STEMdiff™ Forebrain Neuron Differentiation and Maturation Kits

Differentiation and maturation kits for generation of functional forebrain-type neurons from human pluripotent stem cells

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Product Description

STEMdiff™ Forebrain Neuron Differentiation Kit (Catalog #08600/100-1691) is used to generate forebrain neuron precursors from human pluripotent stem cell (hPSC)-derived neural progenitor cells (NPCs) using STEMdiff™ SMADi Neural Induction Kit (Catalog #08581) via either the embryoid body or monolayer protocol. The resulting forebrain neuron precursors can be matured using STEMdiff™ Forebrain Neuron Maturation Kit (Catalog #08605/100-1659) to produce a mixed population of excitatory and inhibitory forebrain-type (FOXG1-positive) cortical neurons. Cells derived using these products are versatile tools for modeling human neurological development and disease, drug screening, toxicity testing, and cell therapy validation.

Product Information

PRODUCT NAME	CATALOG #	SIZE	KIT COMPONENTS
STEMdiff™ Forebrain Neuron Differentiation Kit	08600	100 mL	• STEMdiff™ Forebrain Neuron Differentiation Basal Medium (80 mL) • STEMdiff™ Forebrain Neuron Differentiation Supplement (20 mL)
	100-1691	500 mL	• STEMdiff™ Forebrain Neuron Differentiation Basal Medium (400 mL) • STEMdiff™ Forebrain Neuron Differentiation Supplement (100 mL)
STEMdiff™ Forebrain Neuron Maturation Kit	08605	125 mL	• BrainPhys™ Neuronal Medium (100 mL) • STEMdiff™ Forebrain Neuron Maturation Supplement (25 mL)
	100-1659	625 mL	• BrainPhys™ Neuronal Medium (500 mL) • STEMdiff™ Forebrain Neuron Maturation Supplement (125 mL)

Component Storage and Stability

COMPONENT NAME	COMPONENT #	STORAGE	SHELF LIFE
STEMdiff™ Forebrain Neuron Differentiation Basal Medium	08601/100-1692	Store at 2 - 8°C.	Stable until expiry date (EXP) on label.
STEMdiff™ Forebrain Neuron Differentiation Supplement	08602/100-1693	Store at -20°C.	Stable until expiry date (EXP) on label.
BrainPhys™ Neuronal Medium*	05797/05790	Store at 2 - 8°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Forebrain Neuron Maturation Supplement	08606/100-1690	Store at -20°C.	Stable until expiry date (EXP) on label.

* Protect from light.

NOTE: BrainPhys™ Neuronal Medium may be shipped with ice packs or under ambient conditions. Store at 2 - 8°C upon receipt.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
ACCUTASE™	07920
DMEM/F-12 with 15 mM HEPES	36254
Falcon® Conical Tubes, 15 mL	e.g. 38009
Laminin	Sigma L2020
Poly-L-ornithine solution	Sigma P4957
STEMdiff™ SMADi Neural Induction Kit	08581
STEMdiff™ Neural Progenitor Medium**	05833
Trypan Blue	07050

** Required if starting from expanded or cryopreserved NPCs (section III)

Preparation of Reagents and Materials

A. COATING CULTUREWARE WITH POLY-L-ORNITHINE (PLO) AND LAMININ

Use sterile technique to coat cultureware with PLO and laminin.

1. Dilute the PLO solution in phosphate-buffered saline (PBS) to reach a final concentration of 15 µg/mL.

   For example, add 15 mL of PLO to 85 mL of PBS.
2. Gently mix the diluted PLO solution. Do not vortex.
3. Add PLO solution to tissue culture-treated cultureware to cover the entire growth surface. Refer to Table 1 for recommended coating volumes.
4. Distribute the solution evenly and incubate at 37°C and 5% CO_2 for 2 hours or seal the cultureware (e.g. with Parafilm®) and incubate overnight at 2 - 8°C. Do not let the PLO solution evaporate. Allow stored coated cultureware to warm to room temperature (15 - 25°C) before proceeding to step 6.
5. Prepare a 5 µg/mL working solution of laminin in DMEM/F-12 with 15 mM HEPES. Refer to Table 1 for recommended coating volumes.
6. Gently tilt the PLO-coated cultureware onto one side and allow excess PLO solution to collect at the edge. Remove the excess solution using a serological pipette or by aspiration. Ensure that the coating is not scratched.
7. Wash the PLO-coated vessel twice by pipetting D-PBS gently toward the corner of the cultureware to avoid removing the PLO coating.
8. Remove the PBS from the cultureware and immediately add the laminin solution to cover the entire growth surface. Refer to Table 1 for recommended coating volumes.
9. Incubate at 37°C and 5% CO_2 for 2 hours or seal the cultureware (e.g. with Parafilm®) and incubate overnight at 2 - 8°C. Do not let the laminin solution evaporate.

   NOTE: Using freshly coated cultureware is recommended. However, if not used immediately, coated cultureware can be sealed (e.g. with Parafilm®) and stored at 2 - 8°C in laminin solution for up to 4 days.
10. Warm coated cultureware to 37°C before use.
11. Aspirate laminin solution immediately prior to seeding cells. Do not let the surface dry.

    NOTE: It is not necessary to wash cultureware after removing the laminin solution.

Table 1. Recommended Volumes of PLO and Laminin for Coating Cultureware

CULTUREWARE	APPROXIMATE SURFACE AREA	PLO	LAMININ
96-well plate	0.33 cm^2/well	50 µL/well	50 µL/well
4- or 24-well plate	2 cm^2/well	250 µL/well	250 µL/well
6-well plate	10 cm^2/well	1.5 mL/well	1.5 mL/well
35 mm dish	10 cm^2	1.5 mL	1.5 mL
60 mm dish	20 cm^2	2.5 mL	2.5 mL

B. PREPARATION OF STEMdiff™ FOREBRAIN NEURON DIFFERENTIATION MEDIUM

Use sterile technique to prepare STEMdiff™ Forebrain Neuron Differentiation Medium (Differentiation Basal Medium + Differentiation Supplement). The following example is for preparing 100 mL of medium. If preparing other volumes, adjust accordingly.

1. Thaw Differentiation Supplement at room temperature (15 - 25°C) or at 2 - 8°C overnight. Mix thoroughly.

   NOTE: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately. Do not re-freeze.
2. Add 20 mL of Differentiation Supplement to 80 mL of Differentiation Basal Medium. Mix thoroughly.

   NOTE: If not used immediately, store STEMdiff™ Forebrain Neuron Differentiation Medium at 2 - 8°C for up to 4 weeks. Warm medium to 37°C before use.

C. PREPARATION OF STEMdiff™ FOREBRAIN NEURON MATURATION MEDIUM

Use sterile technique to prepare STEMdiff™ Forebrain Neuron Maturation Medium (BrainPhys™ Neuronal Medium + Maturation Supplement). The following example is for preparing 125 mL of medium. If preparing other volumes, adjust accordingly.

1. Thaw Maturation Supplement at room temperature (15 - 25°C) or at 2 - 8°C overnight. Mix thoroughly.

   NOTE: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately. Do not re-freeze.
2. Add 25 mL of Maturation Supplement to 100 mL of BrainPhys™ Neuronal Medium. Mix thoroughly.

   NOTE: If not used immediately, store STEMdiff™ Forebrain Neuron Maturation Medium at 2 - 8°C for up to 4 weeks. Warm medium to 37°C before use. Protect from light.

Directions for Use

Please read the entire protocol before proceeding. Coat cultureware with PLO/laminin as described in Preparation section A. Use sterile technique when performing the following protocols:

A. Differentiation of NPCs to Forebrain Neuron Precursors
   I. Starting from the EB Neural Induction Protocol
   II. Starting from the Monolayer Neural Induction Protocol
   III. Starting from Expanded or Cryopreserved NPCs
B. Cryopreservation and Thawing of Forebrain Neuron Precursors
C. Neuron Maturation

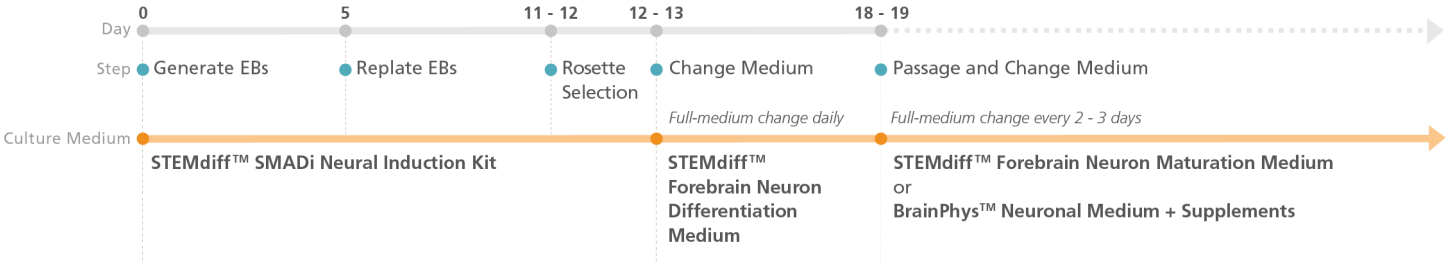
A. DIFFERENTIATION OF NPCs TO FOREBRAIN NEURON PRECURSORS

The following protocols integrate into the STEMdiff™ SMADi Neural Induction Kit (Catalog #08581) embryoid body (EB) protocol after rosette selection (section I), or the monolayer protocol (section II). Alternatively, NPC differentiation may be initiated starting from NPCs that have previously been expanded or cryopreserved (section III).

For complete instructions for generating central nervous system (CNS)-type NPCs using EB formation with the AggreWell™800 plate (Catalog #34811) or using the monolayer protocol, refer to the Technical Manual: Generation and Culture of Neural Progenitor Cells Using the STEMdiff™ Neural System (Document #10000005588), available at www.stemcell.com, or contact us to request a copy.

I. Starting from the EB Neural Induction Protocol

Protocol Diagram



The following instructions are for a single well of a 6-well plate; if using other cultureware, adjust volumes accordingly.

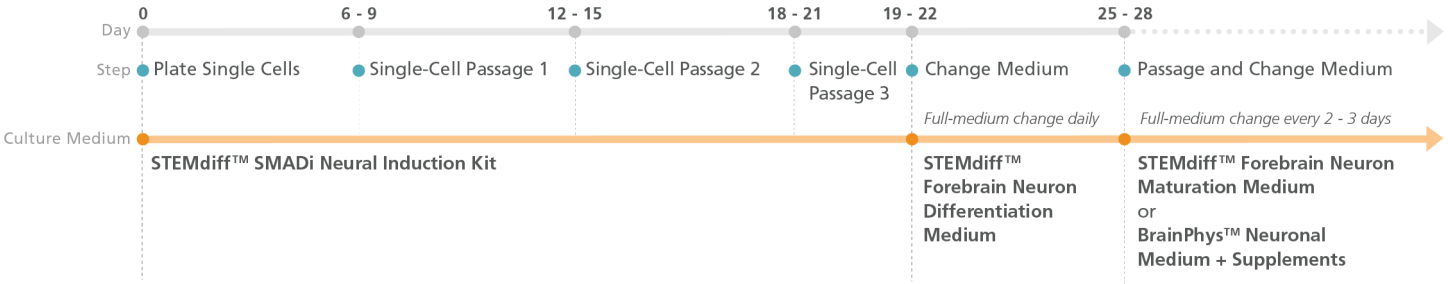
1. **Day 11/12 after EB formation**: Perform neural rosette selection using STEMdiff™ Neural Rosette Selection Reagent (Catalog #05832). Place selected neural rosettes onto a coated well of a 6-well plate containing 2 mL of STEMdiff™ Neural Induction Medium + SMADi. Incubate at 37°C and 5% CO_2 for 24 hours.
2. **Day 12/13 after EB formation**: Aspirate medium and add 2 mL of STEMdiff™ Forebrain Neuron Differentiation Medium (Preparation section B). Incubate at 37°C and 5% CO_2.
3. Perform daily full-medium changes with warm (37°C) STEMdiff™ Forebrain Neuron Differentiation Medium. Incubate at 37°C and 5% CO_2.
4. **Day 18/19 after EB formation**: Forebrain neuron precursors will reach 80 - 90% confluence and will be ready to passage.

For cryopreservation of forebrain neuron precursors, proceed to section B.

If cryopreservation is not required, proceed to section C to passage and seed cells for maturation.

II. Starting from the Monolayer Neural Induction Protocol

Protocol Diagram



The following instructions are for a single well of a 6-well plate; if using other cultureware, adjust volumes accordingly.

1. Day 18 - 21 (Passage 3) of the monolayer protocol: Passage the cells as single cells using ACCUTASE™ as described in the Technical Manual: Generation and Culture of Neural Progenitor Cells Using the STEMdiff™ Neural System (section 6.2).
2. Add cells to a coated well of a 6-well plate at a density of 80,000 - 125,000 cells/cm^2 in 2 mL of STEMdiff™ Neural Induction Medium + SMADi. Incubate at 37°C and 5% CO_2 for 24 hours.

   NOTE: Cell plating density may need to be optimized for each cell line.
3. Day 19 - 22: After 24 hours, aspirate the medium and add 2 mL of STEMdiff™ Forebrain Neuron Differentiation Medium (Preparation section B). Incubate at 37°C and 5% CO_2.
4. Maintain the cultures for 6 days by performing daily full-medium changes with warm (37°C) STEMdiff™ Forebrain Neuron Differentiation Medium. Incubate at 37°C and 5% CO_2.
5. Day 25 - 28: Forebrain neuron precursors will reach 80 - 90% confluence and will be ready to passage.

For cryopreservation of forebrain neuron precursors, proceed to section B.

If cryopreservation is not required, proceed to section C to passage and seed cells for maturation.

III. Starting from Expanded or Cryopreserved NPCs

NPCs expanded in STEMdiff™ Neural Progenitor Medium (Catalog #05833) may be differentiated further to forebrain neurons. For optimal downstream differentiation to forebrain neurons, use NPCs that have been expanded for ≤ 5 passages.

NPCs cryopreserved in STEMdiff™ Neural Progenitor Freezing Medium (Catalog #05838) may be thawed and cultured in STEMdiff™ Neural Progenitor Medium to allow the cells to recover and expand sufficiently before initiating forebrain neuron differentiation.

For complete instructions on passaging, cryopreserving, or thawing NPCs, refer to the Technical Manual: Generation and Culture of Neural Progenitor Cells Using the STEMdiff™ Neural System (sections 6.2, 6.3, or 6.4, respectively), available at www.stemcell.com, or contact us to request a copy.

The following instructions are for passaging previously expanded or cryopreserved NPCs from one well of a 6-well plate and plating them onto a PLO/laminin-coated 6-well plate for forebrain neuron differentiation. If using other cultureware, adjust volumes accordingly.

1. Coat the desired number of wells of a 6-well tissue culture-treated plate with PLO/laminin (Preparation section A).
2. Warm (37°C) sufficient volumes of STEMdiff™ Neural Progenitor Medium and DMEM/F-12 with 15 mM HEPES. Warm ACCUTASE™ to room temperature (15 - 25°C).

   NOTE: For complete instructions on preparing STEMdiff™ Neural Progenitor Medium, refer to the Product Information Sheet (Document #10000003488), available at www.stemcell.com, or contact us to request a copy.
3. Harvest NPCs as follows:
   a. Aspirate medium and add 1 mL of ACCUTASE™ to the well.

      NOTE: If desired, wash the well with 1 mL of DMEM/F-12 with 15 mM HEPES before adding ACCUTASE™.
   b. Incubate at 37°C for 5 - 10 minutes.
   c. Using a 1 mL pipettor, pipette the cell suspension up and down to dislodge the remaining attached cells.
   d. Add 5 mL of DMEM/F-12 with 15 mM HEPES to the well and transfer the NPC suspension to a 15 mL conical tube.
   e. Centrifuge at 300 x g for 5 minutes.
   f. Carefully aspirate the supernatant and resuspend the cells in 1 mL of STEMdiff™ Neural Progenitor Medium.
4. Count viable cells using Trypan Blue and a hemocytometer (e.g. 100-1181).
5. Using a serological pipette or by aspiration, gently remove the matrix solution from the PLO/laminin-coated plate (prepared in step 1). Ensure that the coated surface is not scratched.

6. Plate NPCs onto the PLO/laminin-coated plate at a density of 80,000 - 125,000 cells/cm^2 in 2 mL/well of STEMdiff™ Neural Progenitor Medium.
7. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the NPCs across the surface of the wells.
8. After 24 hours, aspirate the medium and replace with 2 mL/well of warm (37°C) STEMdiff™ Forebrain Neuron Differentiation Medium (Preparation section B). Incubate at 37°C and 5% CO_2.
9. Maintain the cultures for 6 days by performing daily full-medium changes with warm STEMdiff™ Forebrain Neuron Differentiation Medium.
10. After 6 days, cells will reach 80 - 90% confluence and will be ready to passage.

For cryopreservation of forebrain neuron precursors, proceed to section B.

If cryopreservation is not required, proceed to section C to passage and seed cells for maturation.

B. CRYOPRESERVATION AND THAWING OF FOREBRAIN NEURON PRECURSORS

Forebrain neuron precursors may be cryopreserved after 6 days of culture in STEMdiff™ Forebrain Neuron Differentiation Medium. Upon thawing, forebrain neuron precursors must be further cultured in STEMdiff™ Forebrain Neuron Maturation Medium for at least 14 days to mature the cells into terminally differentiated neurons.

Cryopreserving Forebrain Neuron Precursors

The following protocol is for harvesting forebrain neuron precursors from a 6-well plate and cryopreserving in CryoStor® CS10 (Catalog #07930). If using other cultureware, adjust volumes accordingly.

1. Warm a sufficient volume of DMEM/F-12 with 15 mM HEPES and ACCUTASE™ to room temperature (15 - 25°C). Label 2 mL Corning® Cryogenic Vials (e.g. Catalog #100-0091) as needed.
2. Harvest forebrain neuron precursors as follows:
   a. Aspirate medium and add 1 mL of ACCUTASE™ per well.
   b. Incubate the plate at 37°C and 5% CO_2 for 5 - 10 minutes.
   c. Add 5 mL of DMEM/F-12 with 15 mM HEPES to each well. Wash the cells off of the well and transfer the cell suspension to a 15 mL conical tube.
   d. Centrifuge cell suspension at 300 x g for 5 minutes. Remove and discard supernatant.
   e. Resuspend cells in a suitable volume (e.g. 5 mL) of DMEM/F-12 with 15 mM HEPES.
3. Count viable cells using Trypan Blue and a hemocytometer (e.g. Catalog #100-1181).
4. Transfer the cells to be cryopreserved into a new conical tube.
5. Centrifuge the cell suspension at 300 x g for 5 minutes at room temperature.
6. Resuspend cells in cold (4°C) CryoStor® CS10 to a concentration of 1 x 10^6 - 2 x 10^6 cells per mL.
7. Aliquot 1 - 1.5 mL of the cell suspension into each pre-labeled cryogenic vial (prepared in step 1). Place the cryogenic vials into a prepared freezing container.
8. Freeze cells using a standard slow rate-controlled cooling protocol (approximately -1°C/minute) using an isopropanol freezing container (e.g. Nalgene™ Mr. Frosty®) and freeze using or an isopropanol-free container such as Corning® CoolCell® (Catalog #200-0642) and place them overnight in a -80°C freezer.

   NOTE: Long-term storage at -80°C is not recommended.
9. Transfer cryogenic vials to a liquid nitrogen tank for long-term storage.

Thawing Forebrain Neuron Precursors

1. Coat the desired number of wells of a tissue culture-treated plate with PLO/laminin (see Preparation section A).
2. Warm sufficient volumes of DMEM/F-12 with 15 mM HEPES and STEMdiff™ Forebrain Neuron Maturation Medium (see Preparation Section C) to 37°C before starting the protocol to ensure that the thawing procedure is done as quickly as possible.
3. Add 10 mL of warm DMEM/F-12 with 15 mM HEPES to a 15 mL conical tube.
4. Wipe the outside of the vial of cells with 70% ethanol or isopropanol.
5. In a biosafety hood, twist the cap a quarter-turn to relieve internal pressure and then retighten.
6. Quickly thaw the vial of forebrain neuron precursors in a 37°C water bath by gently shaking the cryovial continuously until only a small frozen cell pellet remains. Do not vortex cells.

   NOTE: ThawSTAR® CFT2 Automated Thawing System (Catalog #100-0650) may be used to quickly and efficiently thaw cells. For complete instructions, refer to the Product Information sheet (Document #10000010334), available at www.stemcell.com, or contact us to request a copy.
7. Remove the cryovial from the water bath and wipe it with 70% ethanol or isopropanol.

NOTE: It is important to work quickly in the following steps to ensure high cell viability and recovery.

8. Transfer cells from the cryovial to the tube containing DMEM/F-12 with 15 mM HEPES. Mix gently.
9. Rinse the vials with 1 mL of warm DMEM/F-12 with 15 mM HEPES and add it dropwise to the cells, while gently swirling the 15 mL tube.
10. Centrifuge at 300 x g for 5 minutes at room temperature (15 - 25°C).
11. Aspirate medium, leaving the cell pellet intact.
12. Gently resuspend the cell pellet in 1 mL of STEMdiff™ Forebrain Neuron Maturation Medium.
13. Perform a cell count using Trypan Blue and a hemocytometer (e.g. 100-1181).
14. Proceed to section C step 3 to seed thawed forebrain neuron precursors for neuron maturation.

    NOTE: Post-thaw viability is typically 70 - 90%. If poor cell recovery is observed after plating, 10 µM Y-27632 (Dihydrochloride; Catalog #72302) may be added to the medium during the resuspension and plating steps.

C. NEURON MATURATION

Forebrain neuron precursors must be further cultured in STEMdiff™ Forebrain Neuron Maturation Medium for at least 14 days to mature the cells into terminally differentiated neurons. The following instructions are for passaging forebrain neuron precursors from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly. Ensure that the cells are plated into the final desired cultureware. Neurons are generally not amenable to passaging after the maturation period has started.

1. Coat the desired number of wells of a tissue culture-treated plate with PLO/laminin (see Preparation section A). Warm sufficient volumes of DMEM/F-12 with 15 mM HEPES and STEMdiff™ Forebrain Neuron Maturation Medium (see Preparation section C) to 37°C.
2. Harvest the forebrain neuron precursors as follows:
   a. Aspirate medium and add 1 mL of ACCUTASE™ to the well.
   b. Incubate the plate at 37°C and 5% CO_2 for 5 - 10 minutes.
   c. Add 5 mL of DMEM/F-12 and wash the cells off of the well. Transfer the cell suspension to a 15 mL conical tube.
   d. Centrifuge the cell suspension at 300 x g for 5 minutes. Remove and discard supernatant.
   e. Resuspend the cells in a suitable volume (e.g. 5 mL) of STEMdiff™ Forebrain Neuron Maturation Medium. Perform a cell count using Trypan Blue and a hemocytometer.
3. Using a serological pipette or by aspiration, gently remove the matrix solution from coated cultureware (prepared in step 1). Ensure that the coated surface is not scratched.
4. Seed forebrain neuron precursors at a density of 4 x 10^4 - 8 x 10^4 cells/cm^2 in warm STEMdiff™ Forebrain Neuron Maturation Medium. See Table 2 for recommended volumes.

   NOTE: The seeding density of neuronal precursors should be optimized for the application and cell line. For long-term cultures (> 30 days of maturation) and for immunocytochemistry, seed cells at 1.5 x 10^4 - 3 x 10^4 cells/cm^2. For short-term cultures (< 30 days of maturation), seed cells at 4 x 10^4 - 8 x 10^4 cells/cm^2.

   NOTE: If desired, neurons can be cultured with BrainPhys™ Neuronal Medium (Catalog #05790) + desired supplements (e.g. SM1, N2-A, BDNF, GDNF, Dibutyryl-cAMP, and ascorbic acid, as outlined in the Product Information Sheet for BrainPhys™ Neuronal Medium) in place of STEMdiff™ Forebrain Neuron Maturation Medium.

Table 2. Recommended Volume of STEMdiff™ Forebrain Neuron Maturation Medium for Various Cultureware

CULTUREWARE	VOLUME OF STEMdiff™ FOREBRAIN NEURON MATURATION MEDIUM
96-well plate	100 µL/well
4- or 24-well plate	500 µL/well
6-well plate	2 mL/well
35 mm dish	2 mL
60 mm dish	5 mL

5. Distribute cells evenly. Incubate at 37°C and 5% CO_2.
6. Perform a full-medium change with STEMdiff™ Forebrain Neuron Maturation Medium every 2 - 3 days.

   NOTE: To avoid cell detachment, perform medium changes slowly (e.g. dropwise), pointing the pipette tip toward the wall of the cell culture vessel. If detachment is observed, switch to half-medium changes every other day.
7. Continue maturation of forebrain neurons for a minimum of 14 days. Forebrain neurons can be cultured for up to 12 weeks if additional maturation time is required.

Assessment of Neuronal Differentiation

Neuronal differentiation may be assessed by immunocytochemistry using Anti-Beta-Tubulin III Antibody, Clone TUJ1. The presence of GABAergic neurons can be assessed using anti-GABA antibodies. The presence of synapses can be assessed by evaluating the expression and localization of synapsin.

For evaluating neuronal maturation efficiency, marker expression may be assessed after 14 days of maturation by immunocytochemistry using the following antibodies:

- Anti-FOXG1 Antibody, Polyclonal (Abcam Catalog #ab18259)
- Anti-Beta-Tubulin III Antibody, Clone TUJ1 (Catalog #60052)
- Anti-Human MAP2 Antibody, Polyclonal (Catalog #100-1342)
- Anti-Human Synaptophysin Antibody, Clone 249 (Catalog #100-1345)
- *Anti-Glial Fibrillary Acidic Protein (GFAP) Antibody, Polyclonal (Aves Labs Catalog #GFAP)

* GFAP is an astrocyte marker with low expression in the culture.

When generating NPCs using STEMdiff™ SMADi Neural Induction Kit, an average of 3 NPCs can be expected per input hPSC when following the embryoid body protocol or 27 NPCs per input hPSC when using the monolayer method for the recommended 3 passages. During the 6 days of culture in STEMdiff™ Forebrain Neuron Differentiation Medium, an average of 2-fold expansion is observed. One STEMdiff™ Forebrain Neuron Differentiation Kit can generate an average of 20 million forebrain neuron precursors and one STEMdiff™ Forebrain Neuron Maturation Kit can support maturation of up to 4.5 million neurons for 14 days of culture, depending on the seeding density used.

Related Products

For related products, including specialized cell culture and storage media, supplements, antibodies, cytokines, and small molecules, visit www.stemcell.com/hPSCNCworkflow, or contact us at techsupport@stemcell.com.