STEMdiff™-TF Forebrain Induced Neuron Differentiation Kit

Serum-free differentiation kit for the generation of excitatory glutamatergic forebrain neurons from human ESCs and iPSCs via lipid nanoparticle-based delivery of NGN2

Catalog #100-1678 1 Kit

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Product Description

STEMdiff™-TF Forebrain Induced Neuron Differentiation Kit is serum-free and designed to generate excitatory glutamatergic forebrain neurons from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) within six days. This kit employs a forward programming differentiation approach by using lipid nanoparticle-based delivery of the mRNA encoding transcription factor neurogenin 2 (NGN2) to rapidly and efficiently generate highly pure neurons without genomic integration. The resulting forebrain neurons can be further matured using STEMdiff™ Forebrain Neuron Maturation Kit (Catalog #08605). Cells derived using this kit provide a versatile material for modeling human neurological diseases and are used in drug screening and toxicity testing.

Product Information

The following components are sold as a complete kit (Catalog #100-1678) and are not available for individual sale.

COMPONENT NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
STEMdiff™-TF Forebrain Induced Neuron Basal Medium	100-1679	165 mL	Store at 2 - 8°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™-TF Forebrain Induced Neuron Supplement A	100-2165	230 µL	Store at -80°C.	Stable for 18 months from date of manufacture (MFG) on label.
STEMdiff™-TF Forebrain Induced Neuron Supplement B (50X)	100-1700	4 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.

NOTE: STEMdiff™-TF Forebrain Induced Neuron Basal Medium is shipped with ice packs, while both STEMdiff™-TF Forebrain Induced Neuron Supplement A and STEMdiff™-TF Forebrain Induced Neuron Supplement B (50X) are shipped on dry ice. Components should be stored according to the instructions provided above.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
5-Fluoro-2′-deoxyuridine*	Sigma-Aldrich F0503
24-well flat-bottom plate, tissue-culture treated	e.g. 38021
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
D-PBS (Without Ca++ and Mg++)	37350
DMEM/F-12 with 15 mM HEPES	36254
Falcon® Conical Tubes, 15 mL	e.g. 100-0092
Hausser Scientific™ Bright-Line Hemocytometer	100-1181
mTeSR™ Plus	100-0276
PluriSIn-1	72822
Poly-D-Lysine	Thermo Fisher Scientific A3890401
STEMdiff™ Forebrain Neuron Maturation Kit*	08605
Trypan Blue	07050
TrypLE™ Express Enzyme (1X), phenol red OR ACCUTASE™	Thermo Fisher Scientific 12605010 OR 07920

PRODUCT NAME	CATALOG #
Uridine*	Sigma-Aldrich U3003
Y-27632 (Dihydrochloride)	72302

* These items are only required if the culture is intended to be maintained beyond Day 5 of differentiation.

Preparation of Reagents and Materials

A. COATING CULTUREWARE WITH POLY-D-LYSINE (PDL) AND CORNING® MATRIGEL® HESC-QUALIFIED MATRIX

Use sterile technique to coat tissue culture-treated cultureware with PDL and Corning® Matrigel® hESC-Qualified Matrix.

Poly-D-Lysine (PDL)

1. Use PDL at 100 µg/mL. If needed, dilute PDL solution in phosphate-buffered saline (PBS) to reach a final concentration of 100 µg/mL.
2. Gently mix PDL solution by inverting the bottle several times to ensure homogeneity. Avoid vigorous shaking to prevent foaming.
3. Add PDL solution to tissue culture-treated cultureware to cover the entire growth surface. Refer to Table 1 for recommended coating solution volumes.
4. Distribute the solution evenly and incubate at 37°C and 5% CO_2 for 3 hours.
5. Wash PDL-coated cultureware three times by adding D-PBS (Without Ca++ and Mg++) gently and removing the solution by tilting the cultureware, putting the pipette tip toward the corner of the cultureware to avoid damaging the PDL coating.
6. Allow PDL-coated cultureware to dry out completely by incubating at room temperature (15 - 25°C) for 30 minutes with the lid open in a biosafety cabinet. Do not exceed 30 minutes to avoid contamination.

Corning® Matrigel® hESC-Qualified Matrix

Corning® Matrigel® should be aliquoted and frozen. Consult Matrigel® Certificate of Analysis for the recommended aliquot size ("Dilution Factor") to prepare 25 mL of diluted matrix. Keep on ice at all times when thawing and handling Corning® Matrigel® to prevent it from gelling.

7. Thaw one aliquot of Corning® Matrigel® on ice.
8. Dispense 24 mL of cold DMEM/F-12 with 15 mM HEPES into a 50 mL conical tube and keep on ice.
9. Add thawed Corning® Matrigel® to the cold DMEM/F-12 with 15 mM HEPES (in the 50 mL tube) and mix well. The vial may be washed with cold medium if desired.
10. Immediately use the diluted Corning® Matrigel® solution to coat tissue culture-treated cultureware. Refer to Table 1 for recommended coating volumes.
11. Swirl the cultureware to spread the solution evenly across the surface. If the surface of the cultureware is not fully coated by the Corning® Matrigel® solution, it should not be used.
12. Incubate at room temperature (15 - 25°C) for 1 hour. Do not let the Matrigel solution evaporate.

    NOTE: Using freshly coated cultureware is recommended. However, if not used immediately, the coated cultureware must be sealed (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to one week. Allow stored coated cultureware to come to room temperature before proceeding to step 13.
13. Aspirate Corning® Matrigel® solution immediately prior to seeding cells. Ensure that the coated surface is not scratched. Do not let the surface dry.

    NOTE: It is not necessary to wash cultureware after removing Corning® Matrigel® solution.

Table 1. Recommended Volumes and Seeding Densities for Various Cultureware

CULTUREWARE	APPROXIMATE SURFACE AREA	COATING SOLUTION VOLUME	FINAL CULTURE VOLUME	CELL SEEDING DENSITY	STEMdiff™-TF FOREBRAIN INDUCED NEURON SUPPLEMENT A VOLUME
96-well plate	0.33 cm^2/well	50 µL/well	0.1 mL/well	1.5 - 2.5 x 10^4 cells/well	0.8 µL/well
24-well plate	2 cm^2/well	250 µL/well	0.4 mL/well	1.25 - 1.5 x 10^5 cells/well	1.5 µL/well
12-well plate	4 cm^2/well	500 µL/well	1 mL/well	2.5 - 3 x 10^5 cells/well	3 µL/well
6-well plate	10 cm^2/well	1.5 mL/well	2 mL/well	6.25 - 7.5 x 10^5 cells /well	5.5 µL/well

B. SEEDING MEDIUM

Use sterile technique to prepare seeding medium (mTeSR™ Plus [Catalog #100-0276] + 10 µM Y-27632 [Dihydrochloride]). Prepare appropriate volume of seeding medium to obtain a final concentration of 10 µM Y-27632 (Dihydrochloride). Mix thoroughly. Warm to room temperature before use.

For complete instructions on preparing mTeSR™ Plus, refer to Product Information Sheet (PIS; Document #10000007758), available at www.stemcell.com, or contact us to request a copy.

For complete instructions on preparing Y27632 (Dihydrochloride), refer to the PIS (Document #10000002341), available at www.stemcell.com, or contact us to request a copy.

C. COMPLETE STEMDIFF™-TF FOREBRAIN INDUCED NEURON DIFFERENTIATION MEDIUM B (COMPLETE MEDIUM B)

Use sterile technique to prepare complete STEMdiff™-TF Forebrain Induced Neuron Differentiation Medium B (complete medium B; STEMdiff™-TF Forebrain Induced Neuron Basal Medium + STEMdiff™-TF Forebrain Induced Neuron Supplement B [50X]). The following example is for preparing 50 mL of complete medium B. If preparing other volumes, adjust accordingly.

1. Thaw STEMdiff™-TF Forebrain Induced Neuron Supplement B (50X) at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly by gentle inversion. Do not vortex.

   NOTE: If not used immediately, aliquot and store at -20°C. Do not exceed the supplement's shelf life. Once thawed, use the aliquots immediately. Do not re-freeze.

2. Add 1 mL of STEMdiff™-TF Forebrain Induced Neuron Supplement B (50X) to 49 mL of STEMdiff™-TF Forebrain Induced Neuron Basal Medium. Mix thoroughly by swirling or gentle inversion. Avoid vigorous shaking.

   NOTE: Complete medium B is stable at 2 - 8°C for up to 2 weeks. Warm complete medium B to room temperature before use. Do not freeze. Do not filter complete medium B.

D. COMPLETE STEMDIFF™-TF FOREBRAIN INDUCED NEURON DIFFERENTIATION MEDIUM AB (COMPLETE MEDIUM AB)

Use sterile technique to prepare complete STEMdiff™-TF Forebrain Induced Neuron Differentiation Medium AB (complete medium AB; complete medium B + STEMdiff™-TF Forebrain Induced Neuron Supplement A).

This protocol is for preparing complete medium AB for a single well of a 24-well plate. If using other cultureware, adjust volumes accordingly. Refer to Table 1 for recommended volumes for other cultureware formats.

1. Thaw STEMdiff™-TF Forebrain Induced Neuron Supplement A overnight at 2 - 8°C. Mix thoroughly by gentle pipetting. Do not vortex.

   NOTE: If not used immediately, store at 2 - 8°C for up to 3 weeks. Do not re-freeze. Do not exceed the shelf life of the supplement. For long-term storage, aliquot STEMdiff™-TF Forebrain Induced Neuron Supplement A into tubes. Place tubes in a pre-cooled slow-freezing container (e.g. Corning® CoolCell® LX Cell Freezing Container, Catalog #200-0642) and store the container at -80°C.

2. Add 1.5 µL of STEMdiff™-TF Forebrain Induced Neuron Supplement A to 400 µL of complete medium B for each well of a 24-well plate that will be cultured. Mix thoroughly by pipetting up and down. Do not vortex. Do not filter complete medium AB.

   NOTE: Complete medium AB is stable at 2 - 8°C for up to 2 weeks. Warm complete medium AB to room temperature (15 - 25°C) before use. Mix thoroughly by pipetting up and down. Do not freeze. Do not filter.

E. STEMDIFF™ FOREBRAIN NEURON MATURATION MEDIUM

NOTE: STEMdiff™ Forebrain Neuron Maturation Medium is only required if the culture is intended to continue beyond Day 5.

Use sterile technique to prepare STEMdiff™ Forebrain Neuron Maturation Medium (Catalog #08605; BrainPhys™ Neuronal Medium + STEMdiff™ Forebrain Neuron Maturation Supplement). For complete instructions on preparing STEMdiff™ Forebrain Neuron Maturation Medium, refer to the PIS (Document #10000005464), available at www.stemcell.com, or contact us to request a copy.

Protocol Diagram

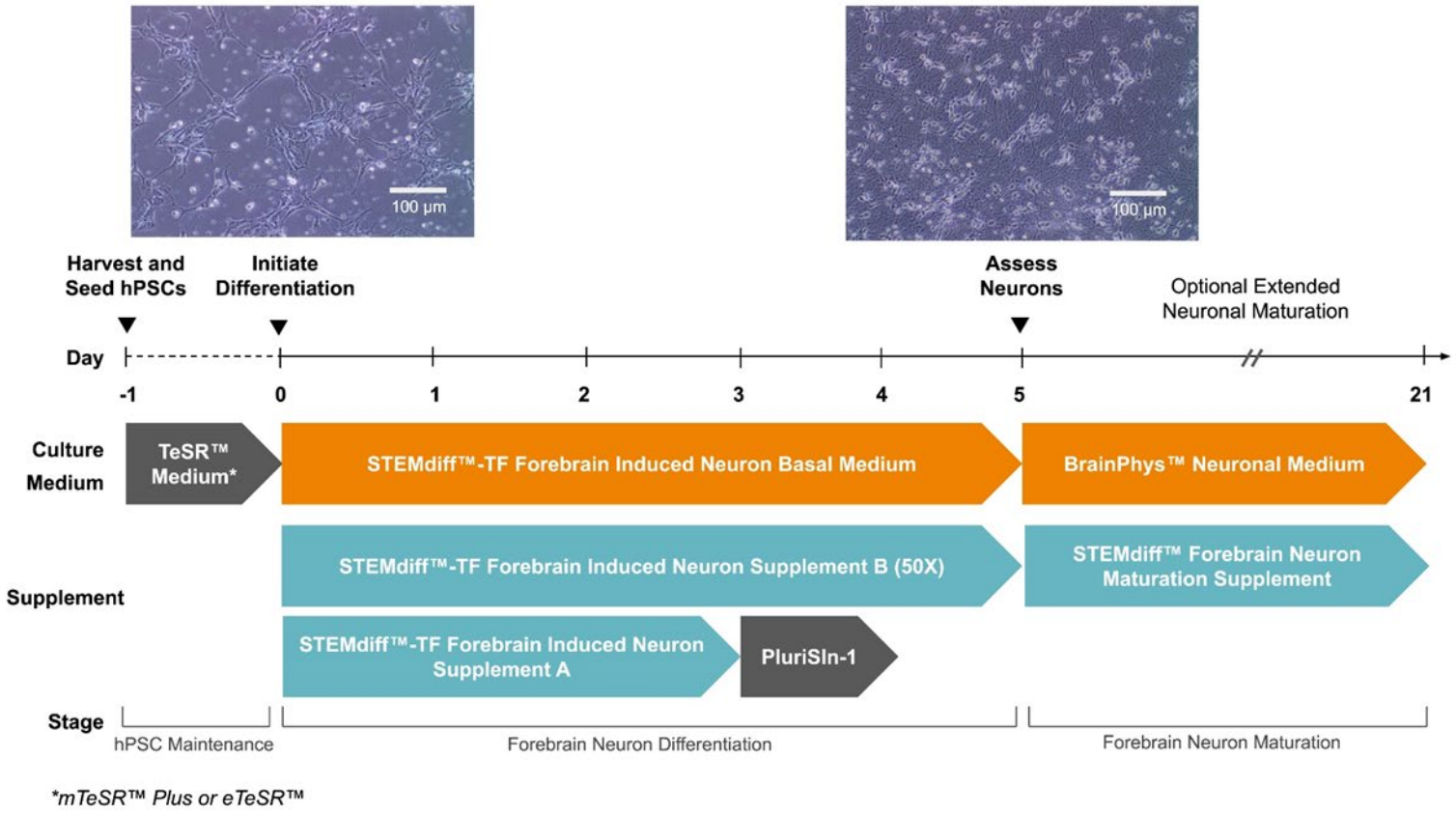

Figure 1. Protocol diagram for STEMdiff™-TF Forebrain Induced Neuron Differentiation Kit

Directions for Use

Please read the entire protocol before proceeding. Use sterile technique when performing the following protocols:

A. Harvesting and seeding hESCs/hiPSCs as single cells (Day -1)

B. Differentiation of hESCs/hiPSCs to neurons (Day 0 - 5)

C. Neuron maturation (Day 5 - 21)

A. Harvesting and Seeding hESCs/hiPSCs as Single Cells (Day -1)

This protocol is for harvesting hESCs/hiPSCs from a single well of a 6-well plate and seeding them into a 24-well plate for differentiation. If using other cultureware, adjust volumes accordingly. Warm cultureware, media, and reagents to room temperature (15 - 25°C) before use. Cells can be previously maintained in mTeSR™ Plus or eTeSR™. For further information on maintaining high-quality hiPSCs, refer to the Technical Manuals for mTeSR™ Plus (Document #10000007757) or eTeSR™ (Document #10000019963), available at www.stemcell.com, or contact us to request a copy.

IMPORTANT: Cell density is a critical parameter for the success of this kit. As culture appearance may vary between users and cell lines, we recommend determining the optimal seeding density by seeding hPSCs at 5 x 10^4 - 1.5 x 10^5 cells/well (i.e. ~2.5 - 7.5 x 10^4 cells/cm^2) in a 24-well plate. For example, seed 5 x 10^4, 7.5 x 10^4, 1 x 10^5, 1.25 x 10^5, and 1.5 x 10^5 cells/well. On Day 0, compare the culture morphology and confluency to the reference images shown in Figure 2. Proceed with differentiation only if the culture appearance closely matches the reference images.

NOTE: The cell densities shown in Figure 2 were established using Healthy Control Human iPSC Line, Female, SCTi003-A, Catalog #200-0511 seeded at 6.25 - 7.5 x 10^4 cells/cm^2. If working with lines that grow at different rates (e.g. disease-model hiPSCs, CRISPR-edited hPSCs, or unusually fast- or slow-growing cells), titrate within the recommended range and adjust as needed to reproduce the Day-0 confluency shown in Figure 2.

NOTE: The recommended cell densities apply to cells maintained in mTeSR™ Plus. If using a different medium, adjust the seeding density to match the appearance shown in Figure 2. For example, when using eTeSR™, a lower density (e.g. 2 - 2.25 x 10^4 cells/cm^2) is advised, as cells cultured in eTeSR™ generally exhibit higher attachment on the seeding day.

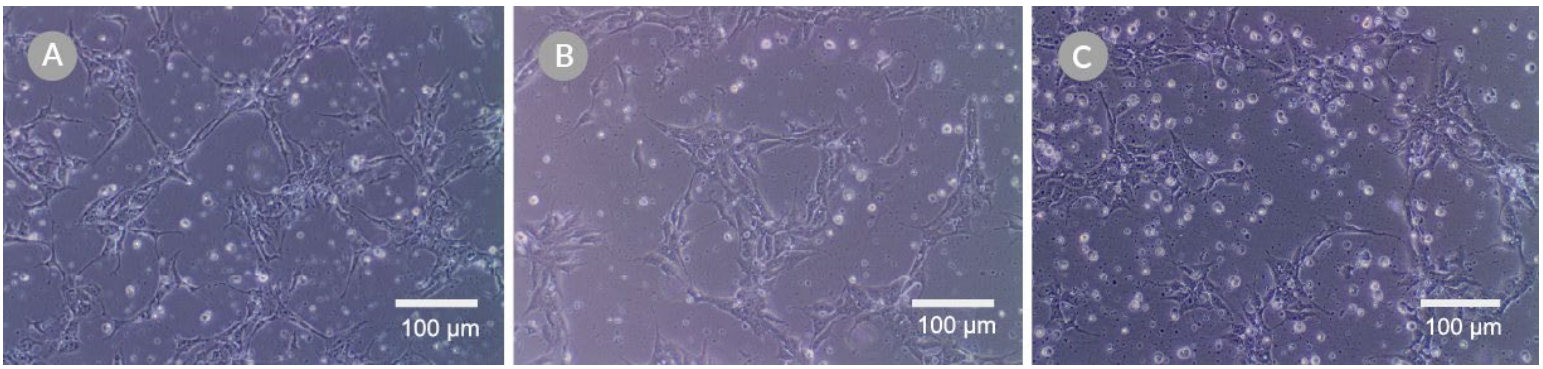

Figure 2. Recommended Cell Density on Day 0

A - C illustrate the recommended cell density on Day 0. If culture appears significantly denser or sparser than A - C, discard the culture, adjust your seeding density, and restart. Maintaining the appropriate cell density is essential to ensure consistent and reliable results with this kit.

1. Coat cultureware with PDL and Corning® Matrigel® as described in Preparation of Reagents and Materials, section A. Warm cultureware to room temperature if it was prepared in advance.
2. Prepare appropriate volume of seeding medium (see Preparation of Reagents and Materials, section B).
3. Use a microscope to visually identify regions of differentiation in the hESC/hiPSC culture. Mark these using a felt tip or lens marker on the bottom of the plate. Remove regions of differentiation by scraping with a pipette tip or by aspiration. Avoid having the culture plate out of the incubator for more than 15 minutes at a time.
4. Wash the well with 2 mL of room temperature D-PBS (Without Ca++ and Mg++). Discard the wash.
5. Add 0.5 mL of TrypLE™ Express Enzyme (1X), phenol red or ACCUTASE™.
6. Incubate at 37°C and 5% CO_2 for 4 - 8 minutes.

   NOTE: The incubation time may vary when using different cell lines and matrices. For cultures maintained on Corning® Matrigel®, 4 - 5 minutes is typically optimal for dissociation.
7. Add 1 mL of seeding medium to the well.
8. Harvest the cells by carefully tilting the plate and gently pipetting up and down with a 1 mL pipette to detach the cells. Transfer the single-cell suspension to a 15 mL conical tube.

   NOTE: Avoid excessive or harsh trituration, as this may adversely impact cell viability. If the cells do not readily detach, a longer incubation time may be required.
9. Centrifuge the cell suspension at 300 x g for 5 minutes.
10. Carefully aspirate the supernatant and gently flick the tube 3 - 5 times to resuspend the cell pellet.
11. Add 1 - 2 mL of seeding medium to the cell pellet. Mix gently by pipetting.
12. Count cells using Trypan Blue and a hemocytometer or an automated cell counting method.
13. Remove the remaining Corning® Matrigel® solution from the culture plate(s) prepared in Step 1. Immediately add 0.4 mL of seeding medium to each well if using a 24-well plate. For other cultureware formats, refer to Table 1 for the recommended volume.
14. Add the cells to each well at 6.25 - 7.5 x 10^4 cells/cm^2 (i.e. 1.25 - 1.5 x 10^5 cells/well for a 24-well plate) or at your determined seeding density. For other cultureware formats, refer to Table 1 for the recommended cell number.
15. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the cells across the surface of the wells.
16. Incubate at 37°C and 5% CO_2. Do not disturb the plate for 24 hours.

B. Differentiation (Day 0 - 5)

This protocol is for differentiating hESCs/hiPSCs in a 24-well plate format. Refer to Table 1 for recommended volumes if using other cultureware. Warm medium and reagents to room temperature (15 - 25°C) before use.

Day 0 - 2

Prepare complete medium B (see Preparation of Reagents and Materials, section C) and complete medium AB (see Preparation of Reagents and Materials, section D). Complete medium AB is required for Day 0 - 2 of differentiation, and complete medium B is required for Day 3 - 5 of differentiation.

1. On Day 0, use a 1 mL pipette tip to gently remove and discard the medium from each well. If using an aspirator, remove the solution by tilting the cultureware and put the tip toward the corner of the cultureware to avoid any contact with the cells and coating matrix.
2. Add 0.4 mL of complete medium AB per well using a serological pipette or 1 mL pipette tip. Avoid disturbing the cells and coating matrix.
3. Incubate at 37°C and 5% CO_2 for 24 hours.
4. Repeat steps 1 - 3 again on Day 1 and 2.

   NOTE: On Day 0, remove the seeding medium from each well completely and replace it with complete medium AB. On Days 1 and 2, avoid removing all the medium; instead, leave a small volume to prevent cells from being exposed to air, as they are more sensitive at this stage.

Day 3

Warm an appropriate volume of complete medium B to room temperature. Add PluriSin-1 to complete medium B at a final concentration of 20 µM. Mix thoroughly.

NOTE: To prepare 10 mM stock solution of PluriSin-1 (213.2 g/mol), resuspend 1 mg in 469 µL of fresh DMSO, aliquot and store aliquots at -20°C to avoid repeated freeze-thaw cycles; protect from prolonged exposure to light.

1. Using a 1 mL pipette tip, gently remove and discard the medium from each well. If using an aspirator, tilt the cultureware and position the tip at the corner of the cultureware to prevent contact with the cells and coating matrices. Be sure to leave a small volume of medium in the wells to prevent the cells from being exposed to air. Gently add 0.4 mL complete medium B with PluriSin-1 per well.
2. Incubate at 37°C and 5% CO_2 for 24 hours.

Day 4 - 5

Warm complete medium B to room temperature.

1. On Day 4, use a 1 mL pipette tip to gently remove and discard the medium from each well. If using an aspirator, tilt the cultureware and position the tip at the corner of the cultureware to prevent contact with the cells and coating matrices. Be sure to leave a small volume of medium in the wells to prevent the cells from being exposed to air.
2. Gently add 0.4 mL complete medium B to each well.
3. Incubate at 37°C and 5% CO_2 for 24 hours.
4. On Day 5, prepare the cells for neuronal differentiation assessment or other intended assays. If continuing the culture beyond this point, proceed to Section C for neuron maturation.

C. Neuron Maturation (Day 5 - 21)

Forebrain neurons may be cultured in STEMdiff™ Forebrain Neuron Maturation Medium for 16 days or longer to further mature the cells. Prepare STEMdiff™ Forebrain Neuron Maturation Medium as described in Preparation of Reagents and Materials, section E. This protocol is for maturing neurons in a 24-well plate format. Refer to Table 1 for recommended volumes if using other cultureware.

1. Using a 1 mL pipette tip, gently remove medium from each well and discard. If using an aspirator, remove the solution by tilting the cultureware and put the tip toward the corner of the cultureware to avoid any contact with the cells and coating matrices. Be sure to leave a small volume of medium in the wells to prevent the cells from being exposed to air.
2. Gently add 0.4 mL STEMdiff™ Forebrain Neuron Maturation Medium to each well.
3. Incubate at 37°C and 5% CO_2.

   NOTE: Perform a full-medium change with STEMdiff™ Forebrain Neuron Maturation Medium every 2 - 3 days. Avoid removing all the medium; leave a small volume to prevent cells from being exposed to air, as they are more sensitive at this stage.

   NOTE: To avoid cell detachment, perform medium changes slowly (e.g. dropwise), pointing the pipette tip toward the wall of the cultureware. If detachment is observed, switch to half-medium changes every other day.
4. By Day 5 , 5 - 10% of proliferating cells can remain. To maintain culture purity beyond this time point, supplement STEMdiff™ Forebrain Neuron Maturation Medium with 2 - 3 µM of 5-Fluoro-2′-deoxyuridine and Uridine (Fdu/U) starting 48 hours after transitioning to maturation medium (i.e. Day 7), followed by at least two additional feedings every 48 hours (i.e. Day 9 and Day 11). If proliferating cells are still present after this, continue supplementing the medium with Fdu/U as needed.

NOTE: Continue maturation of forebrain neurons for 16 days or longer.

Assessment of Neuronal Differentiation

To evaluate neuronal differentiation and maturation efficiency, use the following antibodies to assess Beta-Tubulin III expression on Day 5 and MAP2 and synapsin expression after 16 days of maturation by immunocytochemistry:

- Anti-Beta-Tubulin III antibody, Clone TUJ1 (Catalog #60052)
- Anti-human MAP2 antibody, polyclonal (Catalog #100-1342 or abcam Catalog #ab5392)
- Anti-human synapsin antibody, polyclonal (MilliporeSigma Catalog #AB1543)
- Anti-human FOXG1 antibody, polyclonal (abcam Catalog #ab18259)

Cells are expected to be > 90% Beta III Tubulin-positive by Day 5 and > 90% MAP2-positive and > 80% Synapsin I-positive when maintained in maturation medium for 16 days. Results may vary depending on the cell line used.

Troubleshooting

PROBLEM	RECOMMENDED ACTION
High levels of undifferentiated/ prolifrating cells	• Use high-quality hiPSCs with high viability and minimal differentiation when starting the protocol. • The initial cell density on Day 0 may be too high. Refer to Directions for Use, section A, **"IMPORTANT",** for the optimal cell density. • Ensure STEMdiff™-TF Forebrain Induced Neuron Supplement A is used as per protocol's recommendation; mix it thoroughly by pipeting up and down prior to adding to the wells.
Non-uniform culture/variation among replicates	• Ensure even distribution of the cells during the initial seeding for a uniform culture. • Ensure STEMdiff™-TF Forebrain Induced Neuron Supplement A is used as per protocol's recommendation; thoroughly mix it prior to adding to the wells.
Peeling	• When removing medium, especially when using an aspirator, be careful not to touch the cells. Tilt the cultureware and gently aspirate the medium from the corner to avoid peeling. • Rotate the position of adding/removing the medium, ensuring you don't consistently do this from the same side to prevent peeling. Always leave a small amount of medium in the well to prevent the cells from drying out.
Cell Death	• The initial cell density on Day 0 may be too low. Refer to Directions for Use, section A, **"IMPORTANT",** for guidance on determining the optimal cell density. • Complete medium B and complete medium AB should be stored at 2 - 8°C and must be used within 2 weeks. Using complete medium B and complete medium AB after 2 weeks can result in toxicity. • Complete medium B and complete medium AB are not stable when stored at -20°C and should not be frozen.

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.