TECHNICAL MANUAL

Generation and Culture of Neural Progenitor Cells using the STEMdiff™ Neural System

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, are characterized by their ability to self-renew and differentiate into tissues derived from any of the three embryonic germ layers. This includes endodermal tissues such as intestine, pancreas or liver; mesodermal tissues such as blood or cardiomyocytes; and ectodermal tissues such as the nervous system or skin.[1]

Under specific conditions, human ES and iPS cells can be directed to differentiate into neural progenitor cells (NPCs), which are characterized by their capacity to expand and generate the major differentiated cell types of the central nervous system (CNS): neurons, astrocytes, and oligodendrocytes.

With STEMdiff™ SMADi Neural Induction Kit, NPCs can be generated using either a monolayer culture protocol or an embryoid body (EB) protocol; these two protocols are described in this manual. The addition of STEMdiff™ SMADi Neural Induction Supplement to STEMdiff™ Neural Induction Medium promotes the efficient conversion of human ES and iPS cells to CNS-type NPCs and inhibits the unwanted differentiation of non-CNS-type cells. If desired, STEMdiff™ SMADi Neural Induction Supplement can be omitted and STEMdiff™ Neural Induction Medium used on its own.

The STEMdiff™ Neural System for hPSC-derived NPC research is outlined in Table 1. This is a suite of products specifically designed for the generation, isolation, characterization, expansion, and cryopreservation of hPSC-derived NPCs.

Table 1. The STEMdiff™ Neural System for hPSC-Derived NPC Research

APPLICATIONS	PRODUCT	CATALOG #
Generation of NPCs from hPSCs	STEMdiff™ SMADi Neural Induction Kit • STEMdiff™ Neural Induction Medium (Catalog #05835) • STEMdiff™ SMADi Neural Induction Supplement	08581
Isolation of CNS-type NPCs	STEMdiff™ Neural Rosette Selection Reagent	05832
Expansion of NPCs	STEMdiff™ Neural Progenitor Medium	05833
Cryopreservation of NPCs	STEMdiff™ Neural Progenitor Freezing Medium	05838
Characterization of NPCs	STEMdiff™ Human Neural Progenitor Antibody Panel	69001
Generation of neuronal precursors from NPCs	STEMdiff™ Neuron Differentiation Kit	08500
Maturation of neuronal precursors to generate neurons	STEMdiff™ Neuron Maturation Kit	08510
Generation of dopaminergic neuron precursors from NPCs	STEMdiff™ Dopaminergic Neuron Differentiation Kit	08520
Maturation of dopaminergic neurons	STEMdiff™ Dopaminergic Neuron Maturation Kit	08530
Generation of astrocytic precursors from NPCs	STEMdiff™ Astrocyte Differentiation Kit	08540
Maturation of astrocytic precursors to generate astrocytes	STEMdiff™ Astrocyte Maturation Kit	08550

2.0 Materials, Reagents and Equipment

2.1 Materials Required for Neural Induction Protocols

NEURAL INDUCTION PROTOCOL	PRODUCT	CATALOG #
EB and Monolayer Culture	STEMdiff™ SMADi Neural Induction Kit • STEMdiff™ Neural Induction Medium • STEMdiff™ SMADi Neural Induction Supplement	08581
	Gentle Cell Dissociation Reagent	07174
	DMEM/F-12 with 15 mM HEPES	36254
	D-PBS (Without Ca++ and Mg++)	37350
	Y-27632	72302
	Trypan Blue	07050
	Poly-L-Ornithine	Sigma P4957
	Laminin	Sigma L2020
	Corning® Matrigel® hESC-qualified Matrix	Corning 354277
	Falcon® 6-Well Flat-Bottom Plate, Tissue Culture-Treated	38016
	Falcon® Conical Tubes	38009 (15 mL) OR 38010 (50 mL)
	Falcon® Serological Pipettes, 5 mL	38003
EB	STEMdiff™ Neural Rosette Selection Reagent	05832
	AggreWell™800 24-well Plate	34811
	Anti-Adherence Rinsing Solution	07010
	Wide-bore disposable 1 mL pipette tips	e.g. VWR CA15000-466
	37 µm Reversible Strainer	27215 (small) 27250 (large)

2.2 Materials Required for Neural Progenitor Cell (NPC) Culture

PRODUCT	CATALOG #
STEMdiff™ Neural Progenitor Medium	05833
STEMdiff™ Neural Progenitor Freezing Medium	05838
DMEM/F-12 with 15 mM HEPES	36254
ACCUTASE™	07920
Trypan Blue	07050
Poly-L-Ornithine	Sigma P4957
Laminin	Sigma L2020
Corning® Matrigel® hESC-qualified Matrix	Corning 354277

2.3 Equipment

- Biosafety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor with an adaptor for plate holders
- Pipette-aid with appropriate serological pipettes
- Hemocytometer
- Micropipettor and serological pipettes with appropriate tips
- Inverted microscope
- Isopropanol freezing container
- -150°C freezer or liquid nitrogen (LN_2) vapor tank
- -80°C freezer
- -20°C freezer
- Refrigerator (2 - 8°C)

3.0 Important Parameters for Successful Neural Induction from Human ES and iPS Cells

High-Quality Human ES and iPS Cells

It is critical to start with high-quality human ES and iPS cell cultures for efficient neural induction.

Note: Refer to the Technical Manuals: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #28315) or TeSR™-E8™ (Document #29267) for complete instructions on culturing high-quality ES and iPS cells using these feeder-free maintenance media. These documents are available at www.stemcell.com or contact us at techsupport@stemcell.com to request a copy.

STEMdiff™ SMADi Neural Induction Supplement

The addition of STEMdiff™ SMADi Neural Induction Supplement to STEMdiff™ Neural Induction Medium promotes the efficient conversion of human ES and iPS cells to CNS-type NPCs and inhibits the unwanted differentiation of non-CNS-type cells.

Note: If desired, STEMdiff™ SMADi Neural Induction Supplement can be omitted and STEMdiff™ Neural Induction Medium used on its own.

AggreWell™800

AggreWell™800 24-well Plates are recommended for generating hPSC-derived EBs for neural induction. These plates enable easy generation of uniformly-sized EBs, making differentiation experiments more reproducible. For efficient neural induction in STEMdiff™ Neural Induction Medium, generate EBs consisting of approximately 10,000 cells.

Plating of EBs

Intact EBs should be harvested from AggreWell™800 plates and plated onto matrix-coated plates. Dissociation of EBs into small clusters or single cells can lead to cell death and therefore low NPC yield. It is important to avoid breaking up EBs before plating them onto matrix-coated plates.

Choosing an Appropriate Matrix for Neural Induction

Successful generation and maintenance of human NPCs requires the use of a suitable matrix to allow attachment of neural aggregates. Poly-L-ornithine/laminin or Corning® Matrigel® hESC-Qualified Matrix are recommended for use with the STEMdiff™ Neural System.

Enrichment of CNS-Type NPCs

In the EB protocol, neural rosette selection can be used to isolate CNS-type NPCs from a mixed culture of cells. Manual rosette selection is a laborious and time-consuming process. STEMdiff™ Neural Rosette Selection Reagent allows rapid and efficient isolation of neural rosettes, without harsh enzymatic treatment.

Y-27632 ROCK Inhibitor

ROCK inhibitor Y-27632 has been reported to increase the survival of single hPSCs and to improve EB formation.[2]

4.0 Preparation of Reagents and Materials

4.1 Matrices for Coating Cultureware

4.1.1 Poly-L-Ornithine/Laminin

1. Dilute poly-L-ornithine (PLO) solution in phosphate-buffered saline (PBS) to reach a final concentration of 15 µg/mL.
2. Add the diluted PLO solution to tissue culture-treated cultureware. See Table 2 for recommended coating volumes.
3. Swirl the cultureware to spread the PLO solution evenly across the surface.
4. Incubate at room temperature (15 - 25°C) for at least 2 hours or seal the cultureware (e.g. with Parafilm®) and incubate overnight at 2 - 8°C. Do not let the PLO solution evaporate.
5. Gently remove the PLO solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
6. Wash twice with PBS, followed by a third wash with DMEM/F-12.
7. Dilute laminin in DMEM/F-12 to reach a final concentration of 10 µg/mL.
8. Add the diluted laminin solution to PLO-coated cultureware. See Table 2 for recommended coating volumes.
9. Incubate at room temperature (15 - 25°C) for at least 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: If not used immediately, the cultureware must be sealed to prevent evaporation of the laminin solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 2 weeks after coating. Allow stored coated cultureware to come to room temperature (15 - 25°C) for 30 minutes before plating cells.

4.1.2 Corning® Matrigel®

Corning® Matrigel® hESC-qualified Matrix should be aliquoted and frozen. Consult the Certificate of Analysis supplied with Matrigel® for the recommended aliquot size ("Dilution Factor") to make up 24 mL of diluted matrix. Make sure to always keep Matrigel® on ice when thawing and handling to prevent it from gelling.

1. Thaw 1 aliquot of Matrigel® on ice.
2. Dispense 24 mL of cold DMEM/F-12 into a 50 mL conical tube and keep on ice.
3. Add thawed Matrigel® to the cold DMEM/F-12 and mix well. The vial may be washed with cold medium if desired.
4. Use the diluted Matrigel® solution immediately to coat tissue culture-treated cultureware. See Table 2 for recommended coating volumes.
5. Swirl the cultureware to spread the Matrigel® solution evenly across the surface.
6. Incubate at room temperature (15 - 25°C) for at least 1 hour before use. Do not let the Matrigel® solution evaporate.

   Note: If not used immediately, the cultureware must be sealed to prevent evaporation of the Matrigel® solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 7 days after coating. Allow stored coated cultureware to come to room temperature (15 - 25°C) for 30 minutes before plating cells.

Table 2. Recommended Volumes of Matrix for Coating Cultureware

TISSUE CULTURE-TREATED CULTUREWARE	VOLUME OF DILUTED MATRIX
24-well plate	300 µL/well
12-well plate	500 µL/well
6-well plate	1 mL/well
100 mm dish	6 mL/dish
T-25 cm^2 flask	3 mL/flask
T-75 cm^2 flask	8 mL/flask

4.2 Preparation of Media

The addition of STEMdiff™ SMADi Neural Induction Supplement to STEMdiff™ Neural Induction Medium promotes the efficient conversion of human ES and iPS cells to CNS-type NPCs and inhibits the unwanted differentiation of non-CNS-type cells. However, if desired, STEMdiff™ SMADi Neural Induction Supplement can be omitted and STEMdiff™ Neural Induction Medium used on its own; protocol changes are noted where applicable.

Prepare **either** STEMdiff™ Neural Induction Medium + SMADi (section 4.2.1) **or** STEMdiff™ Neural Induction Medium (without SMADi) (section 4.2.2).

4.2.1 STEMdiff™ Neural Induction Medium + SMADi

Use sterile techniques when preparing STEMdiff™ Neural Induction Medium + SMADi (STEMdiff™ Neural Induction Medium + STEMdiff™ SMADi Neural Induction Supplement). The following example is for preparing approximately 250 mL of medium. If preparing other volumes, adjust accordingly.

1. Thaw STEMdiff™ Neural Induction Medium and STEMdiff™ SMADi Neural Induction Supplement at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.

2. Add 0.5 mL of STEMdiff™ SMADi Neural Induction Supplement to 250 mL of STEMdiff™ Neural Induction Medium. Mix thoroughly. Warm medium to room temperature (15 - 25°C) before use.

   Note: If not used immediately, store at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C. Do not exceed the shelf life of the individual components. After thawing the aliquots, use immediately. Do not re-freeze.

4.2.2 STEMdiff™ Neural Induction Medium (Without SMADi)

Use sterile techniques when preparing STEMdiff™ Neural Induction Medium.

Thaw STEMdiff™ Neural Induction Medium at room temperature (15 - 25°C) or overnight at 2 - 8°C.

NOTE: If not used immediately, store at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C. Do not exceed the expiry date (EXP) as indicated on the label. After thawing the aliquots, use immediately. Do not re-freeze.

4.3 AggreWell™800 Plates

The following instructions are for preparing 1 well of an AggreWell™800 24-well Plate. If using more wells, adjust volumes accordingly.

1. Pre-treat wells with Anti-Adherence Rinsing Solution as follows:
   a. Add 500 µL of Anti-Adherence Rinsing Solution to each well to be used.
   b. Centrifuge plate at 1300 x *g* for 5 minutes in a swinging bucket rotor fitted with plate holders.

      NOTE: Plates must be well balanced. Prepare a balance plate using a standard plate filled with water to match the weight and position of the AggreWell™ plate.
   c. Observe plate under a microscope to ensure that bubbles have been removed from microwells. If bubbles remain trapped in any microwells, centrifuge at 1300 x *g* for an additional 5 minutes.
   d. Aspirate Anti-Adherence Rinsing Solution from the wells.
2. Add Y-27632 to STEMdiff™ Neural Induction Medium + SMADi to obtain a final concentration of 10 µM.
3. Warm (37°C) STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632. Add 1 mL per well of the plate prepared in step 1. Set the plate aside until use.

   Note: Do not remove medium at time of use.

5.0 Neural Induction: Generating NPCs from Human ES and iPS Cells

STEMdiff™ Neural Induction Medium + SMADi (section 4.2.1) can be used to generate CNS-type NPCs from human ES and iPS cells using either an EB protocol (Figure 1A; section 5.1) or monolayer culture protocol (Figure 1B; section 5.2). Some of the key differences between these two protocols are outlined in Table 3. The addition of STEMdiff™ SMADi Neural Induction Supplement to STEMdiff™ Neural Induction Medium promotes efficient conversion of human ES and iPS cells to CNS-type NPCs and inhibits the unwanted differentiation of non-CNS-type cells.

Resulting NPCs can be characterized by immunolabeling using the STEMdiff™ Human Neural Progenitor Antibody Panel, which includes primary antibodies that are immunoreactive toward marker proteins highly expressed either by NPCs (Nestin, PAX6, SOX1) or by undifferentiated human ES and iPS cells (OCT4/OCT3). For complete instructions on using this antibody panel, refer to Document #28137 available at www.stemcell.com or contact us to request a copy.

NPCs can be differentiated further to neurons and glial cells using lineage-specific STEMdiff™ differentiation and maturation kits (see Table 1). For instructions for use of these differentiation and maturation media, refer to the corresponding Product Information Sheet (PIS; Document #DX20341 [neuron], DX20343 [dopaminergic neuron], and DX20345 [astrocyte]) available at www.stemcell.com or contact us to request a copy. The differentiation protocols integrate into the EB neural induction protocol; for best results, read the instructions in the PIS before initiating neural induction.

Table 3. Comparison of Embryoid Body and Monolayer Culture Protocols

	NEURAL INDUCTION PROTOCOL	
	EMBRYOID BODY	MONOLAYER CULTURE
DAYS TO SINGLE-CELL NPCs	16 - 19	6 - 9
METHOD TO CONFIRM NEURAL INDUCTION	Visual inspection or phenotypic characterization	Phenotypic characterization only
NEURAL ROSETTE SELECTION	Yes	No
hPSC FEEDER-FREE MAINTENANCE MEDIUM COMPATIBILITY	mTeSR™1 or *TeSR™-E8™	mTeSR™1 or TeSR™-E8™

*When using TeSR™-E8™-maintained cells in the embryoid body protocol, the addition of STEMdiff™ SMADi Neural Induction Supplement to STEMdiff™ Neural Induction Medium is highly recommended.

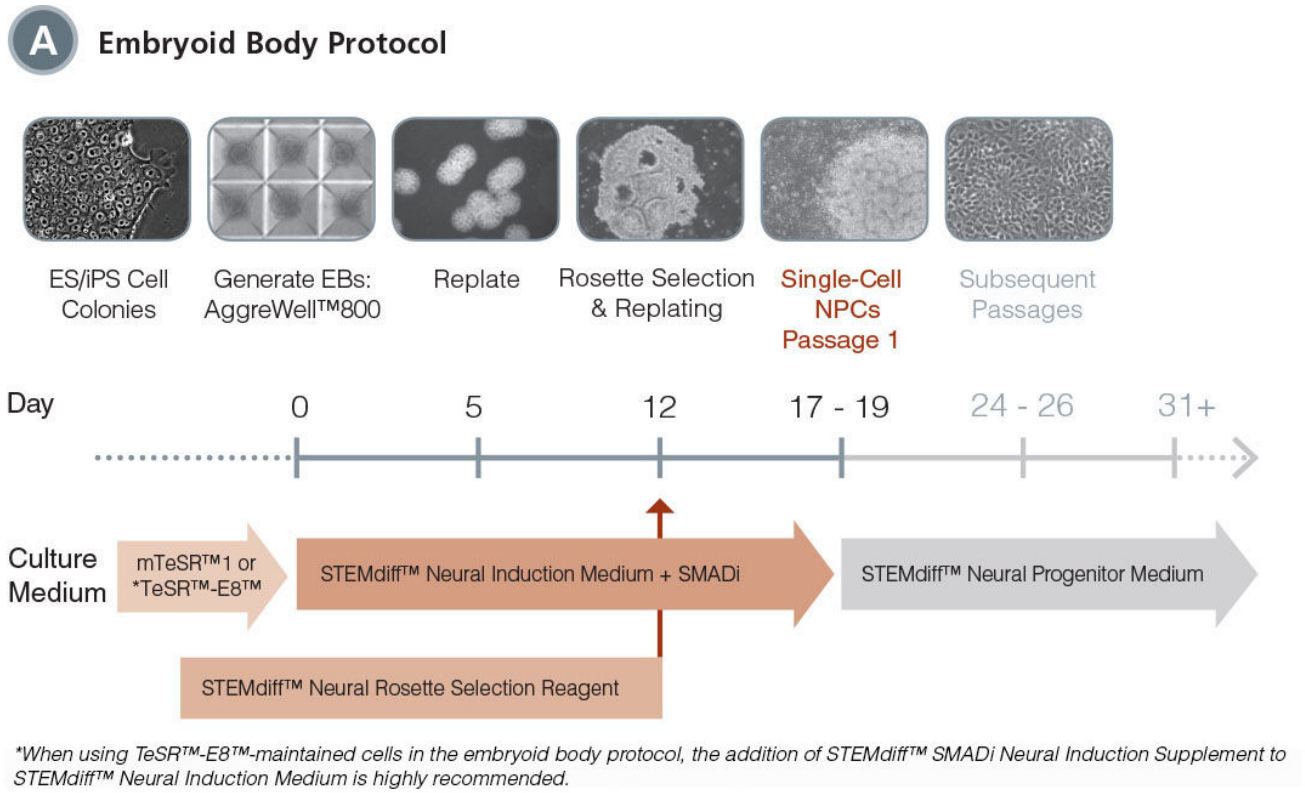

Figure 1. Schematic of Embryoid Body and Monolayer Culture Protocols. For NPC culture using STEMdiff™ Neural Progenitor Medium, refer to section 6.0. For downstream differentiation to neurons and glia using STEMdiff™ Neuron Differentiation Kit, STEMdiff™ Dopaminergic Neuron Differentiation Kit, or STEMdiff™ Astrocyte Differentiation Kit, refer to the corresponding PIS (Document #DX20341, DX20343, or DX20345, respectively) available at www.stemcell.com or contact us to request a copy.

5.1 EB Protocol

Day 0: Generating EBs Using AggreWell™800 Plates

The following instructions are for generating a single-cell suspension of human ES and iPS cells previously cultured in mTeSR™1 or *TeSR™-E8™ in a 100 mm dish and then plating cells into 1 well of an AggreWell™800 24-well Plate. If using other cultureware or number of wells, adjust volumes accordingly. 3 x 10^6 cells will be required for each well of an AggreWell™800 24-well Plate, resulting in 10,000 cells per microwell.

*When using TeSR™-E8™-maintained cells in the embryoid body protocol, the addition of STEMdiff™ SMADi Neural Induction Supplement to STEMdiff™ Neural Induction Medium is highly recommended.

Note: Addition of Y-27632 to STEMdiff™ Neural Induction Medium + SMADi is only required on Day 0.

1. Prepare 1 well of an AggreWell™800 24-well Plate (section 4.3).
2. Add Y-27632 to STEMdiff™ Neural Induction Medium + SMADi to obtain a final concentration of 10 µM.
3. Warm (37°C) sufficient volumes of STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632, Gentle Cell Dissociation Reagent, D-PBS (Without Ca++ and Mg++), and DMEM/F-12.
4. Use a microscope to visually identify regions of differentiation in the human ES and iPS cell culture. Mark these using a felt tip or lens marker on the bottom of the 100 mm dish. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
5. Wash the dish once with 5 - 10 mL of sterile PBS.
6. Aspirate and add 3 mL of Gentle Cell Dissociation Reagent.
7. Incubate at 37°C for 8 - 10 minutes.

   Note: The incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents, therefore dissociation should be monitored under the microscope until the optimal time is determined.
8. Dislodge cells by pipetting up and down 3 - 5 times using a micropipettor. Collect the cells into a 15 mL or 50 mL conical tube using a 5 mL serological pipette and ensure that any remaining cell aggregates are broken up into single cells.
9. Wash the dish with 10 mL of DMEM/F-12 and add to the tube containing the single-cell suspension.
10. Count viable cells using Trypan Blue and a hemocytometer.
11. Centrifuge at 300 x *g* for 5 - 10 minutes.
12. Carefully aspirate the supernatant and resuspend cells in STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632 to obtain a final concentration of 3 x 10^6 cells/mL.
13. Add 1 mL of the single-cell suspension (e.g. 3 x 10^6 cells) to a single well of the AggreWell™800 plate prepared in step 1. This will result in 10,000 cells/microwell.

    Note: Ensure that newly plated cells are evenly dispersed across the entire surface of the well by gently pipetting up and down several times.
14. Centrifuge the AggreWell™800 plate at 100 x *g* for 3 minutes. This will capture the cells in the microwells.

    Note: Plates must be balanced. It is recommended to balance the plate against a standard 24-well plate filled with water to match the weight and position of the AggreWell™800 plate.
15. Examine the AggreWell™800 plate under a microscope to ensure that cells are evenly distributed among the microwells.
16. Incubate cells at 37°C.

Day 1 - 4: Partial Medium Change

Note: On Day 1, uniform EBs should be visible in the AggreWell™800 well. At least 50% of cells within the microwell should be incorporated into the EBs. See section 8.0 for troubleshooting suggestions.

Perform a daily partial (3/4) medium change using the following protocol on Days 1 - 4. The following instructions are for performing a partial medium change in a single well of an AggreWell™800 24-well Plate.

1. Warm (37°C) a sufficient volume of STEMdiff™ Neural Induction Medium + SMADi.
2. Carefully remove the AggreWell™800 plate from the incubator, taking care not to disturb the contents.
3. Gently remove 1.5 mL of the medium from the well using a 1 mL pipettor and discard.

   Note: Do not disturb the EBs. Keep the pipette tip towards the upper surface of the medium in the well while removing the medium.
4. Slowly add 1.5 mL of STEMdiff™ Neural Induction Medium + SMADi to the well using a 1 mL pipettor.

   Note: It is important not to disturb the EBs. Do NOT add the medium directly onto the surface of the well. Support the pipette tip by slightly touching the side of the well at the surface level of the remaining medium inside the well. This will allow for a more controlled release of the medium. Release the medium very slowly into the well by setting the pipette-aid to "gravity" or "slow". Quick release of medium will dislodge the EBs from the wells.
5. Incubate at 37°C.
6. Repeat steps 1 - 5 until Day 4.

Day 5: Replating EBs

The following instructions are for harvesting EBs from a single well of an AggreWell™800 24-well Plate and plating them onto a matrix-coated well of a tissue culture-treated 6-well plate. If using multiple wells or other cultureware, adjust volumes accordingly.

Note: If you are using STEMdiff™ Dopaminergic Neuron Differentiation Kit to generate dopaminergic neuron precursors, the neural induction protocol is modified at this stage; refer to the PIS for the kit (Document #DX20343) for detailed instructions.

1. Coat 1 well of a 6-well tissue culture-treated plate with either PLO/laminin (section 4.1.1) or Corning® Matrigel® (section 4.1.2).
2. Warm (37°C) sufficient volumes of DMEM/F-12 and STEMdiff™ Neural Induction Medium + SMADi.
3. Place a 37 µm Reversible Strainer on top of a 50 mL conical tube. Label the tube "waste".

   Note: The arrow on the reversible strainer should point upwards. Use a new strainer and a new tube for each AggreWell™800 well to be harvested.
4. Remove the medium from the EB-containing well and firmly expel it into the well using a 1 mL pipettor with a wide-bore tip. This will dislodge the EBs from the AggreWell™800 well.
5. Using the same wide-bore tip, aspirate the EB suspension and filter it through the 37 µm Reversible Strainer. EBs will remain on top of the strainer and single cells will flow through into the waste tube.
6. Draw up 1 mL of DMEM/F-12 using the wide-bore tip and firmly expel it into the same AggreWell™800 well. While EBs are in suspension, quickly transfer the EB suspension into the strainer from step 5.
7. Repeat step 6 until all EBs have been removed from the well. One or two repeats should be sufficient to dislodge all EBs. Examine the well under a microscope to ensure that all EBs have been removed.
8. Invert the strainer over a new 50 mL conical tube and add 2 mL of STEMdiff™ Neural Induction Medium + SMADi onto the strainer to collect all the EBs.
9. Gently remove the matrix solution from the 6-well plate (prepared in step 1) using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.

10. Plate the EB suspension (from step 8) into a single well of the matrix-coated 6-well plate using a wide-bore tip or a serological pipette. This will avoid breaking up the EBs.
11. Place the 6-well plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the EBs across the surface of the wells.

Day 6 - 11: Medium Change

Perform a daily full medium change with warm (37°C) STEMdiff™ Neural Induction Medium + SMADi. Use 2 mL of medium per well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

Day 8: Determining % Neural Induction

Examine the morphology of the culture and estimate the percentage of neural induction per well. EBs will have spread out and neural rosettes should be clearly visible. Follow the instructions below to calculate % Neural Induction.

Note: If neural induction calculated on Day 8 is < 75%, neural rosette selection may be inefficient. Refer to section 8.0 for troubleshooting suggestions.

1. Count all attached EBs.
2. Count attached EBs in which 50% or more of the area of each individual aggregate is filled with neural rosettes. See Figure 2A for a representative image of EBs with 100% neural rosettes and Figure 2B for a representative image of EBs with less than 50% neural rosettes.
3. Quantify neural rosette induction using the following formula:

$$\%\ Neural\ Induction = \frac{\#\ of\ EBs\ with \geq 50\%\ neural\ rosettes}{Total\ \#\ of\ EBs}\ x\ 100$$

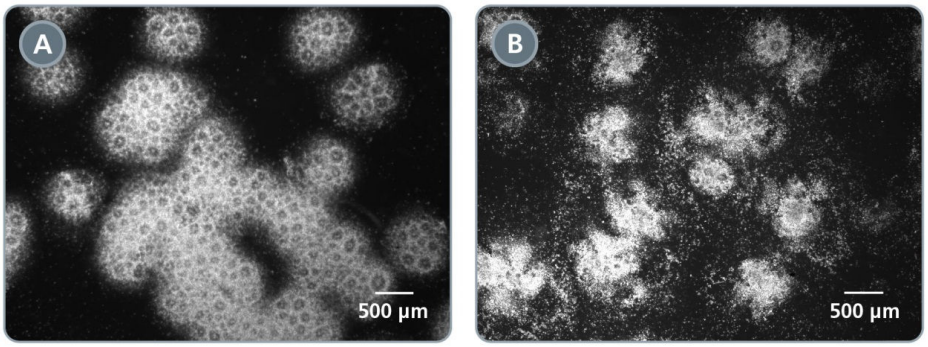

Figure 2. Determining % Neural Induction by Assessing Neural Rosette Formation in EBs.
Neural induction was calculated to be (A) 100% and (B) 39.2%.

Day 12: Neural Rosette Selection and Replating

The following instructions are for selecting neural rosettes from a single well of a 6-well plate and plating them onto a matrix-coated well of a new 6-well tissue culture-treated plate. If using other cultureware or number of wells, adjust volumes accordingly.

Note: If you are using STEMdiff™ Neuron Differentiation Kit or STEMdiff™ Astrocyte Differentiation Kit to generate neuronal or astrocytic precursors, respectively, the neural induction protocol is modified at this stage; refer to the corresponding kit PIS (Document #DX20341 or DX20345) for detailed instructions.

1. Coat 1 well of a tissue culture-treated 6-well plate with either PLO/laminin (section 4.1.1) or Corning® Matrigel® (section 4.1.2).
2. Warm (37°C) sufficient volumes of STEMdiff™ Neural Rosette Selection Reagent, STEMdiff™ Neural Induction Medium + SMADi, and DMEM/F-12.
3. Aspirate medium from the neural rosette-containing well and add 1 mL of DMEM/F-12 to wash the cells.
4. Aspirate DMEM/F-12 and add 1 mL of STEMdiff™ Neural Rosette Selection Reagent.
5. Incubate at 37°C for 1.5 hours (if using SMADi) or 1 hour (if using STEMdiff™ Neural Induction Medium *without* SMADi).

   Note: This incubation time may need to be optimized. Refer to section 8.0 for troubleshooting suggestions.
6. Carefully remove and discard the STEMdiff™ Neural Rosette Selection Reagent using a pipettor.
7. Using a 1 mL pipettor with a standard tip, draw up 1 mL of DMEM/F-12 and firmly expel it into the well, aiming specifically at the rosette clusters. This will dislodge the neural rosettes from the well.
8. Add the neural rosette suspension to a 15 mL conical tube. Do not further resuspend the neural rosettes, in order to minimize breaking up the clusters.
9. Repeat steps 7 and 8 until the majority of the neural rosette clusters have been collected, as determined by examination under a microscope. See Figure 3 for representative examples.

   Note: If the majority of neural rosettes have not detached, refer to section 8.0 for troubleshooting suggestions.

   Note: To avoid contamination with non-CNS-type cells, do not over-select. It is preferable to leave some rosettes behind, slightly sacrificing yield for higher purity.
10. Centrifuge at 350 x *g* for 5 minutes.
11. Carefully aspirate the supernatant and add 2 mL of STEMdiff™ Neural Induction Medium + SMADi. Gently resuspend the neural rosettes by pipetting slowly up and down 1 - 2 times using a 1 mL pipettor.
12. Gently remove the matrix solution from the new 6-well plate (prepared in step 1) using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
13. Add the neural rosette suspension (2 mL) to a single well of the matrix-coated 6-well plate.
14. 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 neural rosettes across the surface of the wells.

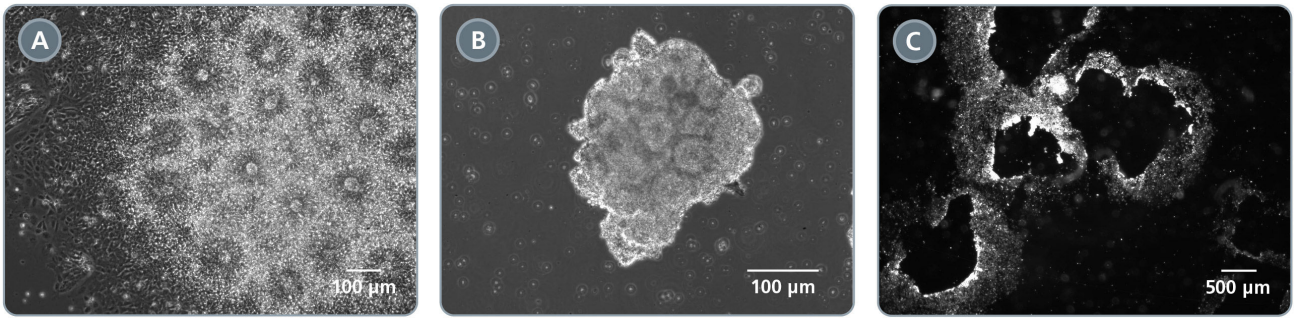

Figure 3. Selective Detachment of Neural Rosettes Using STEMdiff™ Neural Rosette Selection Reagent. Examples of neural rosettes (A) before and (B) after incubation with STEMdiff™ Neural Rosette Selection Reagent. (C) After selection, all neural rosettes have been removed.

Day 13 - 17 (or 19): Medium Change

Perform a daily full medium change with warm (37°C) STEMdiff™ Neural Induction Medium + SMADi until cultures are ready to be passaged. Use 2 mL/well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

Selected rosette-containing clusters will attach and NPC outgrowths will form a monolayer between the clusters. NPCs are ready for passage 1 when cultures are approximately 80 - 90% confluent (typically at about Day 17 - 19; see Figure 4A for a representative image). See section 6.0 for detailed instructions on how to passage and maintain NPCs using STEMdiff™ Neural Progenitor Medium.

*Note: On **Day 13**, high levels of cell death are typically observed. This does not affect the final yield of NPCs. NPCs will eventually start filling the gaps between larger rosette-containing clusters within 2 - 5 days.*

5.2 Monolayer Culture Protocol

Day 0: Plating Single Cells

The following instructions are for generating CNS-type NPCs from human ES and iPS cells previously cultured in mTeSR™1 or TeSR™-E8™ in a 100 mm dish, and then plating them onto a single well of a tissue culture-treated 6-well plate. If using other cultureware or number of wells, adjust volumes accordingly.

Note: Addition of Y-27632 to STEMdiff™ Neural Induction Medium + SMADi is only required on Day 0.

1. Coat 1 well of a tissue culture-treated 6-well plate with either PLO/laminin (section 4.1.1) or Corning® Matrigel® (section 4.1.2).
2. Add Y-27632 to STEMdiff™ Neural Induction Medium + SMADi to obtain a final concentration of 10 µM.
3. Warm (37°C) sufficient volumes of STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632, Gentle Cell Dissociation Reagent, D-PBS (Without Ca++ and Mg++), and DMEM/F-12.
4. Use a microscope to visually identify regions of differentiation in the human ES and iPS cell culture. Mark these using a felt tip or lens marker on the bottom of the 100 mm dish. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
5. Wash the dish once with 5 - 10 mL of sterile PBS.
6. Aspirate and add 3 mL of Gentle Cell Dissociation Reagent.
7. Incubate at 37°C for 8 - 10 minutes.

   Note: The incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents; monitor dissociation under the microscope until the optimal time is determined.
8. Dislodge cells by pipetting up and down 3 - 5 times using a micropipettor. Collect the cells into a 15 mL or 50 mL conical tube using a 5 mL serological pipette and ensure that any remaining cell aggregates are broken up into single cells.
9. Wash the dish with 10 mL of DMEM/F-12 and add to the tube containing the single-cell suspension.
10. Count viable cells using Trypan Blue and a hemocytometer.
11. Centrifuge cells at 300 x *g* for 5 - 10 minutes.
12. Carefully aspirate the supernatant and resuspend cells in STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632 to obtain a final concentration of 1 x 10^6 cells/mL (i.e. 2 x 10^5 cells/cm^2).

    Note: Plating densities of 2 - 2.5 x 10^5 cells/cm^2 are recommended.
13. Gently remove the matrix solution from the 6-well plate (prepared in step 1) using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
14. Add 2 mL of cell suspension (2 x 10^6 cells/well) to a single well of the matrix-coated 6-well plate.
15. 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 neural rosettes across the surface of the wells.

Day 1 - 6 (or 9): Medium Change

Perform a daily full medium change with warm (37°C) STEMdiff™ Neural Induction Medium + SMADi until cultures are ready to be passaged (i.e. when cells are approximately 80 - 90% confluent, typically after approximately 7 days of culture). Use 2 mL/well of a 6-well plate.

Note: Addition of Y-27632 to STEMdiff™ Neural Induction Medium + SMADi is not required when performing daily medium changes.

Day 6 - 9: Passage Cells

NPCs are ready for passage 1 when cultures are approximately 80 - 90% confluent (typically Day 6 - 9).

The following are instructions for passaging cells from 1 well of a 6-well plate and plating them onto a matrix-coated well of a new 6-well plate. If using other cultureware, adjust volumes accordingly.

1. Coat 1 well of a tissue culture-treated 6-well plate with either PLO/laminin (section 4.1.1) or Corning® Matrigel® (section 4.1.2).
2. Add Y-27632 to STEMdiff™ Neural Induction Medium + SMADi to obtain a final concentration of 10 µM.
3. Warm (37°C) sufficient volumes of STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632, DMEM/F-12, and ACCUTASE™.

   Optional: Wash cells to be passaged with 1 mL of DMEM/F-12.
4. Aspirate medium from the NPC-containing well and add 1 mL of ACCUTASE™.
5. Incubate at 37°C for 5 - 10 minutes.
6. Dislodge remaining attached cells by pipetting up and down using a 1 mL pipettor.
7. Add 5 mL of DMEM/F-12 to the well and transfer the NPC suspension to a 15 mL conical tube.
8. Centrifuge at 300 x *g* for 5 minutes.
9. Carefully aspirate the supernatant and add 1 mL of complete STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632.
10. Count viable cells using Trypan Blue and a hemocytometer.
11. Gently remove the matrix solution from the new 6-well plate (prepared in step 1) using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
12. Plate cells at desired density (e.g. 1.5 - 2 x 10^5 cells/cm^2) in 2 mL of STEMdiff™ Neural Induction Medium + SMADi into a single well of the new matrix-coated 6-well plate.
13. 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.
14. Perform a daily full medium change using STEMdiff™ Neural Induction Medium + SMADi.

    Note: Y-27632 is not required in the medium when performing daily medium changes.
15. Visually assess cultures to monitor growth and to determine timing of the next passage (i.e. when cells are approximately 80 - 90% confluent, typically after approximately 5 - 7 days of culture).

Note: It is recommended to passage NPCs using the above protocol one more time. When NPCs are ready for passage 3, continue to section 6.0 and passage using STEMdiff™ Neural Progenitor Medium.

6.0 Neural Progenitor Cell (NPC) Culture

STEMdiff™ Neural Progenitor Medium is optimized for the expansion of NPCs generated using STEMdiff™ Neural Induction Medium using either the EB protocol (section 5.1) or the monolayer culture protocol (section 5.2). NPCs cultured in STEMdiff™ Neural Progenitor Medium display typical NPC morphology and express markers that are indicative of CNS-type NPCs, such as PAX6 and SOX1. These markers can be characterized using the STEMdiff™ Human Neural Progenitor Antibody Panel. In addition, NPCs generated using STEMdiff™ Neural Induction Medium or expanded using STEMdiff™ Neural Progenitor Medium can be cryopreserved using STEMdiff™ Neural Progenitor Freezing Medium (section 6.3).

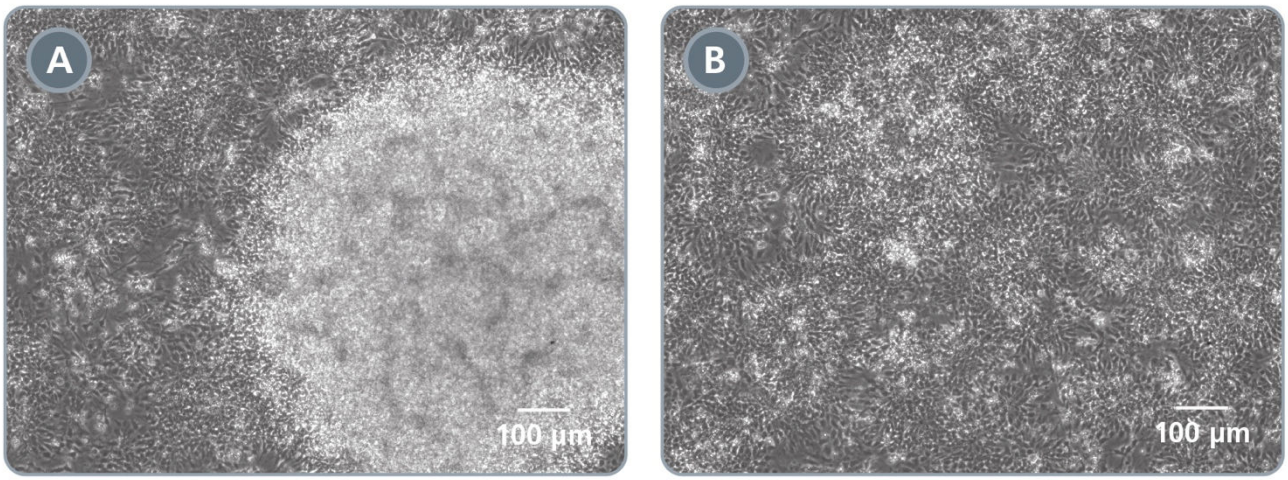

Figure 4. Confluent NPC Cultures Ready to be Passaged using STEMdiff™ Neural Progenitor Medium. Examples of NPCs obtained after the (A) EB protocol (section 5.1) and (B) the monolayer culture protocol (section 5.2). Cells should be approximately 80 - 90% confluent before passaging.

6.1 Complete STEMdiff™ Neural Progenitor Medium

Use sterile techniques to prepare complete STEMdiff™ Neural Progenitor Medium (Basal Medium [#05834] + Supplement A [#05836] + Supplement B [#05837]). The following example is for preparing approximately 100 mL of complete STEMdiff™ Neural Progenitor Medium. If preparing other volumes, adjust accordingly.

1. Thaw STEMdiff™ Neural Progenitor Basal Medium at room temperature (15 - 25°C) or overnight at 2 - 8°C.

   Note: If not used immediately, store at 2 - 8°C for up to 3 weeks. Alternatively, aliquot and store at -20°C. Do not exceed the shelf life of the basal medium. After thawing the aliquoted basal medium, use immediately. Do not re-freeze.

2. Thaw STEMdiff™ Neural Progenitor Supplement A (50X) and Supplement B (1000X) at room temperature (15 - 25°C) or at 2 - 8°C.

   Note: Once thawed, use immediately or aliquot and store at -20°C. Do not exceed the shelf life of the supplements. After thawing the aliquoted supplements, use immediately. Do not re-freeze.

3. Add 2 mL of STEMdiff™ Neural Progenitor Supplement A and 100 µL of STEMdiff™ Neural Progenitor Supplement B to 98 mL of STEMdiff™ Neural Progenitor Basal Medium. Mix well.

   Note: If not used immediately, store complete STEMdiff™ Neural Progenitor Medium at 2 - 8°C for up to 2 weeks. Do not freeze complete medium.

6.2 Passaging Protocol

NPCs are ready for passage when cultures are approximately 80 - 90% confluent (see Figure 4).

Note: For monolayer neural induction passage 1 and 2, follow passaging procedures in section 5.2.

The following are instructions for passaging cells from 1 well of a 6-well plate and plating them onto a matrix-coated well of a new 6-well plate. If using other cultureware, adjust volumes accordingly.

1. Coat 1 well of a new 6-well tissue culture-treated plate with either PLO/laminin (section 4.1.1) or Corning® Matrigel® (section 4.1.2).
2. Warm (37°C) sufficient volumes of complete STEMdiff™ Neural Progenitor Medium (section 6.1), DMEM/F-12, and ACCUTASE™.

   Optional: Wash cells to be passaged with 1 mL of DMEM/F-12.
3. Aspirate medium and add 1 mL of ACCUTASE™.
4. Incubate at 37°C for 5 - 10 minutes.
5. Dislodge remaining attached cells by pipetting up and down using a 1 mL pipettor.
6. Add 5 mL of DMEM/F-12 to the well and transfer the NPC suspension to a 15 mL conical tube.
7. Centrifuge at 300 x *g* for 5 minutes.
8. Carefully aspirate the supernatant and add 1 mL of complete STEMdiff™ Neural Progenitor Medium.
9. Count viable cells using Trypan Blue and a hemocytometer.
10. Gently remove the matrix solution from the new plate (prepared in step 1) using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
11. Plate cells at desired density (e.g. 1.25 x 10^5 cells/cm^2) in 2 mL of complete STEMdiff™ Neural Progenitor Medium onto the new matrix-coated plate.
12. 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.
13. Perform daily medium changes using complete STEMdiff™ Neural Progenitor Medium.
14. Visually assess cultures to monitor growth and to determine timing of the next passage (i.e. when cells are approximately 80 - 90% confluent, typically after approximately 7 days of culture).

6.3 Freezing Protocol

1. Prepare a single-cell suspension of NPCs using a passaging protocol of your choice. For example, follow instructions in section 6.2 steps 1 - 6.
2. Count viable cells using Trypan Blue and a hemocytometer.
3. Centrifuge cells at 300 x *g* for 5 minutes.
4. Carefully aspirate the supernatant and resuspend cell pellet at 2 - 4 x 10^6 cells/mL using cold (2 - 8°C) STEMdiff™ Neural Progenitor Freezing Medium.
5. Transfer 1 mL of cell suspension into each cryovial.
6. Freeze cells using a standard slow rate-controlled cooling protocol that reduces temperatures at a rate of approximately -1°C/min, followed by long-term storage at -135°C (liquid nitrogen) or colder. Long-term storage at -80°C is not recommended.

6.4 Thawing Protocol

In general, 1 frozen vial containing 2 - 4 x 10^6 NPCs can be successfully thawed into 1 well of a tissue culture-treated 6-well plate.

1. Coat 1 well of a 6-well tissue culture-treated plate with either PLO/laminin (section 4.1.1) or Corning® Matrigel® (section 4.1.2).
2. Warm (37°C) DMEM/F-12 and medium (e.g. complete STEMdiff™ Neural Progenitor Medium) 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 to a 15 mL conical tube.
4. Quickly thaw cells in a 37°C water bath by gently shaking the cryovial continuously until only a small frozen cell pellet remains.
5. Remove the cryovial from the water bath and wipe it with 70% ethanol or isopropanol.
6. Transfer cells from the cryovial to the tube containing DMEM/F-12. Mix gently.
7. Centrifuge cells at 300 x *g* for 5 minutes.
8. Aspirate medium, leaving the cell pellet intact. Gently resuspend the cell pellet in 2 mL of medium (e.g. complete STEMdiff™ Neural Progenitor Medium).
9. Gently remove the matrix solution from the 6-well plate (prepared in step 1) using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
10. Add cells to 1 well of the matrix-coated 6-well plate.
11. 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.

7.0 Differentiation of Neural Progenitor Cells to Generate Neurons and Astrocytes

NPCs can be differentiated further to neurons and glial cells using lineage-specific STEMdiff™ differentiation and maturation kits (see Table 1). For instructions for using these differentiation and maturation media, refer to the corresponding Product Information Sheet (PIS; Document #DX20341 [neuron], DX20343 [dopaminergic neuron], and DX20345 [astrocyte]) available at www.stemcell.com or contact us to request a copy. The differentiation protocols integrate into the EB neural induction protocol; for best results, read the instructions in the PIS before initiating neural induction.

8.0 Troubleshooting

8.1 Generating NPCs Using the EB Protocol

PROBLEM	SOLUTION
Air bubbles are present in microwells of the AggreWell™800 plate prior to adding hPSCs	• Ensure that the AggreWell™800 plate was prepared prior to use, as described in section 4.3.
Uneven distribution of hPSCs into all microwells	• Immediately after adding the hPSC suspension, pipette several times with a micropipettor and 1 mL pipette tip, to generate an evenly distributed cell suspension.
hPSCs overflow microwell capacity	• Do not exceed 20,000 cells per microwell. The recommended number of cells per microwell is 10,000.
Poor incorporation of hPSCs into EBs	• Ensure that high-quality hPSCs are used when starting the protocol.
Poor survival of hPSCs on Day 1	• Ensure that Y-27632 has been added at a final concentration of 10 µM when plating the single-cell suspension of hPSCs into the AggreWell™800 plate.
Difficulty removing EBs from microwells of the AggreWell™800 plate	• Ensure that the microwells have been pre-coated with Anti-Adherence Rinsing Solution (Catalog #07010) on Day 0 (section 4.3).
EBs dislodge from microwells of the AggreWell™800 plate during feeding	• Ensure that the microwells are not disturbed or shaken when moving back and forth from incubator. • Ensure that the pipette-aid is set to the lowest flow setting and that the pipette tip is touching the side of the well for the slowest possible medium release. • Choose one region of the microwell in which to place the pipette tip for each feed. This will minimize the number of EBs that become dislodged.
EBs break up when removing from microwells	• Ensure that a wide-bore tip is used instead of a regular 1 mL pipette tip when dislodging and transferring the EBs. • Ensure that high-quality hPSCs are used when starting the protocol.
EBs do not attach on Day 6	• Ensure that the cultureware is coated with appropriate matrix (section 4.1) prior to plating the EBs on Day 5.
Neural induction is < 75%	• Ensure that high-quality hPSCs are used when starting the protocol.
Poor survival of cells within neural rosettes	• Perform daily medium changes with STEMdiff™ Neural Induction Medium + SMADi. • Ensure that high-quality hPSCs are used when starting the protocol. • If issues persist: 1. Culture EBs for a longer time period in AggreWell™800 plates (e.g. 7 days instead of 5 days). 2. Isolate neural rosettes using STEMdiff™ Neural Rosette Selection Reagent at an earlier timepoint (e.g. Day 10 or 11 instead of Day 12).
Excessive cell death occurs after lifting and re-plating neural rosettes using STEMdiff™ Neural Rosette Selection Reagent (after Day 12)	• Cell death is to be expected after the treatment with STEMdiff™ Neural Rosette Selection Reagent due to low survival of single-cell NPCs. Single-cell NPCs should fill in the gaps between larger clumps after a few days and re-form rosettes. Ensure that neural rosettes are plated at a high density.
Difficulty isolating neural rosettes	• On Day 12, add STEMdiff™ Neural Rosette Selection Reagent to the well and incubate at 37°C for an additional 20 - 30 minutes.

PROBLEM	SOLUTION
Poor differentiation of NPCs and/or detection of non-CNS type NPCs	• When using STEMdiff™ Neural Rosette Selection Reagent on Day 12, isolate the neural rosettes and avoid collecting surrounding cells. • Ensure that high-quality hPSCs are used when starting the protocol. • If issues persist: 1. Culture EBs for a longer time period in AggreWell™800 plates (e.g. 7 days instead of 5 days). 2. Isolate neural rosettes using STEMdiff™ Neural Rosette Selection Reagent at an earlier time point (e.g. Day 10 or 11 instead of Day 12).

8.2 Generating NPCs Using the Monolayer Culture Protocol

PROBLEM	SOLUTION
hPSCs are confluent on Day 1	• Check cell count prior to plating hPSCs on Day 0. • Decrease plating density of hPSCs on Day 0.
No neural rosettes are visible	• Typically neural rosettes are not observed due to high cell density. For assessment of neural induction, characterize expression of markers such as PAX6 (e.g. using STEMdiff™ Human Neural Progenitor Antibody Panel).
Neural induction is not complete by Day 6 in the monolayer protocol or Day 7 of the EB protocol	• Timing of neural induction may need to be optimized for individual hPSCs.
Cell death is observed when single-cell suspension of hPSCs is plated	• It is normal to observe cell death on Day 1 - 3 of the monolayer protocol. Remaining attached cells will proliferate to confluency by Day 6 - 9.
Low attachment or cell death after passage 1	• Ensure that 10 µM Y-27632 is added when passaging NPCs using STEMdiff™ Neural Induction Medium + SMADi on Day 6 - 9.

9.0 References

1. Itskovitz-Eldor J et al. (2000) Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol Med 6(2): 88–95.
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