations across the world please visit www.millipore.com/offices

## Introduction

EMD Millipore's Human Dopaminergic Neurogenesis Kit (Cat No. SCR135) contains the necessary growth factors and complete differentiation media (Cat No. SCM110 and SCM111) needed to differentiate human ES/iPS cells into dopaminergic neurons using a 21 day differentiation protocol. The protocol has been validated with 4 human ES and iPS lines for efficient dopaminergic neuronal differentiation.

H1: 30% TH positive neurons
H9: 50-70% TH positive neurons.
Two Human iPS cell lines: ~ 50% TH positive neurons.

The Dopaminergic Differentiation Growth Factor Sampler (SCR128) contains a collection of five validated growth factors that are routinely used to induce differentiation of human pluripotent ES/iPS cells to dopaminergic neurons along with a reliable high affinity antibody marker, anti-tyrosine hydroxylase, to aid in quantifying the percentage yields from differentiation experiments. Included in the kit are the following:

- 2 vials of human recombinant Sonic Hedgehog (Shh) (5 μg/vial)
- 1 vial of human recombinant FGF-8 (5 μg/vial)
- 1 vial of human recombinant BDNF (2 μg/vial)
- 1 vial of human recombinant GDNF (2 μg/vial)
- 1 vial of human recombinant TGF-β-III (2 μg/vial)
- 1 vial of anti-tyrosine hydroxylase, rabbit polyclonal (20 μL)

The Human ES/iPS Neural Induction Medium (Cat. No. SCM110) and Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111) are robust and user-friendly media that contain the necessary media and reagents for the rapid production of expandable neural progenitor cells and terminally differentiated neurons from pluripotent human ES and iPS cells using small molecule inducers.

*For Research Use Only; Not for use in diagnostic procedures*

## Kit Components

**Dopaminergic Differentiation Growth Factor Sampler (Cat. No. SCR128)**

1. Sonic HedgeHog (Shh), Human Recombinant (Part No. GF174-5UG): Two 5 μg vials. Lyophilized powder. Store at -20°C.

2. Fibroblast Growth Factor-8 (FGF-8), Human Recombinant (Part No. GF110-5UG): One 5 μg vial. Lyophilized powder. Store at -20°C.

3. Brain Derived Neurotrophic Factor (BDNF), Human Recombinant (Part No. GF029-2UG): One 2 μg vial. Lyophilized powder. Store at -20°C.

4. Glial Derived Neurotrophic Factor (GDNF), Human Recombinant (Part No. GF030-2UG): One 2 μg vial. Lyophilized powder. Store at -20°C.

5. Transforming Growth Factor βIII (TGF-β-III), Human Recombinant (Part No. GF176-2UG): One 2 μg vial. Lyophilized powder. Store at -20°C.

6. Anti-Tyrosine Hydroxylase antibody, rabbit polyclonal (Part No. AB152-20UL): One 20 μL vial. Store at -20°C.

**Human ES/iPS Neural Induction Medium (Cat. No. SCM110)**

1. Neural Induction Basal Medium (Part No. CS210991). One 125 mL bottle.
2. Neural Supplement 1 (50X) (Part No. CS210992). One 2.5 mL bottle.
3. Neuro2 Medium Supplement (Part No. SCM012-S). One 1.25 mL bottle.
4. GSK3 and TGFβR Inhibitor Cocktail (1000X) (Part No. CS210983-150UL). One 150 µL vial.
5. AMPK Inhibitor (1000X) (Part No. CS210995-60UL). One 60 µL vial.

**Human ES/iPS Neuronal Differentiation Medium: (Cat. No. SCM111)**

1. Neuronal Differentiation Basal Medium (Part No. CS211007). One 100 mL bottle.
2. Neural Supplement 1 (50X) (Part No. CS210992). One 2.5 mL bottle.
3. Adenosine 3', 5'-cyclic Monophosphate, N6, O2'-Dibutyryl-, Sodium Salt (Cat. No. 28745-25mg). One vial containing 25 mg of lyophilized powder.
4. Ascorbic Acid 2-Phosphate (Part No. 2004011). One 0.5 mL vial of 100 mM solution

## Materials Not Supplied but required

1. Tissue culture-wares and supplies.
2. Medium used to culture hES/iPS cells. For example, DMEM/F12 (Cat. No. DF-041-B) supplemented with 20% KnockOut™ Serum Replacement (LIFE Cat. No. 10828010), 1% non-essential amino acids (Cat. No. TMS-001-C), 2 mM glutamine (Cat. No. TMS-002-C), 0.1mM β-mercaptoethanol (Cat. No. ES-007-E) and 8 ng/mL FGF-2 (Cat. No. GF003).
3. Serum free hES/iPS culture medium. For example, PluriSTEM™ (Cat. No. SCM130)
4. EmbyroMax® ES Cell-Qualified Penicillin-Streptomycin Solution, 100X (Cat. No. TMS-AB2-C) (Optional).
5. Basement membrane protein extracts such as Matrigel (BD Cat. No. 356234)
6. Phosphate-Buffered Saline (1X PBS) (Cat. No. BSS-1005-B)
7. EmbryoMax® 0.1% Gelatin Solution (Cat. No. ES-006-B)
8. EmbryoMax ES Cell-Qualified Ultra Pure Water, sterile $H_20$, 500 mL (Cat. No. TMS-006-B)
9. Accutase (Cat. No. SCR005)
10. Rock Inhibitor, Y-27632 (Cat. No. SCM075)
11. Laminin (Cat. No. CC095)
12. Poly-L-ornithine (Cat. No. A-004-C)
13. Fixative (e.g. 4% Paraformaldehyde in 1X PBS)
14. Blocking Solution (5% normal donkey serum, 5% BSA ±0.1% Triton X-100 in 1X PBS)
15. Fluorescent-labeled secondary antibodies. Donkey anti-mouse IgG, Cy3-conjugated (Cat. No. AP192C) and donkey anti-rabbit IgG, Cy3-conjugated (Cat. No. AP182C)
16. 4'-6-Diamidino-2-phenylindole (DAPI) / PBS solution

17. Scepter Handheld Automated Cell Counter (Cat. No. PHCC00000) or Hemacytometer
18. Millicell EZ SLIDE 8-well glass, sterile (Cat. No. PEZGS0896)
19. ENStem-A™ Neural Expansion Medium (Cat. no. SCM004)

## Storage

- Growth factors are provide as a lyophilized power and while stable at room temperature, is best stored desiccated below 0°C. After a quick spin, the lyophilized powder can be reconstituted to 0.1 – 1.0 mg/mL with water or other buffered solutions. 0.1% bovine serum albumin (BSA) or human serum albumin (HSA) can be added to the reconstituted growth factor for better stability. Reconstituted growth factors should be stored in undiluted aliquots at -20°C for up to six months. Avoid repeated freeze/thaw cycles.

- Anti-tyrosine hydroxylase antibody should be stored at -20°C to -70°C in undiluted aliquots for up to 6 months after date of receipt. Avoid repeated freeze/thaw cycles.

- All components in SCM110 and SCM111 should be stored at -20°C. Good for 3 months from date of receipt or until expiration date on bottle when reagents are handled and stored appropriately.

## Human ES/iPS Neural Induction Medium (Cat. No. SCM110)

| **Description** | Human ES/iPS Neural Induction Medium (Cat. No. SCM110) |
|---|---|
| **Kit Components** | 1. Neural Induction Basal Medium (Part No. CS210991). One 125 mL bottle.<br>2. Neural Supplement 1 (50X) (Part No. CS210992). One 2.5 mL bottle.<br>3. Neuro2 Medium Supplement (Part No. SCM012-S). One 1.25 mL bottle.<br>4. GSK3 and TGFβR Inhibitor Cocktail (1000X) (Part No. CS210983-150UL). One 150 µL vial.<br>5. AMPK inhibitor (1000X) (Part No. CS210995-60UL). One 60 µL vial. |
| **Storage & Stability** | Store at -20°C. Good for 3 months from date of receipt or until expiration date on bottle when reagents are handled and stored appropriately. **Please note:** The supplemented ES/iPS Neural Induction Medium should be stored at 2 to 8°C for up to 4 weeks. |
| **Applications** | Human ES/iPS Neural Induction Medium is a defined, serum-free medium that has been optimized to induce neural differentiation from human ES and iPS cells. |

### Quality Control

Each lot is tested for the following parameters:

Sterility testing: Negative for bacterial and fungal growth

pH: 7.2 ± 0.2

### Media Preparation

All media components should be stored at -20°C until ready to be used. Thaw media components at 4°C overnight or at room temperature for 4 hours before assembling the complete media. Neural Supplement 1 (50X) and Neuro 2 Medium Supplement may be thawed at room temperature for 30 minutes before assembling the complete media.

Prepare Human ES/iPS Neural Induction Medium 1 and 2 (NIM 1 and 2)

**Human ES/iPS Neural Induction Medium I (NIM 1):**

49.0 mL Neural Induction Basal Medium (Part No.CS210991)
1.0 mL Neural Supplement 1 (50X) (Part No. CS210992)
0.5 mL Neuro 2 Medium Supplement (Part No. SCM012-S)
50 µL GSK3 and TGFβR Inhibitor Cocktail (1000X) (Part No. CS210983-150UL)
50 µL AMPK Inhibitor (1000X) (Part No. CS210995-60UL)

~50 mL Total volume

Mix thoroughly in a 50 mL conical tube. Store at 2 – 8°C for up to 4 weeks.

**Optional:** While not necessary, antibiotics may be added to the medium. If desired, add 0.5 mL 100X Penicillin-Streptomycin Solution (Cat. No. TMS-AB2-C, not included in the kit). Complete medium may be sterile filtered using a 0.22 µm filter (Cat. No. SCGP00525, not included in the kit).

**Human ES/iPS Neural Induction Medium 2 (NIM 2):**

49.0 mL Neural Induction Basal Medium (Part No. CS210991)
1.0 mL Neural Supplement 1 (50X) (Part No. CS210992)
0.5 mL Neuro2 Medium Supplement (Part No. SCM012-S)
50 µL GSK3 and TGFβR Inhibitor Cocktail (1000X) (Part No. CS210983-150UL)

~50 mL Total volume

Mix thoroughly in a 50 mL conical tube. Store at 2-8°C for up to 4 weeks. Supplemented with sonic hedgehog to final 200ng/mL and FGF-8 to final 100ng/mL for mid-brain cell fate differentiation.

**Optional:** While not necessary, antibiotics may be added to the medium. If desired, add 0.5 mL 100X Penicillin-Streptomycin Solution (Cat. No. TMS-AB2-C, not included in the kit). Complete medium may be sterile filtered using a 0.22 µm filter (Cat. No. SCGP00525, not included in the kit).

## Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111)

| **Description** | Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111) |
|---|---|
| **Kit Components** | 1. Neuronal Differentiation Basal Medium (Part No. CS211007). One 100 mL bottle.<br>2. Neural Supplement 1 (50X) (Part No. CS210992). One 2.5 mL bottle.<br>3. Adenosine 3', 5'-cyclic Monophosphate, N6, O2'-Dibutyryl-, Sodium Salt (Cat. No. 28745-25mg). One vial containing 25 mg lyophilized powder.<br>4. Ascorbic Acid 2-Phosphate (Part No. 2004011). One 0.5 mL vial of 100 mM solution. |
| **Storage & Stability** | Store at -20°C. Good for 3 months from date of receipt or until expiration date on bottle when reagents are handled and stored appropriately. **Please note:** The supplemented ES/iPS Neuronal Differentiation Medium should be stored at 2 to 8°C for up to 1 week. |
| **Applications** | βIII-tubulin positive neurons may be observed after 14 days of differentiation. |

### Quality Control

Each lot is tested for the following parameters:

Sterility testing: Negative for bacterial and fungal growth

pH: 7.2 ± 0.2

### Media Preparation

All media components should be stored at -20°C until ready to be used. Thaw media components at 4°C overnight or at room temperature for 4 hours before assembling the complete media. After thawing, the Neuronal Differentiation Basal Medium, Neural Supplement 1 (50X) and Ascorbic Acid 2-Phosphate may be stored separately at 2 – 8°C for up to 1 month. For optimal results, the Complete Human ES/iPS Neuronal Differentiation Medium should always be prepared fresh.

1. Prepare a 0.5M stock of Dibutyryl cyclic AMP (1000X): Reconstitute Adenosine 3', 5'-cyclic Monophosphate, N6, O2'-Dibutyryl-, Sodium Salt (Dibutyryl cyclic-AMP) with 100 µL of sterile water. Aliquot into 25 µL per vial and store the aliquots at -20ºC for up to 3 months.

2. To make 50 mL Human ES/iPS Neuronal Differentiation Medium (NDM):

   **Human ES/iPS Neuronal Differentiation Medium (NDM)**

   49.0 mL Neuronal Differentiation Basal Medium (Part No. CS211007)
   1.0 mL Neural Supplement1 (50X) (Part No. CS210992)
   50 µL 0.5M Dibutyryl cyclic-AMP solution (1000X)
   100 µL 100mM Ascorbic Acid 2- Phosphate solution (Part No. 2004011)

   ~ 50 mL Total Volume

   Mix thoroughly in a 50 mL conical tube. Store at 2 – 8°C for up to 1 week.

3. Supplemented with BDNF to final 20ng/mL, GDNF to final 20ng/mL and TGFβIII to final 1ng/mL of dopaminergic neuronal cell differentiation.

**Optional:** While not necessary, antibiotics may be added to the medium. If desired, add 0.5 mL 100X Penicillin-Streptomycin Solution (Cat. No. TMS-AB2-C, not included in the kit) to 50mL medium. Complete medium may be sterile filtered using a 0.22 µm filter (Cat. No. SCGP00525, not included in the kit).

## Dopaminergic Differentiation Protocol (for 6-well plates)

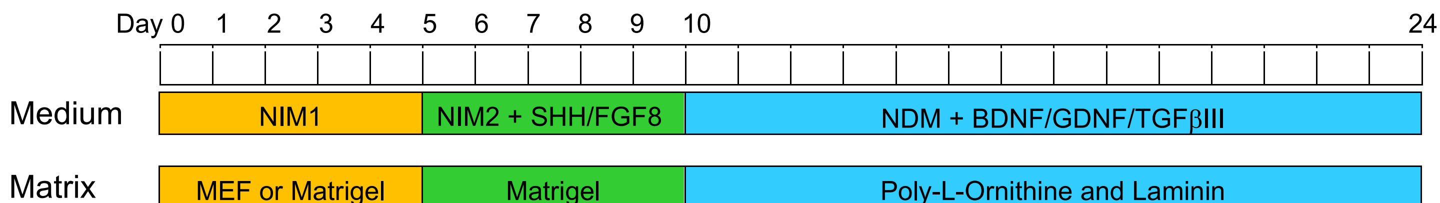


Figure 1 Schematic time course for SCR135 dopaminergic neurogenesis

## Preparation of Coated Plates

**Table 1: Volumes recommended for coating cultureware:**

| CultureWare | Coating Volume (mL) | Cell Culture Volume (mL) | Surface Area ($cm^2$) |
|---|---|---|---|
| 96 well plate | 0.1 mL/well | 0.2 mL/well | 0.34 |
| 24 well plate | 0.5 mL/well | 1 mL/well | 2.0 |
| 6 well plate | 2 mL/well | 3 mL/well | 9.6 |
| T25 flask | 3 mL | 5 mL | 25 |
| T75 flask | 8 mL | 20 mL | 75 |

**MATRIGEL COATING:**

Neural induction of Human ES/iPS cells and expansion of the resulting neural progenitor cells require cultureware that are coated with Matrigel.

1. Thaw Matrigel on ice or at 2 – 8ºC overnight. Keep on ice and use pre-cooled medium and pipettes to avoid gelling of the ECM gel. **IMPORTANT: Do not thaw Matrigel at temperatures higher than 15ºC to avoid gelling.**
2. Dilute the Matrigel 1:20 with cold DMEM medium. For example, to every 0.5 mL Matrigel, add 9.5 mL cold DMEM medium for a total volume of 10 mL. Scale accordingly to the volumes required.
3. Cover the cultureware with the recommended volumes (see Table 1). Incubate at room temperature for 1 hour or 2 – 8ºC overnight.

   ***Note**: If not used immediately, Matrigel coated cultureware should be sealed with parafilm to prevent evaporation and can be stored at 2 – 8ºC for up to one week or stored frozen at -20ºC for up to 3 months.*

4. Prior to seeding the cells, bring the plate back to room temperature, remove the coating solution and rinse with 1X PBS to remove residual ECMs. **IMPORTANT: Do not allow the coating to dry out.**

### POLY-L-ORNITHINE AND LAMININ COATING:

For terminal differentiation, Human ES/iPS derived neural progenitor cells should be cultured on poly-L-ornithine and laminin coated cultureware before initiating terminal differentiation. The following procedures are recommended.

1. Prepare coating solution by diluting Poly-L-Ornithine solution (0.1mg/mL, Cat. No. A-004-C) 1:10 with PBS to make final 10 µg/mL solution.

2. Add sufficient volumes of poly-L-ornithine solution to cover the whole surface of the tissue cultureware. Refer to Table 1 for recommended volumes. Incubate at room temperature for one hour or 2 – 8ºC overnight.

3. Thaw laminin (Cat. No. CC095), provided at 1 mg/mL on ice. Using sterile 1X PBS (Cat. No. BSS-1006-B), dilute the laminin to a final concentration of 10 µg/mL. Sterilize with a 0.22 µm filter.

4. Aspirate the poly-L-ornithine solution. Add sufficient volume of 10 µg/mL laminin solution to cover the whole surface of the tissue cultureware. Refer to Table 1 for recommended volumes. Incubate at room temperature for one hour or 2 – 8ºC overnight.

5. Coated plates and flasks can be stored in the laminin solution at -20ºC for up to 6 months. The plates should be wrapped in plastic saran wrap before storage at -20ºC.

6. Just before use, aspirate the laminin solution in the coated cultureware and wash once with 1X PBS. Add Human ES/iPS Neuronal Differentiation Medium to the tissue cultureware. **IMPORTANT: Do not allow plates or wells to dry out as it may result in uneven cell attachment.**

---

## Step 2: Neural Induction with NIM 1 and NIM 2 Medium (SCM110)

The protocol is based on the culture of human ES/iPS cells in a 6 well plate. Volumes should be adjusted to the tissue cultureware being used. The protocol can accommodate both feeder-based and feeder-free cultures of human ES/iPS cells without the need to optimize cell density.

1. Prepare high quality human ES/iPS by passaging healthy undifferentiated colonies onto a freshly cultured mouse embryonic fibroblast feeder plate in KOSR based medium or in feeder-free conditions using Matrigel coated plates and feeder-free, serum free based medium. Manual passaging is recommended in this step to maximize the purity of undifferentiated colonies and to minimize the contamination from spontaneously differentiated cells. Alternatively, spontaneously differentiated areas may be removed from the culture.

2. Approximately 48 to 72 hours after passaging, remove the growth medium and change to NIM 1 medium at 0.3 $mL/cm^2$, (i.e. 3 mL per well of a 6 well plate). This is considered Day 0 of induction.

3. Change with fresh NIM 1 medium every other day (i.e. on day 2 and day 4) for 5 days.

4. On day 4 or 5, prepare Matrigel coated plates. Please refer to section on “Preparation of Coated Plates”. Matrigel coating should be at 1:20 dilution.

5. On day 5, carefully aspirate the NIM 1 medium. Rinse the culture with 2 mL of 1X D-PBS w/o $Ca^{2+}$ & $Mg^{2+}$ buffer (Cat. No. BSS-1006-B). Observe the confluency of the plate. Three-dimensional growth of hES/iPS on the edges or in the center of the colony may be observed. Determine the passaging ratio to use. If the confluency of the plate is less than 50%, use a 1:1 split ratio. If the culture plate is 50 to 75% confluent, use a 1:2 split ratio. If the confluency is higher than 75%, use a 1:3 split ratio. The edges of colonies may lift off as the MEF culture may no longer be able to support cell attachment.

6. Carefully aspirate the 1X D-PBS w/o $Ca^{2+}$ & $Mg^{2+}$ buffer (Cat. No. BSS-1006-B) from each well. Add 1 mL 1X D-PBS w/o $Ca^{2+}$ & $Mg^{2+}$ buffer to each well and incubate at 37ºC for 5 minutes. After 5 minutes, use a p1000 pipette to dislodge the colonies from the plate and transfer the colonies to a 15 mL conical tube. Rinse well(s) with an additional 1 mL 1X D-PBS w/o $Ca^{2+}$ & $Mg^{2+}$ buffer to collect and add residual colonies to the cell suspension in the 15 mL conical tube.

   ***Note:*** *The use of the p1000 pipette will generate small clumps. Do not dissociate into single cell suspension as this will lead to high cell death.*

7. Centrifuge at 200 rpm for 5 minutes.

8. Remove the supernatant and resuspend the cell pellet in 2 mL NIM 2. Set aside.

9. In the meantime, remove the coating solution from a new Matrigel coated 6-well plate (1:20 Matrigel dilution) and add 2 mL NIM 2 supplemented with 200ng/mL sonic hedgehog (Shh) and 100ng/mL FGF-8 medium to each well.

10. Depending upon the state of confluency determined from step 5, plate the cells suspension from step 8 at a 1:1 to a 1:3 split ratio (i.e., from one well of a 6 well plate to one to three wells of freshly coated Matrigel plate (1:20 Matrigel dilution).

11. Change with fresh NIM 2 supplemented with 200ng/mL SHH and 100ng/mL FGF8 medium every other day for an additional 5 days. Total induction = 10 days.

12. On day 10, a high percentage of cells should have a neural rosette structure (See Figure 1). The relative degree of neural rosette structures may vary between cell lines and subclones. Rinse the cells with 1 mL/well Accutase (Cat. No. SCR005). Aspirate and add 1 mL/well Accutase and incubate at 37ºC for 5 minutes to dissociate the cells.

13. Transfer the single cell suspension to a 15 mL conical tube with a p1000 pipette and rinse the plate with 1 mL NIM 2 supplemented with 200ng/mL SHH and 100ng/mL FGF-8 to collect any residual cells. Combine both cell suspensions and centrifuge at 1000 rpm for 5 minutes.

14. Remove the supernatant. The cell pellet may be resuspended in cryopreservation medium (Cat. No. SCM011) for future experiment or resuspend in NDM (Cat. No. SCM111) supplemented with 20ng/mL BDNF, 20ng/mL GDNF and 1ng/mL TGFβIII and continue the differentiation following Step 3 protocol.

## Step 3: Neuronal differentiation with Human ES/iPS Neuronal Differentiation Medium (SCM111)

The protocol is based on the terminal differentiation of Human ES/iPS derived dopaminergic NPCs in 24 well plates.

1. Coat 24 well plates with poly-L-ornithine and laminin. Please refer to the section "**Preparation of Coated Plates**".
2. Prepare the Human ES/iPS Neuronal Differentiation Medium. Please refer to the section "**Media Preparation**" on pg.
3. Before using, remove the poly-L-ornithine and laminin coating solution and add 0.5 mL Complete ENStem-A Expansion Medium containing 1X Glutamine and 20 ng/mL FGF-2 to each well.
4. Plate human ES/iPS derived neural progenitor cells at 1 – 2 $x10^4$ $cells/cm^2$ or 20,000 to 40,000 cells per well of a 24 well plate in Complete ENStem-A Expansion Medium containing 1X glutamine and 20 ng/mL FGF-2. Total volume per well = 1 mL. At this plating density, cells should be 50 – 70% confluent by the next day. Incubate overnight at 37ºC.
5. The next day, carefully remove approximately 80% of the medium (i.e. this corresponds to 0.8 mL volume) and replace with 0.8 mL per well of fresh Complete Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111). Total volume per well = 1 mL.

   ***Note:*** *Subsequent media changes should always be done at 80% volume (i.e. 0.8 mL) to avoid perturbation to cell attachment during media changes*.
6. Change with fresh Complete Human ES/iPS Neuronal Differentiation Medium every other day for 10 to 14 days. Significant neurite outgrowth should be observed after 9 days of differentiation.
7. Cells can be fixed and stored at 2 to 8ºC in 1X PBS with 0.1% sodium azide for up to 3 months.

## Immunostaining Protocol

1. Fix the cells by incubation in 4% freshly prepared paraformaldehyde in 1X PBS for 15 min at room temperature.
2. Carefully aspirate the fixative and rinse four times (5 – 10 min each) with 1X PBS. Prepare the blocking solution (e.g. 5% BSA, 5% normal donkey serum, 0.1-0.2% TX-100 in 1X PBS).
3. Carefully remove the 1X PBS wash and add the blocking solution. Incubate at room temperature for 2 hours or overnight at 4ºC. **IMPORTANT: Do not shake the cells.**
4. Dilute the primary anti-tyrosine hydroxylase antibody (provided) to 1:400 in the blocking solution.
5. In a separate control well, add equivalent concentrations of normal rabbit serum in blocking solution.
6. Carefully remove the blocking solution from each well and add the appropriate diluted primary antibody to each well. Incubate at room temperature for 4 hours or 2 to 8 º C overnight. **IMPORTANT: Do not shake.**

7. Remove the primary antibody solution. Wash the cells four times with blocking solution (5 minutes each wash).

8. Dilute secondary antibodies to 1 to 5 μg/mL concentration (1:200 to 1:1000 dilutions) with the blocking solution.

9. Remove the last wash and add the appropriate diluted secondary antibody to each well. Incubate at room temperature for 1 hour. Cover the plate with tinfoil to protect from the light.

10. Remove the secondary antibody solution. Wash 4 times (5 minutes each) with 1X PBS.

11. Prepare the DAPI dye: Dilute the DAPI with 1X PBS to 1 μg/mL (1:10,000 dilution)

12. Remove the last wash, add DAPI staining solution and incubate at room temperature for 15 minutes.

13. Remove the DAPI solution; wash twice with 1X PBS.

14. If using glass coverslips, mount the coverslip onto glass slides using anti-fading mounting solution (e.g. DABCO/PVA)

15. Visualize the cell staining with a fluorescent microscope. ***Note:*** Be sure to use the correct filter to visualize fluorescent-labeled cells.

## Results

The following are representative results obtained by using the SCR135 differentiated H9 human ESCs and STEMCCA human iPS cells to dopaminergic cells.

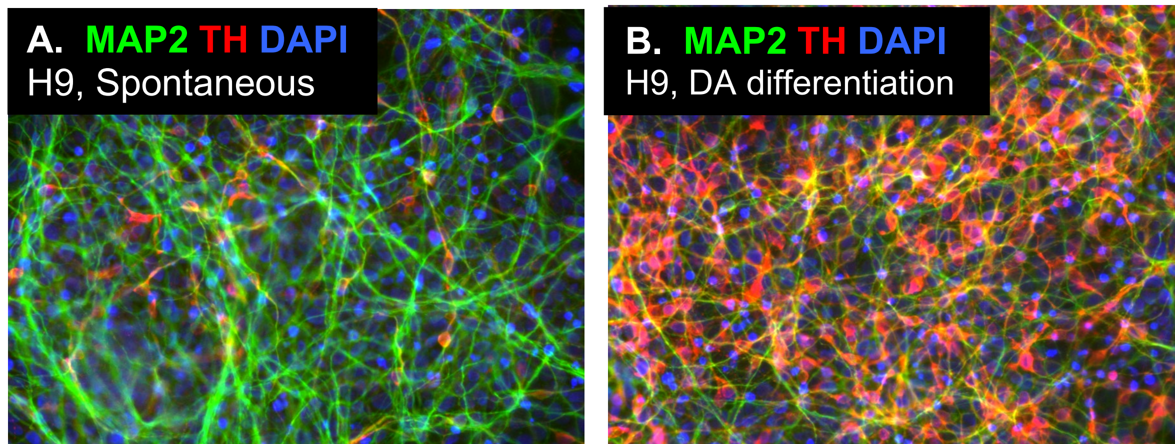


Figure 2 Human ES cell line, H9, derived neural progenitors were subjected to either spontaneous differentiation via growth factor withdrawal (**A**) or directed differentiation to dopaminergic neurons using SCR135 (**B**). Cells were fixed and stained with neuronal marker, MAP2 (MAB3418) and dopaminergic neuron marker, TH (AB152) at 1:400 dilution and counter stained with nuclei dye, DAPI. An elevated level of TH positive cells was observed when cells were differentiated using the Dopaminergic Differentiation Growth Factor Sampler Kit.

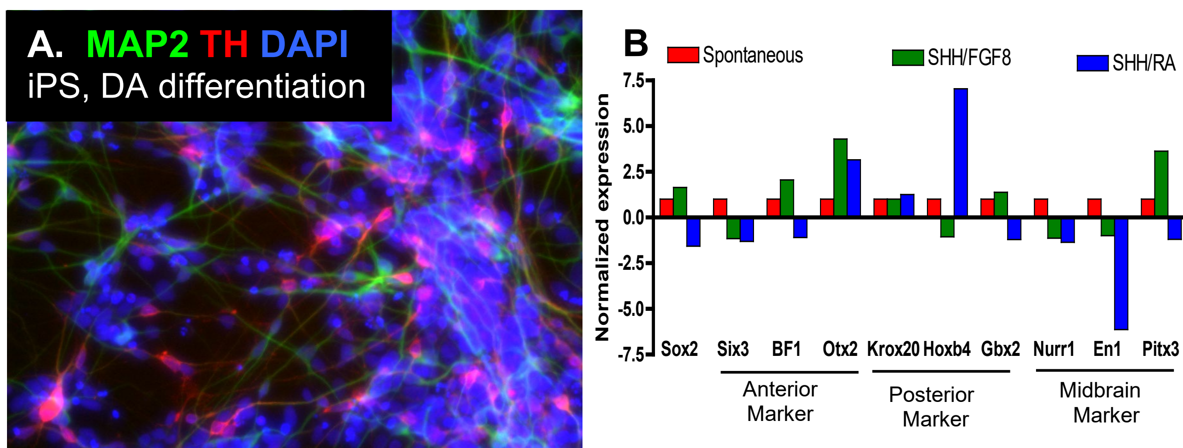


Figure 3 STEMCCA derived iPS cells were directed to dopaminergic neural lineage with SCR135. 40 to 50% of neuronal cells stained positive for tyrosine hydroxylase (TH) at day 24 (**A**). (**B**) After 5 day of NIM1 induction, cells were cultured in either NIM2 (spontaneous), or NIM2 with 200ng/mL sonic hedgehog, 100ng/mL FGF8 (SHH/FGF8), or 200ng/mL sonic hedgehog, 1μM retinoic acid (SHH/RA) for 10 days to prime cells for midbrain or hindbrain fate. After 10 days, cells were harvested and the gene expression pattern were analyzed by qRT-PCR. Fold of expression was calculated based on ΔCt value (normalized with GAPDH expression) then normalized with the spontaneous sample (spontaneous = 1). Cells shows higher midbrain and anterior brain marker expression and reduced posterior brain marker expression in the SHH/FGF8 culture.

## References

1. Perrier A. L., Tabar, V., Barberi, T., Rubio, M. E., Bruses, J., Topf, N., Harrison, N. L., and Studer, L. (2004) Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci USA* **101(34)**: 12543-8.

2. Yan, Y., Yang, D., Zarnowska, E. D., Du, Z., Werbel, B., Valliere, C., Pearce, R. A., Thomson, J. A., and Zhang, S. C. (2005) Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells* **23(6)**: 781-90.

3. Cho, M. S., Lee, Y. E., Kim, J. Y., Chung, S., Cho, Y. H., Kim, D. S., Kang, S. M., Lee, H., Kim, M. H., Kim, J. H., Leem, J. W., Oh, S. K., Choi, Y. M., Hwang, D. Y., Chang, J. W., Kim, D. W. (2008) Highly efficient and large-scale generation of functional dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci USA* **105(9)**: 3392-7.

4. Kriks, S. and Studer, L. (2009) Protocols for generating ES cell-derived dopamine neurons. *Adv Exp Med Bio*. **651**:101-11.

5. Nguyen, H. N., Byers, B., Cord, B., Shcheglovitov, A., Byrne, J., Gujar, P., Kee, K., Schule, B., Dolmetsch R. E., Langston W., Palmer, T. D., and Pera R. R. (2011) LRRK2 mutant iPSC-derived DA neurons demonstrate increased susceptibility to oxidative stress. *Cell Stem Cell* **8(3)**: 267-80.

## Warranty

**EMD Millipore Corporation** ("EMD Millipore") warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of one year from shipment of the products. **EMD MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of EMD Millipore products appearing in EMD Millipore's published catalogues and product literature may not be altered except by express written agreement signed by an officer of EMD Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, EMD Millipore Corporation's sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies EMD Millipore Corporation promptly of any such breach. If after exercising reasonable efforts, EMD Millipore Corporation is unable to repair or replace the product or part, then EDM Millipore shall refund to the Company all monies paid for such applicable Product. **EMD MILLIPORE CORPORATION SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY COMPANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**

Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.

(c) 2009 - 2014: Merck KGaA, Darmstadt, Germany. The M mark is a registered trademark of Merck KGaA, Darmstadt, Germany. All rights reserved. No part of these works may be reproduced in any form without permission in writing.

**Cat No. SCR135**

2020/July
Version 4.0:
Document Number: SCR128MAN