TECHNICAL MANUAL

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

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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, hPSCs 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 hPSCs 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 designed for the generation, isolation, 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

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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
	6-well flat-bottom plate, tissue culture-treated	e.g. 38016
	96-well half-area high-content imaging microplate	Corning 4680
	70% (vol/vol) ethanol	Sigma 793213
	Conical tubes	e.g. 38009 (15 mL) OR 38010 (50 mL)
	Serological pipettes, 5 mL	e.g. 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) or 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

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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
- Hemocytometer
- Pipettor (e.g. Catalog #38058) 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)
- High-content platform equipment:
    - Plate washer with multiple syringe capacity (washing and liquid dispensing)
    - Plate loader (precise dispensing 0.5 - 2500 µL)
    - Plate centrifuge
    - Automated incubator
    - Liquid handler
    - Robotic arm
    - High-content microscope

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3.0 Important Parameters for Successful Neural Induction From hPSCs

High-Quality hPSCs

It is critical to start with high-quality hPSC cultures for efficient neural induction.

Note: Refer to the Technical Manuals for mTeSR™1, mTeSR™ Plus, or TeSR™-E8™ for complete instructions on culturing high-quality hPSCs 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 hPSCs 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 embryoid bodies (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 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]

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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 with 15 mM HEPES.
7. Dilute laminin in DMEM/F-12 with 15 mM HEPES 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.

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4.1.2 Corning® Matrigel®

Corning® Matrigel® hESC-Qualified Matrix should be aliquoted and frozen. Consult the Matrigel® Certificate of Analysis for the recommended aliquot size ("Dilution Factor") to prepare 25 mL of diluted matrix. Ensure to always keep Matrigel® on ice when thawing and handling to prevent it from gelling.

1. Thaw one aliquot of Matrigel® on ice.
2. Dispense 25 mL of cold DMEM/F-12 with 15 mM HEPES 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. Immediately use the diluted Matrigel® solution 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.

   Note: If the cultureware surface is not fully coated by the Matrigel® solution, it should not be used for hPSC culture.
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
96-well plate	50 µL/well
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 hPSCs 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).

Note: If STEMdiff™ Neural Induction Medium is received thawed, immediately place at -20°C or aliquot and store at -20°C. Product performance will not be affected. For thawing instructions, refer to section 4.2.1 or 4.2.2.

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4.2.1 STEMdiff™ Neural Induction Medium + SMADi

Use sterile technique 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.

   Note: If not used immediately, aliquot and store at -20°C. Do not exceed the product shelf life. Alternatively, thawed Neural Induction Medium may be stored at 2 - 8°C for up to 2 weeks. After thawing aliquots, use immediately. Do not re-freeze.

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 aliquots, use immediately. Do not re-freeze.

4.2.2 STEMdiff™ Neural Induction Medium (Without SMADi)

Use sterile technique 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 aliquots, use immediately. Do not re-freeze.

4.3 AggreWell™800 Plates

The following instructions are for preparing one 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.

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5.0 Neural Induction: Generating NPCs From hPSCs

STEMdiff™ Neural Induction Medium + SMADi (section 4.2.1) can be used to generate CNS-type NPCs from hPSCs using either an embryoid body (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 hPSCs to CNS-type NPCs and inhibits the unwanted differentiation of non-CNS-type cells.

Table 3. Comparison of Embryoid Body and Monolayer Culture Protocols

	NEURAL INDUCTION PROTOCOL	
	EMBRYOID BODY (EB)	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, mTeSR™ Plus, or **TeSR™-E8™	mTeSR™1, mTeSR™ Plus, or TeSR™-E8™

*For NPCs generated using the monolayer culture protocol, culture for 18 - 21 days (3 passages) prior to downstream differentiation for best results.

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

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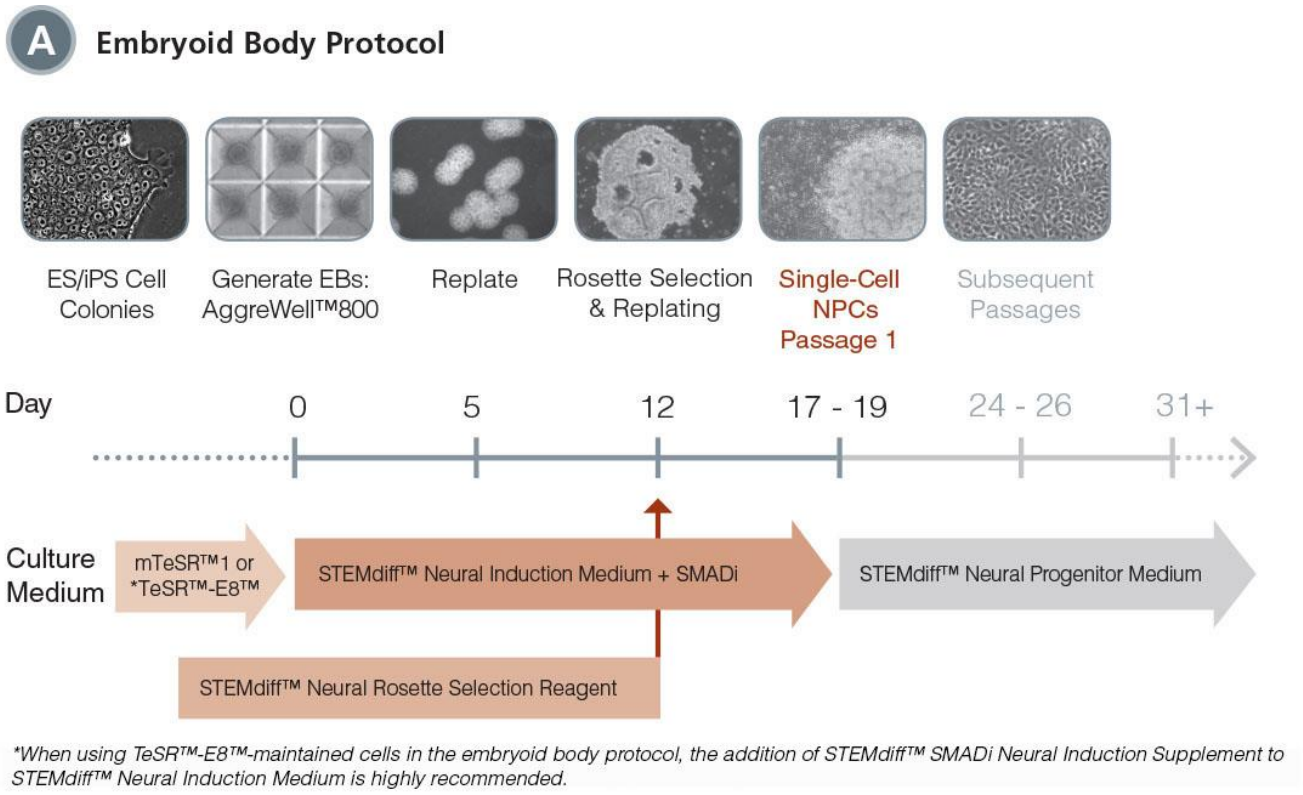

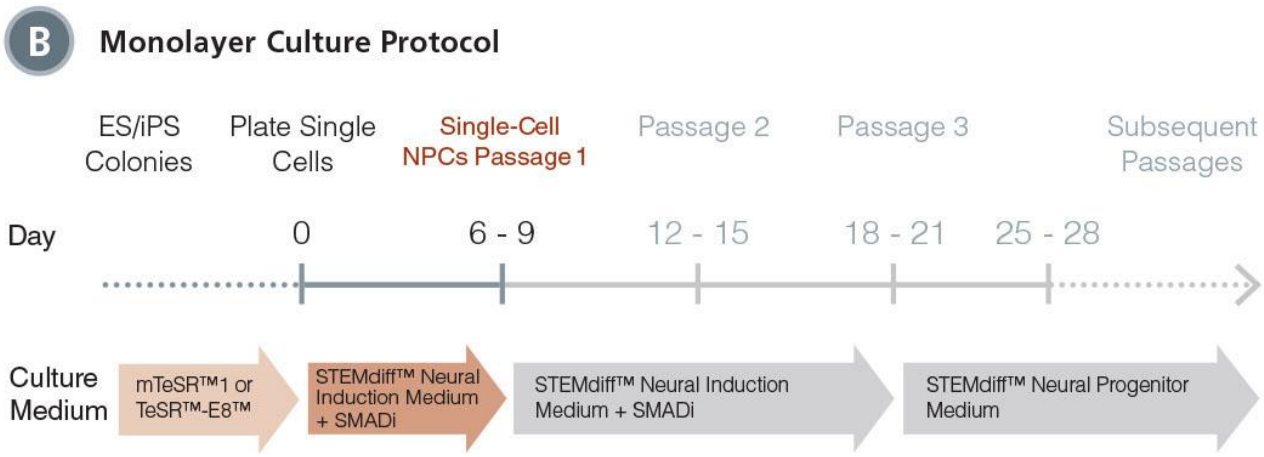

Figure 1. Schematic of Embryoid Body and Monolayer Culture Protocols

Note: mTeSR™ Plus may be used instead of mTeSR™1 or TeSR™-E8™ for culturing hPSCs.

For NPC culture using STEMdiff™ Neural Progenitor Medium, refer to section 6.0.

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5.1 EB Protocol

Day 0: Generating EBs Using AggreWell™800 Plates

The following instructions are for generating a single-cell suspension of hPSCs previously cultured in mTeSR™1, mTeSR™ Plus, or *TeSR™-E8™ in a 100 mm dish and then plating cells into one 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 EB 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 one 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 with 15 mM HEPES.
4. Use a microscope to visually identify regions of differentiation in the hPSC 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. Using a pipettor, pipette the cell suspension up and down 3 - 5 times to dislodge remaining attached cells. Using a 5 mL serological pipette, collect the cells into a 15 mL or 50 mL conical tube. Ensure that any remaining cell aggregates are broken up into single cells.
9. Wash the dish with 10 mL of DMEM/F-12 with 15 mM HEPES 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.

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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 7.0 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. Using a 1 mL pipettor, gently remove 1.5 mL of the medium from the well and discard.

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

   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.

1. Coat one 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 with 15 mM HEPES 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 with 15 mM HEPES 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 into the tube.
9. Gently remove the matrix solution from the 6-well plate (prepared in step 0) using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.

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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 7.0 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:

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

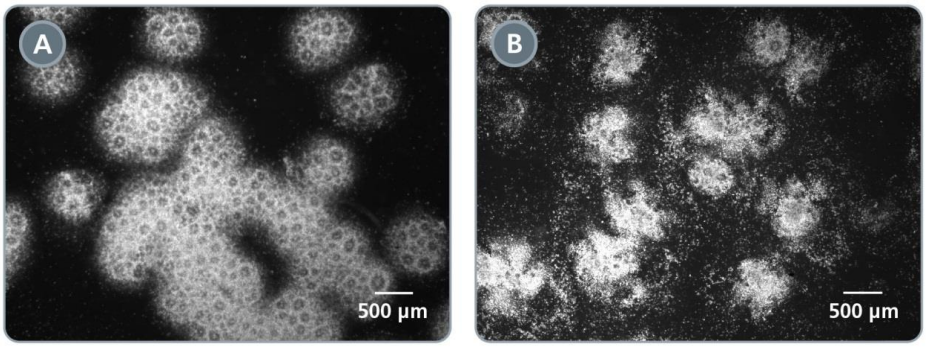

Figure 2. Determining % Neural Induction by Assessing Neural Rosette Formation in EBs

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.

1. Coat one well of a tissue culture-treated 6-well plate with either PLO/laminin (section 4.1.1) or Corning® Matrigel® (section 4.1.2).

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2. Warm (37°C) sufficient volumes of STEMdiff™ Neural Rosette Selection Reagent, STEMdiff™ Neural Induction Medium + SMADi, and DMEM/F-12 with 15 mM HEPES.
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 7.0 for troubleshooting suggestions.
6. Using a pipettor, carefully remove and discard STEMdiff™ Neural Rosette Selection Reagent.
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 7.0 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. Using a 1 mL pipettor, gently resuspend the neural rosettes by pipetting slowly up and down 1 - 2 times.
12. Using a serological pipette or by aspiration, gently remove the matrix solution from the new 6-well plate (prepared in step 1). 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.

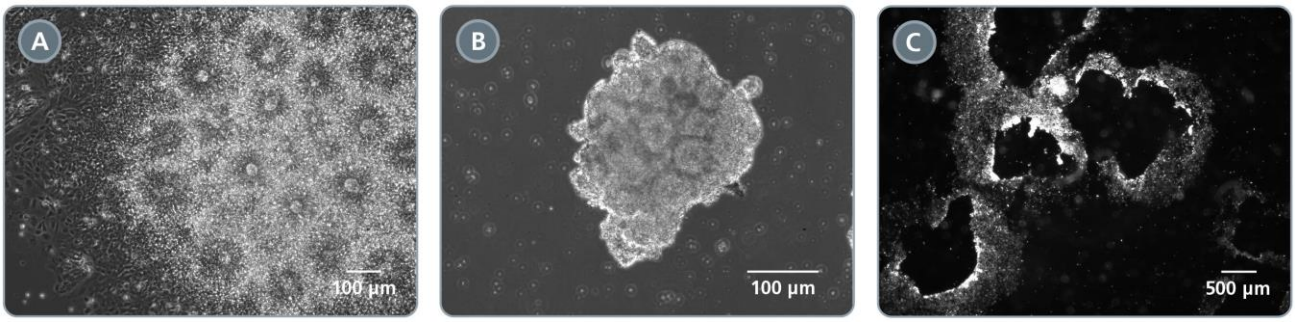

Figure 3. Selective Detachment of Neural Rosettes Using STEMdiff™ Neural Rosette Selection Reagent. Examples of neural rosettes **(A)** before and **(B)** after incubation with STEMdiff™ Neural Rosette Selection Reagent. **(C)** After selection, all neural rosettes have been removed.

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.

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Selected rosette-containing clusters will attach, and NPC outgrowths will form a monolayer between the clusters. NPCs are ready for passage 1 at approximately 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 hPSCs previously cultured in mTeSR™1, mTeSR™ Plus, 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 one 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 with 15 mM HEPES.
4. Use a microscope to visually identify regions of differentiation in the hPSC 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. Aspirate.
6. Add 3 mL of Gentle Cell Dissociation Reagent. 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.
7. Using a pipettor, pipette the cell suspension up and down 3 - 5 times to dislodge remaining attached cells. Collect the cells into a 15 mL or 50 mL conical tube using a 5 mL serological pipette. Ensure that any remaining cell aggregates are broken up into single cells.
8. Wash the dish with 10 mL of DMEM/F-12 and add to the tube containing the single-cell suspension.
9. Count viable cells using Trypan Blue and a hemocytometer.
10. Centrifuge cells at 300 x *g* for 5 - 10 minutes.
11. 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.
12. Using a serological pipette or by aspiration, gently remove the matrix solution from the 6-well plate (prepared in step 1). Ensure that the coated surface is not scratched.
13. Add 2 mL of cell suspension (2 x 10^6 cells/well) 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.

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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 (after approximately 7 days of culture; see Figure 4B).
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 typically ready for passage 1 on Day 6 - 9, depending on the rate of cell growth.

The following are instructions for passaging cells from one 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 one 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 with 15 mM HEPES, 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. Using a 1 mL pipettor, pipette the cell suspension up and down to dislodge remaining attached cells.
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. Using a serological pipette or by aspiration, gently remove the matrix solution from the new 6-well plate (prepared in step 1). 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 (after approximately 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.

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5.2.1 Automated High-Content Monolayer Protocol for NPC Differentiation From hPSCs

Day 0: Plating Single Cells

The following instructions are for generating CNS-type NPCs from hPSCs previously cultured in mTeSR™1, mTeSR™ Plus, or TeSR™-E8™ in a 100 mm dish, and then plating them onto a tissue culture-treated 96-well half-area high-content imaging 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. Add Y-27632 to STEMdiff™ Neural Induction Medium + SMADi to obtain a final concentration of 10 µM.
2. Prepare a medium plate using a 96-well plate with conical bottom with sufficient medium for 50 µL per well of the 96-well experimental plate, using STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632.
3. Prepare sufficient amounts (enough for dispensing volume plus priming and dead volume of dispensing equipment) of Corning® Matrigel® and place it on ice.
4. Coat the experimental plate using a microplate dispenser (e.g. Multidrop or plate washer's dispensing cassette). Dispense 50 µL of Matrigel® in each well.
5. Wash the cassette and tubing of the microplate dispenser with 70% (vol/vol) ethanol immediately after dispensing to avoid clogging.
6. Centrifuge the plate at 300 x *g* for 30 seconds to remove bubbles.
7. Incubate at room temperature (15 - 25°C) for 1 hour.
8. Remove Matrigel® using a plate washer with an aspiration manifold head. Leave some Matrigel® in each well.
9. Wash the aspiration manifold head with 70% (vol/vol) ethanol immediately after aspiration.
10. Using a microplate dispenser, dispense 50 µL of STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632 from the medium plate (prepared in step 2) into each well of the experimental plate to prevent drying out of the coated surface.
11. Prepare a single-cell suspension of hPSCs in STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632 (refer to section 5.2 [Day 0] steps 4 - 8).
12. Count viable cells using Trypan Blue and a hemocytometer.
13. Centrifuge at 300 x *g* for 5 minutes.
14. Carefully aspirate the supernatant and resuspend cells in STEMdiff™ Neural Induction Medium + SMADi + 10 µM Y-27632 to obtain a final concentration of 0.2 - 1.2 x 10^6 cells/mL.
15. Filter cells through a 37 µm cell strainer.
16. Add 25 - 75 µL of cell suspension (1.6 - 3.2 x 10^4 cells) to a single coated well of the 96-well plate. Incubate at 37°C.
    Note: Plating density of 1 - 2 x 10^5 cells/cm^2 is recommended with 25 - 75 µL of cell suspension per well.

Day 1 - 6 (or 9): Medium Change

Perform a daily full-medium change until cultures are ready to be fixed (after approximately 7 days of culture; see Figure 4B), as follows:

1. Using the plate washer, remove medium from the well.
2. Add 75 - 125 µL of warm (37°C) STEMdiff™ Neural Induction Medium + SMADi per well.
   Note: Addition of Y-27632 to STEMdiff™ Neural Induction Medium + SMADi is not required when performing daily medium changes.
3. Incubate at 37°C.

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6.0 NPC Culture

STEMdiff™ Neural Progenitor Medium is optimized for the expansion of NPCs generated using STEMdiff™ Neural Induction Medium using either the embryoid body (EB) protocol (section 5.1) or the monolayer culture protocol (section 5.2). See Figure 4 for examples of NPC cultures ready for passaging using STEMdiff™ Neural Progenitor Medium.

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, and have a low or negative expression of β-tubulin III (can be determined using Anti-Beta-Tubulin III Antibody, Clone TUJ1, Catalog # 60052). 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).

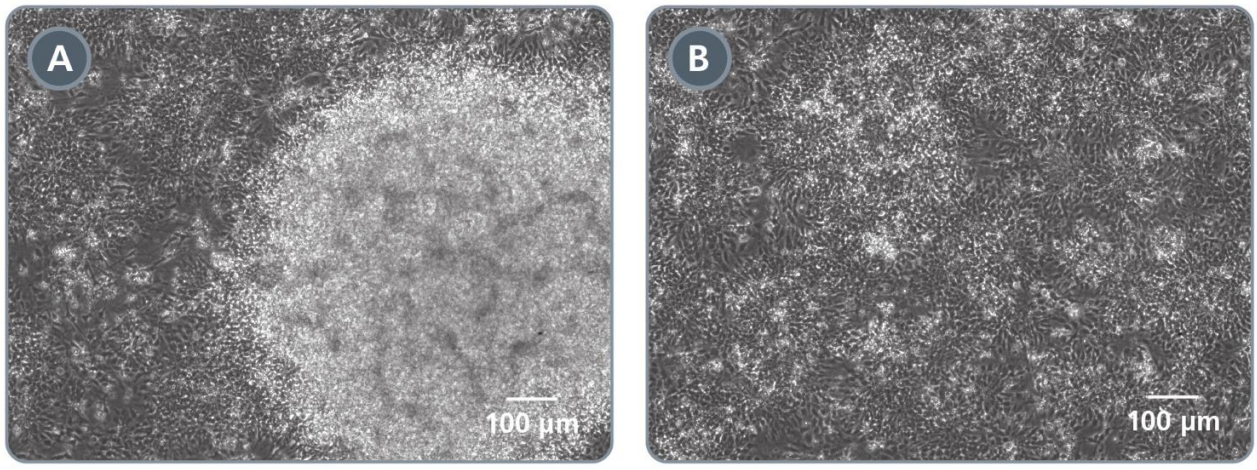

Figure 4. NPC Cultures Ready to be Passaged Using STEMdiff™ Neural Progenitor Medium
Examples of NPCs obtained after the **(A)** EB protocol (section 5.1) and **(B)** the monolayer culture protocol (section 5.2).

6.1 Preparation of STEMdiff™ Neural Progenitor Medium

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

Note: If STEMdiff™ Neural Progenitor Basal Medium is received thawed, immediately place at -20°C or aliquot and store at -20°C. Product performance will not be affected.

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 medium. After thawing aliquots, 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 medium at 2 - 8°C for up to 2 weeks. Do not freeze complete medium.

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6.2 Passaging NPCs Using STEMdiff™ Neural Progenitor Medium

Refer to Figure 4 for examples of NPC cultures ready for passaging using STEMdiff™ Neural Progenitor Medium.

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

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

1. Coat one 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 STEMdiff™ Neural Progenitor Medium (section 6.1), DMEM/F-12 with 15 mM HEPES, 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. Using a 1 mL pipettor, pipette the cell suspension up and down to dislodge remaining attached cells.
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 STEMdiff™ Neural Progenitor Medium.
9. Count viable cells using Trypan Blue and a hemocytometer.
10. Using a serological pipette or by aspiration, gently remove the matrix solution from the new plate (prepared in step 1). 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 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 STEMdiff™ Neural Progenitor Medium.
14. Visually assess cultures to monitor growth and to determine timing of the next passage (after approximately 7 days of culture).

6.3 Cryopreserving NPCs Using STEMdiff™ Neural Progenitor Freezing Medium

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. Cryopreserve 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.

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6.4 Thawing NPCs

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

1. Coat one 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 with 15 mM HEPES 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 with 15 mM HEPES 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. STEMdiff™ Neural Progenitor Medium).
9. Using a serological pipette or by aspiration, gently remove the matrix solution from the 6-well plate (prepared in step 1). Ensure that the coated surface is not scratched.
10. Add cells to one 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.

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7.0 Troubleshooting

7.1 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 pipettor 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 to use high-quality hPSCs 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-treated with Anti-Adherence Rinsing Solution 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 to use high-quality hPSCs 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. Check for chromosomal abnormalities by karyotyping, or use the Genetic Analysis Kit (Catalog #07550) to check for small-scale amplifications.

Poor survival of cells within neural rosettes	• Perform daily medium changes with STEMdiff™ Neural Induction Medium + SMADi.
	• Ensure to use high-quality hPSCs when starting the protocol.
	• If issues persist:
	  ○ Culture EBs for a longer time period in AggreWell™800 plates (e.g. 7 days instead of 5 days).
	  ○ 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.

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PROBLEM	SOLUTION
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.
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 to use high-quality hPSCs when starting the protocol. • If issues persist: o Culture EBs for a longer time period in AggreWell™800 plates (e.g. 7 days instead of 5 days). o Isolate neural rosettes using STEMdiff™ Neural Rosette Selection Reagent at an earlier timepoint (e.g. Day 10 or 11 instead of Day 12).

7.2 Generating NPCs Using the Monolayer Culture Protocol

PROBLEM	SOLUTION
Unsure if NPCs are ready for passage	• NPCs will pack together nicely if allowed to grow. Keep feeding the culture for 6 - 7 days; NPCs can get very dense. As long as there is no cell death, the culture can appear more than 100% confluent and still remain healthy.
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.
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.

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TECHNICAL MANUAL

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

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