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Induction of Neural Stem Cells from Human Pluripotent Stem Cells Using PSC Neural Induction Medium

Publication Number MAN0008031 **Revision** A.0

Introduction

Human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are excellent resources for studying cell fate specification, disease modeling and drug screening. The first important step in producing various neural cells from PSCs is the induction of PSCs to neural stem cells (NSCs). Conventional methods of NSC derivation from human PSCs involving embryoid body (EB) formation or co-cultures with stromal cell lines have several disadvantages, including a time-consuming protocol and variability in the quality of resulting NSCs. We have developed a serum-free neural induction medium which can differentiate human PSCs into NSCs in one week with high efficiency, but without the laborious processes of EB formation and mechanical NSC isolation.

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from www.lifetechnologies.com/support.

Materials needed

- PSC Neural Induction Medium (consists of Neurobasal® Medium and Neural Induction Supplement, 50X (Cat. no. A1647801)
- Advanced™ DMEM/F-12 (Cat. no. 12634) (needed for NSC expansion)
- Geltrex® LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Cat. no. A1413301 or A1413302)
- Distilled water (Cat. no. 15230)
- ROCK Inhibitor Y27632 (Sigma-Aldrich, Cat. no. Y0503)
- DPBS without $CaCl_2$ and $MgCl_2$ (Cat. no. 14190)
- StemPro® Accutase® Cell Dissociation Reagent (Cat. no. A11105)
- Dimethyl sulfoxide (DMSO), Sterile (Sigma-Aldrich, Cat. no. D2650)
- Cell scraper (Fisher Scientific, Cat. no. 08-771-1A)
- 15-mL and 50-mL sterile polypropylene conical tubes
- 6-well culture plates (Fisher Scientific, Cat. no. 08-772-1B)
- Thermo Scientific® Nunc™ Lab-Tek™ Chamber Slide System, 4-well (Fisher Scientific, Cat. no. 1256521)
- Pasteur glass pipettes
- 5-, 10-, 25-, and 50-mL sterile pipettes
- 100-µm strainer (Fisher Scientific, Cat. no. 08-771-19)
- Cryovial® vials
- Nalgene® Mr. Frosty® Freezing Container (Fisher Scientific, Cat. no. 15-350-50)

- 37°C humidified cell culture incubator with 5% CO_2
- Liquid nitrogen storage
- Centrifuge
- 37°C water bath

Immunocytochemistry reagents:

- Molecular Probes® Human Neural Stem Cell Immunocytochemistry Kit (Cat. no. A24354)

  Note: This complete immunocytochemistry kit contains primary antibodies for four common NSC markers (Nestin, PAX6, SOX1, and SOX2), a matching set of Alexa Fluor®-labeled secondary antibodies, a nuclear DNA stain (DAPI), and all of the buffers necessary for performing the staining protocol.

- OCT4 Rabbit Monoclonal Antibody (Cat. no. A13998)
- 4-well chamber slide (Fisher Scientific, Cat. no. 12-565-17)
- Cover glass, 24 × 50 mm (Fisher Scientific, Cat. no. 22-050-232)
- (*Optional*) ProLong® Gold Antifade Reagent (Cat. no. P36930)

If you are working with individual antibodies, the following primary antibodies are recommended:

- Rabbit anti-Oct4 IgG antibody, 1:100 dilution (Cat. no. A13998)
- Goat anti-Sox1 IgG antibody, 1:100 dilution (R&D systems, Cat. no. AF3369)
- Mouse anti-Sox2 IgG2a antibody, 1:100 dilution (R&D systems, Cat. no. MAB2018)
- Mouse anti-Nestin IgG1 antibody, 1:1000 dilution (BD Bioscience, Cat. no. 611658)

Prepare media and materials

PSC Neural Induction Medium (for 500 mL)

1. To prepare 500 mL of complete PSC Neural Induction Medium, aseptically mix the following components:

Neurobasal® Medium	490 mL
Neural Induction Supplement	10 mL

2. Complete PSC Neural Induction Medium can be stored at 2–8°C in the dark for up to 2 weeks. Warm the Neural Induction Medium in a 37°C water bath for 5–10 minutes before using. Do not warm the Neural Induction Medium in a 37°C water bath for longer than 10 minutes, as this may cause degradation of the medium.

   Note: Neural Induction Supplement can be thawed at 2–8°C overnight or quickly in a 37°C water bath for about 5 minutes, and then aliquoted and frozen at –5°C to –20°C to allow for preparation of smaller volumes of complete medium. Avoid repeated thawing and freezing of the Neural Induction Supplement.

Neural Expansion Medium (for 100 mL)

1. To prepare 100 mL of complete Neural Expansion Medium, aseptically mix the following components:

Neurobasal® Medium	49 mL
Advanced™ DMEM/F-12	49 mL
Neural Induction Supplement	2 mL

2. Complete Neural Expansion Medium can be stored at 2–8°C in the dark for up to 2 weeks. Warm the Neural Expansion Medium in a 37°C water bath for 5–10 minutes before using.

ROCK Inhibitor Y27632 Solution (5 mM)

1. To prepare 5 mM ROCK inhibitor Y27632 solution, aseptically mix the following components:

Y27632	1 mg
Distilled water	0.625 mL

2. After dissolving, filter through a 0.22 µm filter, aliquot 20–50 µL into sterile tubes, and store at –5°C to –20°C in the dark for up to 1 year. Thawed Y27632 solution can be kept at 4°C for up to 1 week.

   Note: The Molecular Weight of Y27632 is 320.26, however variation in molecular weight may occur between lots depending on the water content.

Coat culture vessels with Geltrex® LDEV-Free, hESC-Qualified Basement Membrane Matrix

1. Thaw a vial of Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix at 2–8°C overnight.

   Note: Thawed Geltrex® matrix can be aliquoted and then frozen at –5°C to –20°C, or stored at 2–8°C for up to 2 weeks. Avoid repeated thawing and freezing.

2. To create a working solution, dilute the thawed Geltrex® matrix solution 1:100 with cold Neurobasal® Medium or DMEM/F12 on ice.
3. Quickly cover the whole surface of each culture vessel with the Geltrex® matrix solution (refer to Table 1).
4. Incubate the culture vessels in a 37°C, 5% CO_2 incubator for 1 hour.
5. The culture vessels are now ready for use. Just before use, aspirate the diluted Geltrex® solution from the culture vessels. Cells can be plated directly onto the Geltrex® matrix-coated culture vessels without rinsing. Coated culture vessels can also be stored at 2–8°C for up to one week. When storing, seal culture vessels with Parafilm® laboratory film to prevent drying. Before using, warm up the coated culture vessels stored at 2–8°C at room temperature for 30 minutes.

Table 1 Required volume of Geltrex® matrix solution

Culture vessel	Approximate surface area (cm^2)	Diluted Geltrex® matrix volume (mL)
4-well chamber slide	1.8 cm^2/well	0.3 mL/chamber
6-well plate	9.6 cm^2/well	1 mL/well
35-mm dish	11.8 cm^2	1 mL
60-mm dish	20 cm^2	2 mL
100-mm dish	60 cm^2	5 mL

Methods

Culture and prepare human PSCs for neural induction

1. Start with high quality human PSCs (with minimal or no differentiated colonies) cultured in feeder-free conditions such as in Essential 8™ Medium on Vitronectin or Geltrex substrate, or in StemPro® hESC SFM on Geltrex® matrix. For PSC culturing protocols, visit https://www.lifetechnologies.com/us/en/home/references/protocols/cell-culture/stem-cell-protocols/ipsc-protocols.html.

IMPORTANT! The quality of the PSCs (with minimal or no differentiated colonies) is **critical** for efficient neural induction. Remove any differentiated and partially differentiated colonies before passaging PSCs. Differentiated colonies can be marked by using a Nikon® microscopy object marker (Nikon Instruments Inc., Cat. no. MBW10020) with a Nikon® microscopy C-OA 15 mm objective adapter (Nikon Instruments Inc., Cat. no. MXA20750).

Note: Human PSCs cultured on mouse embryonic fibroblasts can also be used for neural induction.

2. Coat 6-well plates with the same coating material on which your PSCs are cultured.

3. When the PSCs reach ~70–80% confluency, dislodge PSCs to generate cell clumps for passaging by following the appropriate PSC subculture protocol. Follow steps 3a–e to estimate the cell concentration of the suspension of PSC clumps before plating.
   a. Generate a PSC cell suspension, then transfer a portion of the cell suspension to a 15-mL conical tube (for example, transfer 1 mL of a 6 mL PSC suspension prepared from one well of a 6-well plate) to estimate the total cell number of the PSC cell suspension.
   b. Centrifuge the 15-mL conical tube with the cells at 200 × *g* for 3 minutes and aspirate the supernatant.
   c. Add 1 mL of pre-warmed StemPro® Accutase® Cell Dissociation Reagent to the 15-mL conical tube containing the cells, then incubate for 5 minutes at 37°C.
   d. Vigorously pipet the cells up and down with a 1 mL pipette 5 times to dissociate the cells into a single cell suspension.
   e. Determine the total cell number using your preferred method. If the total cell number in 1 mL StemPro® Accutase® reagent is 1×10^6, the total number of cells in the remaining 5 mL of PSC suspension is: $1 \times 10^6 \times 5 = 5 \times 10^6$.

4. Aspirate the coating solution and add 2.5 mL PSC culture medium into each well of coated 6-well plates.
5. Gently shake the conical tube containing the PSC cell suspension and plate the PSCs into each well at 2.5×10^5–3×10^5 PSCs per well. For example, add 0.25–0.3 mL of PSC suspension to each well if the concentration of PSC suspension is 1×10^6 cells/mL.
6. Move the plates in several quick back-and-forth and side-to-side motions to disperse the cells across the surface, then gently place the plates in a CO_2 incubator.

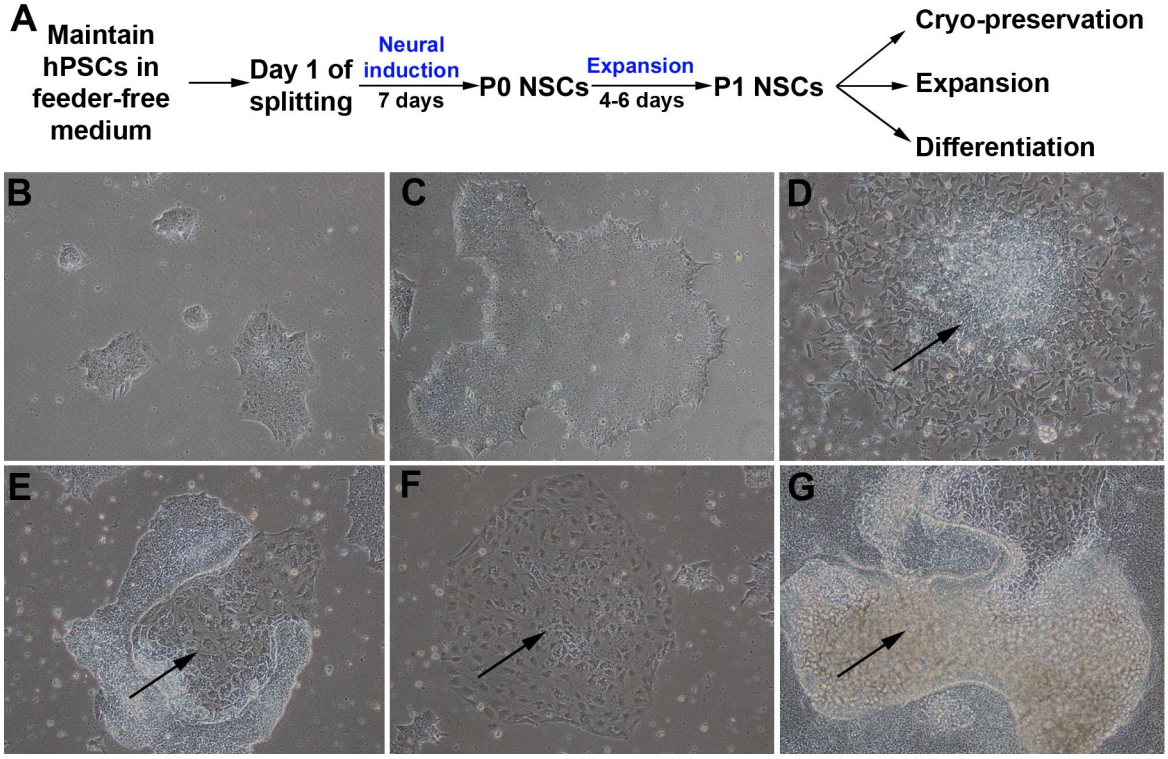
   Note: The split ratio varies depending on the confluence of PSCs before splitting and variability in PSC lines. Neural induction starts on day 1 of PSC splitting (about 24 hours after passaging). The starting density of PSCs should be about 15–25% confluency. When passaging PSCs, cells should be plated as small clumps and not as a single cell suspension. Avoid plating PSC as single cells as that can lead to increased cell death.

   Note: To prevent cell death, you may treat the cells overnight with 10 µM of ROCK inhibitor Y27632 by adding it to the PSC medium at the time of splitting.

Neural induction

The NSC derivation workflow is shown in **Fig. 1 A**.

Figure 1. A: Workflow of NSC derivation from PSCs. **B:** Human PSCs cultured in feeder-free condition on day 1 of splitting with 15–25% confluency. **C:** Cells under neural differentiation on day 2 of neural induction. **D–G:** Representative images of non-neural differentiation (indicated by arrows) on day 2 of induction, due to the use of poor quality starter PSCs during neural induction.

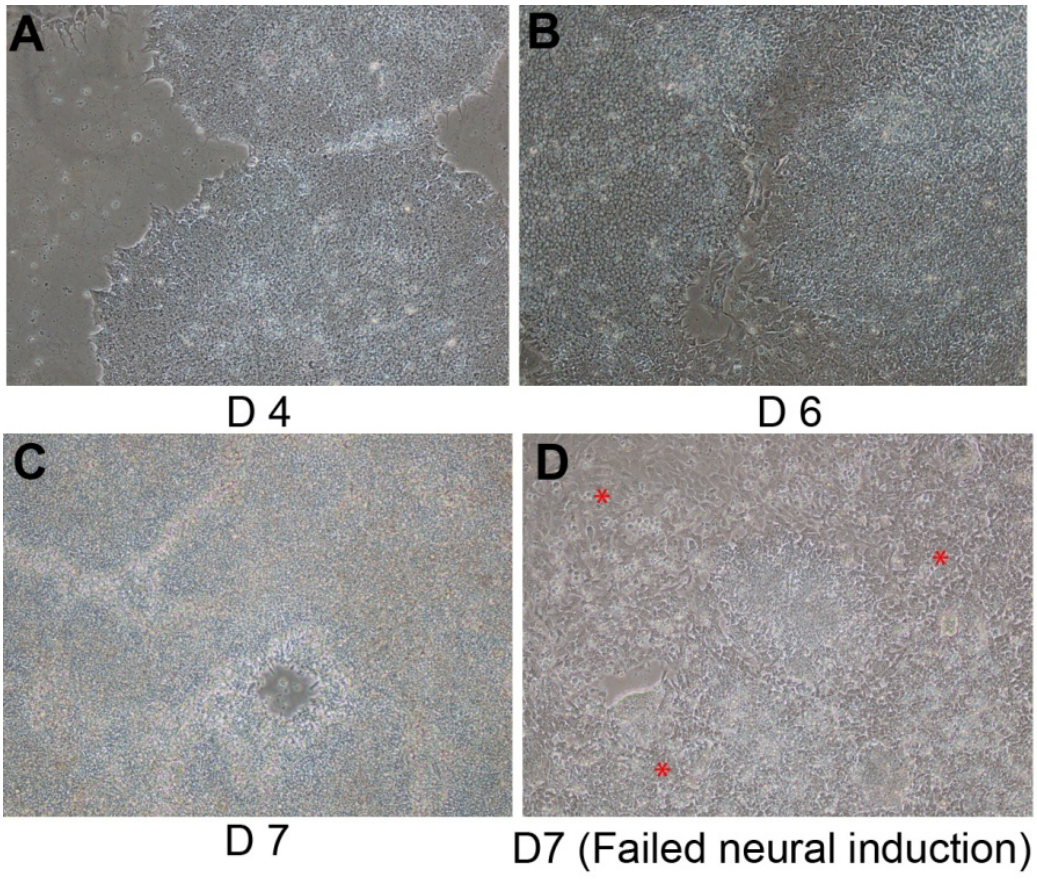


1. On day 1 of PSC splitting (15–25% confluency, see **Fig. 1 B**), aspirate the spent medium to remove non-attached cells, and add 2.5 mL pre-warmed complete PSC Neural Induction Medium to each well of the 6-well plates. Return the plates to the incubator.
2. On day 2 of neural induction, confirm that the morphology of cell colonies is uniform (see **Fig. 1 C**)**:**
   - If the quality of PSCs is good before neural induction, the morphology of the cells should appear as in **Fig. 1 C**.
   - If the quality of starter PSC is poor, the cultures will be dominated by a number of non-neural colonies as shown in **Fig. 1 D–G**. In this case, there are two options:
     - If there are few areas in dish exhibiting this morphology, cultures can be continued after removing such non-neural differentiated colonies. To remove non-neural differentiated colonies, mark all non-neural differentiation colonies with a microscopy marker. Tilt the plate and remove all unwanted colonies at the upper half of the well by using a Pasteur glass pipette to aspirate the cells from the marked colonies. Turn the plate 180 degrees, and repeat. Perform this step one well at a time to prevent cells from becoming too stressed without medium.
     - If there are a large number of such non-neural colonies, it is recommended to discard the cultures and start with high quality PSCs.

3. On day 2 (about 48 hours after switching to PSC Neural Induction Medium), change the medium by aspirating spent medium from each well. Add 2.5 mL of pre-warmed complete Neural Induction Medium per well.
4. On day 4 of neural induction, cells will be reaching confluency **(Fig. 2 A).** Mark all colonies if any non-neural differentiation is noticed, and remove such unwanted colonies with a Pasteur glass pipette. Aspirate the spent medium from each well. Add 5 mL of pre-warmed complete PSC Neural Induction Medium per well.
5. On day 6 of neural induction, cells should be near maximal confluence (**Fig. 2 B**). Remove any non-neural differentiated cells and add 5 mL of complete PSC Neural Induction Medium into each well.

Note: Due to high cell density in the culture from day 4 onwards, doubling the volume of PSC Neural Induction Medium is very critical for cell nutrition. Also, minimal cell death should be observed from days 4 to 7 after neural induction. If the color of cells turns brownish with many floating cells during days 4 to 7 of neural induction, it indicates that the starting density of PSCs was too high. In this case, change the Neural Induction Medium every day using 5 mL per well.

Figure 2. A: The morphology of cells on Day 4, **B:** Day 6, and **C:** Day 7 of neural induction. **D:** The morphology of cells on day 7 of culture due to omission of key components in the neural induction supplement. * indicates flat non-neural cells.



Harvest and expand P0 NSC

On day 7 of neural induction (**Fig. 2C**), NSCs (P0) are ready to be harvested and expanded.

1. Prepare Geltrex® matrix-coated vessels before performing the next steps.
2. Use a Pasteur glass pipette to aspirate the spent PSC Neural Induction Medium from the 6-well plate(s) to be passaged.
3. Gently add 2 mL of DPBS without $CaCl_2$ and $MgCl_2$ to each well of a 6-well plate and aspirate the DPBS to rinse the cells.

   Note: Add DPBS towards the wall of the well to avoid cell detachment.

4. Add 1 mL of pre-warmed StemPro® Accutase® Cell Dissociation Reagent to each well of the 6-well plates and incubate for 5–8 minutes at 37°C until most cells detach from the surface of the culture vessels.
5. Use a cell scraper to detach the cells from the surface of the plates.
6. Use a 5-mL pipette to transfer the cell clumps to a 15- or 50-mL conical tube.
7. Add 1 mL of DPBS to each well of the 6-well plates to collect residual cells, then transfer the cell suspension to the conical tube.
8. Gently pipet the cell suspension up and down 3 times with a 5-mL or 10-mL pipette to break up the cell clumps.
9. Pass the cell suspension through a 100-µm strainer and centrifuge the cells at 300 × *g* for 4 minutes.
10. Aspirate the supernatant, re-suspend the cells with DPBS (3–5 mL of DPBS for all cells from 1 well of a 6-well plate), then centrifuge the cells at 300 × *g* for 4 minutes.
11. Aspirate the supernatant, re-suspend the cells with pre-warmed complete Neural Expansion Medium (for example, use 1 mL for all cells from 1 well of a 6-well plate).
12. Determine the cell concentration using your preferred method.
13. Dilute the cell suspension with pre-warmed complete Neural Expansion Medium to 2×10^5–4×10^5 cells/mL.
14. Add ROCK inhibitor Y27632 to the cell suspension to a final concentration of 5 µM.
15. Aspirate the Geltrex® matrix solution from the Geltrex® matrix-coated vessels and add the diluted cell suspension to each culture plate/dish to plate the cells at a density of 0.5×10^5 –1×10^5 cells/cm^2 as shown in Table 2.

Table 2 Required cell suspension volume

Culture vessel	Approximate surface area (cm^2)	Cell suspension volume (mL)
4-well chamber slide	1.8 cm^2/well	0.46 mL/chamber
6-well plate	9.6 cm^2/well	2.5 mL/well
35-mm dish	11.8 cm^2	3 mL
60-mm dish	20 cm^2	5 mL
100-mm dish	60 cm^2	15 mL

16. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse the cells across the surface, then gently place the vessels in the incubator.

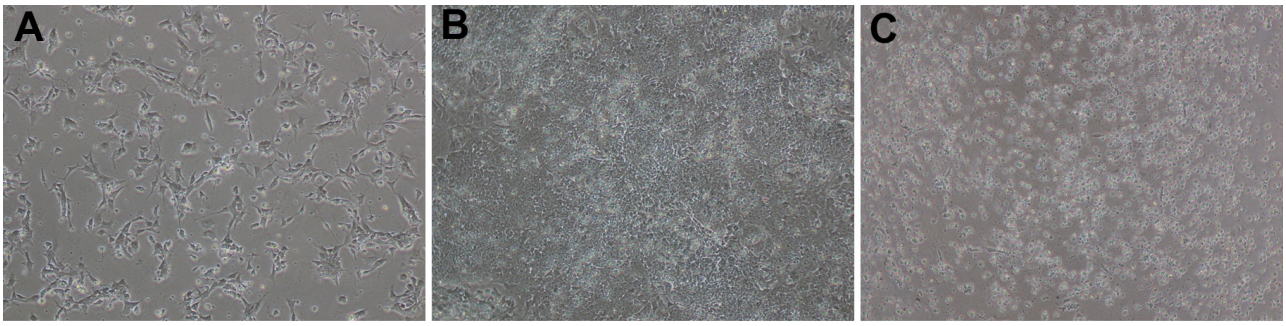
    Note: Avoid splashing the medium on the outsides of the well to avoid contamination.

17. After overnight incubation, change to complete Neural Expansion Medium to eliminate the ROCK inhibitor Y27632. Continue to exchange the Neural Expansion Medium every other day thereafter. Do not add ROCK inhibitor Y27632 during this step.

18. Usually, NSCs reach confluency on days 4–6 after plating (Fig. 3A, B). When NSCs reach confluency, they can be further expanded in complete Neural Expansion Medium. Expanded NSCs can be cryopreserved (see protocol below) or differentiated into specific neural cell types following the protocol of your choice.

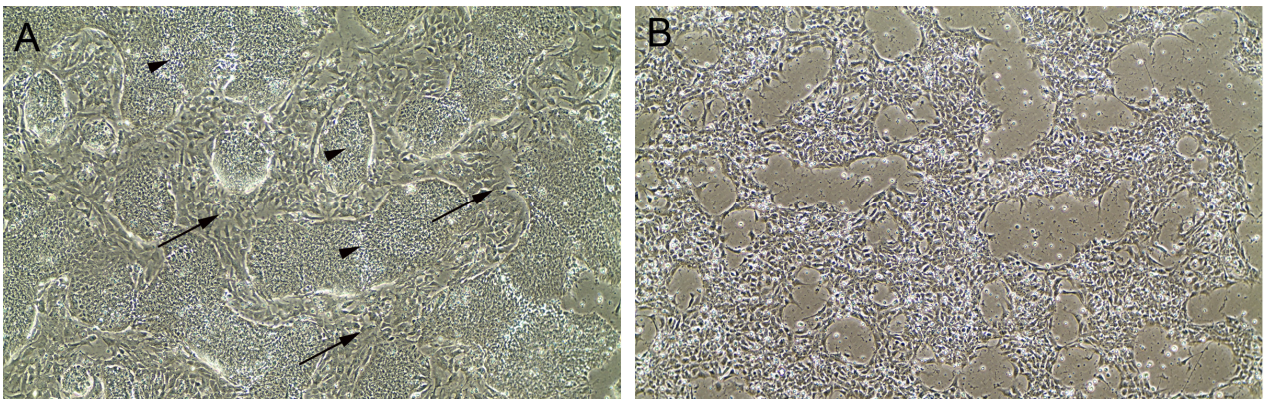
    Note: After dissociation of P0–P4 NSCs, the overnight treatment with the ROCK inhibitor Y27632 at a final concentration of 5 µM is required at the time of plating to prevent cell death for both **expansion** and **differentiation** into glial and neuronal cells (**Fig. 3C**).

Figure 3. A: P0 NSCs plated at a density of 1×10^5 cells/cm^2 on day 1 of plating with Y27632 treatment. **B:** NSCs reach confluence on day 5 of plating. **C:** Without Y27632 treatment, intensive cell death takes place on day 1 of NSC plating.



IMPORTANT! If you observe heterogeneous cell morphology (**Fig. 4A**) with the contamination of non-neural cells during NSC expansion, follow the procedure in Appendix B to diminish the number of non-neural cells (**Fig. 4B**).

Figure 4. A: P1 NSCs at day 4 of P0 NSC plating showed a heterogeneous morphology with flat non-neural cells (indicated by arrows) and compact NSCs (indicated by arrowheads) **B:** P2 NSCs at day 2 of re-plating of P1 NSCs showed a relative homogenous NSC morphology after the sequential treatment with StemPro® Accutase® Cell Dissociation Reagent as described in Appendix B.



Cryopreserve NSCs

1. Passage NSCs to at least P1 before cryopreservation.
2. When NSCs reach confluency, warm the appropriate amount of StemPro® Accutase® Cell Dissociation Reagent in a 37°C water bath according to Table 3.

Table 3 Required StemPro® Accutase® Cell Dissociation Reagent volume

Culture vessel	Approximate surface area (cm^2)	StemPro® Accutase® reagent volume (mL)
6-well plate	9.6 cm^2/well	1 mL/well
35-mm dish	11.8 cm^2	1 mL
60-mm dish	20 cm^2	2 mL
100-mm dish	60 cm^2	5 mL

3. Aspirate the spent medium and add pre-warmed StemPro® Accutase® reagent into culture vessels according to Table 3.
4. Incubate for 3–5 minutes at 37°C until all cells detach from the surface of culture vessels.
5. Transfer the cells into a 15- or 50-mL conical tube using a 5-mL pipette.
6. Add the appropriate amount of DPBS to each well of the vessel (for example, add 1 mL to 1 well of a 6-well plate) to collect the residual cells, then add the cell suspension to the tube.
7. Triturate the cell suspension 3 times with a 5-mL or 10-mL pipette to break up the cell clumps.
8. Centrifuge the cells at 300 × *g* for 4 minutes and aspirate the supernatant.
9. Re-suspend the cells with the appropriate amount of DPBS and centrifuge the cells at 300 × *g* for 4 minutes.
10. Aspirate the supernatant and re-suspend the cells with the appropriate amount of Neural Expansion Medium.
11. Determine the cell concentration using your preferred method.
12. Dilute the cell suspension with Neural Expansion Medium to 2×10^6–4×10^6 cells/mL.
13. Add the same volume of Neural Expansion Medium containing 20% DMSO.
14. Allocate 1 mL cell suspension into each cryotube and freeze cells at –80°C overnight in Nalgene® Mr. Frosty® Freezing Containers with isopropanol.
15. After freezing cells overnight, transfer the cells into a liquid nitrogen tank for long term storage.

Recover cryopreserved NSCs

⚠ **WARNING!** When working with liquid nitrogen and for thawing of cryotubes, follow your institutional guidelines. ANSI Z87.1 certified safety glasses and LN2 rated cryo-gloves are recommended.

1. Prepare Geltrex® matrix-coated dishes as previously described.
2. Remove cryotubes of NSCs from the liquid nitrogen storage tank using metal forceps.
3. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
4. When only an ice crystal remains, remove the vial from the water bath.

5. Spray the outside of the vial with 70% ethanol and place it in the cell culture hood.
6. Pipet the cells gently into a sterile 15-mL conical tube using a 1-mL pipette.
7. Add 1 mL of DPBS into the vial to collect the resident cells. Transfer the DPBS from the vial and add it drop-by-drop to the 15-mL conical tube. While adding, gently move the tube back and forth to mix the NSCs to reduce osmotic shock to cells.
8. Centrifuge the cells at 300 × *g* for 5 minutes and aspirate the supernatant.
9. Resuspend the cell pellet in DPBS. Centrifuge at 300 × *g* for 5 minutes and aspirate the supernatant.
10. Re-suspend the cell pellet in the appropriate amount (for example, 1 mL for all NSCs from 1 vial) of pre-warmed Neural Expansion Medium.
11. Determine the cell concentration using your preferred method.
12. Dilute the cell suspension with pre-warmed Neural Expansion Medium into a solution containing 2×10^5 – 4×10^5 cells/mL.

    Note: If the NSCs were cryo-preserved before passage 4, addition of the ROCK inhibitor Y27632 solution at a final concentration of 5 µM into the cell suspension is required to prevent cell death.

13. Aspirate the Geltrex® matrix solution from the Geltrex® matrix-coated vessels and add the appropriate amount of diluted cell suspension into each culture vessel to plate cells at the density of 0.5×10^5 – 1×10^5 cells/cm^2 (see Table 4).

    Table 4 Required volume of cell suspension

Culture vessel	Approximate surface area (cm^2)	Cell suspension volume (mL)
4-well chamber slide	1.8 cm^2/well	0.46 mL/chamber
6-well plate	9.6 cm^2/well	2.5 mL/well
60-mm dish	20 cm^2	5 mL
100-mm dish	60 cm^2	15 mL
T25 Flask	25 cm^2	6.5 mL
T75 Flask	75 cm^2	19.5 mL

14. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse the cells across the surface, then gently place the vessels in the incubator.

    Note: Avoid splashing the medium to the outsides of the well to avoid any possible contamination.

15. On day 2 of cell plating, exchange the Neural Expansion Medium. Continue to exchange the medium every other day until the NSCs reach confluency and are ready for further expansion.

    Note: If NSCs were under P4, the overnight treatment with the ROCK inhibitor Y27632 is required at the time of NSC plating to prevent cell death. At day 1 after NSC plating, replace the medium with complete Neural Expansion Medium to eliminate Y27632 from the culture. Do not add ROCK inhibitor Y27632 during this step.

Stain NSCs for pluripotent (Oct4) and NSC (Nestin, Pax6, Sox1, and Sox2) markers

Note: We recommend using the Molecular Probes® Human Neural Stem Cell Immunocytochemistry Kit (Cat. no. A24354). This complete immunocytochemistry kit contains primary antibodies for four common NSC markers (Nestin, Pax6, Sox1, and Sox2), a matching set of Alexa Fluor®-labeled secondary antibodies, a nuclear DNA stain (DAPI), and all of the buffers necessary for performing the staining protocol. In addition, pluripotency marker Oct4 (Cat. no. A13998), which is compatible with the reagents in the NSC immuncytochemistry kit, can be used as a negative control marker for NSCs.

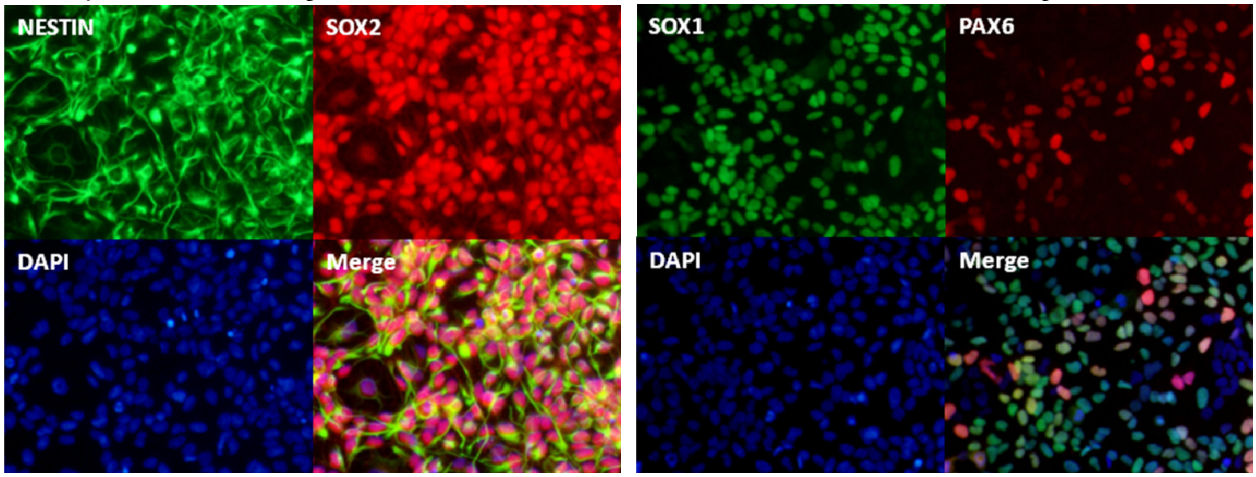
1. Plate the dissociated NSCs at a density of 1×10^5–3×10^5 cells/cm^2 into 4-well chamber slides according to the NSC expansion protocol.

   Note: For P0–P4 NSCs, treatment with 5 µM ROCK inhibitor Y27632 is essential to prevent cell death.

2. Follow the protocol for the Molecular Probes® Human Neural Stem Cell Immunocytochemistry Kit to complete staining. If you are working with individual antibodies, following the procedure in Appendix A or use the protocol of your choice.
3. After staining is completed, apply a suitable quantity (about 1 drop) of ProLong® Gold Antifade Reagent to the space of each well, cover with a coverslip, and air dry the slide in the dark overnight before imaging.

Figure 5. Neural stem cells derived from an iPSC line using PSC Neural Induction Medium (Cat. no. A1647801) were stained for NSC markers Nestin and Sox2 (antibody combination 1) or Sox1 and Pax6 (antibody combination 2) and nuclear DNA (DAPI) using the Neural Stem Cell Immunocytochemistry Kit (Cat. no. A24354).

Antibody combination 1 (left): Nestin and Sox2 with additional DAPI (nuclear DNA) staining.
Antibody combination 2 (right): Sox1 and Pax6 with additional DAPI (nuclear DNA) staining.



Note: Please note that not all NSC will stain positive for PAX6, this is normal and to be expected (i.e., only NSCs that possess forebrain fate commitment will stain positive). In our experience ~15–50% of NSCs generated using PSC Neural Induction Medium stain positive for Pax6.

Troubleshooting

The table below lists some potential problems and solutions that may help you troubleshoot your neural induction experiments.

Observation	Recommended action
The starting density of human PSCs is too low or too high.	Due to variable parameters such as the confluence of PSCs, cell clump passaging, cell attachment and the property of different PSC lines, it may be difficult to determine the splitting ratio. By following the protocol to estimate cell number of PSC clumps before plating, PSC should be 15–25% confluent on day 1 of PSC plating (Day 0 of neural induction).
Too many colonies with non-neural differentiation morphology in culture plate.	Select and maintain high quality of PSCs before starting neural induction.
Cell detachment during neural induction.	For PSCs cultured in Essential 8™ Medium, cells may detach during neural induction. To prevent cell detachment, coat culture plate with 10 µg/mL of Vitronectin when splitting PSCs.
Extensive cell death during the late stage of neural induction.	Check whether cells are over-confluent. If so, change PSC Neural Induction Medium every day using 5 mL per well of a 6-well plate.
Extensive cell death after plating dissociated NSCs for expansion and differentiation.	Check whether ROCK inhibitor Y27632 is added into cell suspension at final concentration of 5 µM if NSCs are under P4. NSCs from some human PSC lines may be more sensitive to dissociation. For NSCs derived from those PSC lines, the overnight treatment with Y27632 after P4 decreases cell death after re-plating.
NSCs reach confluence in 2–3 days after passaging.	NSCs derived from some human PSC lines may have an increased proliferation rate. In this case, decrease NSC plating density to 5×10^4 cells/cm^2.
Extensive cell death after thawing and plating cryopreserved NSCs.	Check whether NSCs are treated with the ROCK inhibitor Y27632 after plating if NSCs are cryopreserved under P4.
Abnormal staining pattern of NSCs stained with antibodies against neural markers.	Antibodies which are not stored or handled properly may result in loss of staining quality and should be replaced. Note that Nestin should stain filaments in the cytoplasm whereas Pax6, Sox1, and Sox2 should stain the nucleus. Oct4 should stain the nucleus of pluripotent cells, but not NSCs.

Frequently Asked Questions

Question	Answer
Does PSC Neural Induction Medium work for both human ESCs and iPSCs?	Yes, PSC Neural Induction Medium works for both human ESCs and iPSCs.
Does PSC Neural Induction Medium work for human PSCs cultured on feeders (mouse embryonic fibroblasts, MEF) and feeder-free conditions?	PSC Neural Induction Medium has been tested on human PSCs cultured in both feeder-free and feeder-based conditions. To eliminate MEF contamination, we strongly recommend starting neural induction by using human PSCs cultured in feeder-free conditions such as Essential 8™ Medium.
Which format of cultures should I use for neural induction?	Neural induction can be started with human PSCs cultured on 6-well plate or culture dishes. We do not recommend starting neural induction by using PSCs cultured in flasks because it is difficult to remove non-neural differentiated colonies from PSCs in flasks.
Can PSC Neural Induction Medium convert mouse PSCs into NSCs?	PSC Neural Induction Medium has not been tested on mouse PSCs and it is not recommended for the conversion of mouse PSCs into NSCs.
Can NSCs derived by PSC Neural Induction Medium be differentiated into cells in the PNS?	No. NSCs derived by PSC Neural Induction Medium can be differentiated into neurons and glial cells in the CNS. Sensory, sympathetic and parasympathetic neurons, as well as Schwann cells in the PNS are derived from precursor cells of the neural crest.

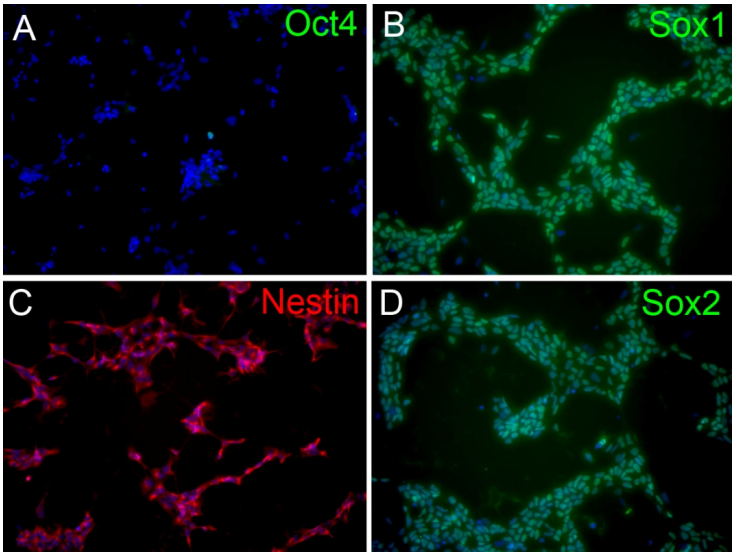
Appendix A: Stain NSCs for pluripotent (Oct4) and NSC (Sox1, Nestin and Sox2) markers — Single antibody/marker protocol

1. Plate the dissociated NSCs at a density of 1×10^5–3×10^5 cells/cm^2 into 4-well chamber slides according to the NSC expansion protocol.
   Note: For P0–P4 NSCs, treatment with 5 µM ROCK inhibitor Y27632 is essential to prevent cell death.
2. At day 1 of NSC plating, aspirate the spent medium and rinse the cells once with 0.5 mL of DPBS per well.
   Note: Add the DPBS toward the wall of the well to avoid cell detachment.
3. Aspirate the DPBS and add 0.5 mL of 4% paraformaldehyde (PFA) into each well to fix the cells. Incubate for 15 minutes at room temperature.
4. Aspirate the PFA solution and rinse the cells 3 times with 0.5 mL DPBS per well, with 5 minutes per rinse (3 × 5 minutes) at room temperature.
   Note: Fixed cells can be stored in 0.5–1 mL DPBS/per well, wrapped in Parafilm® laboratory film at 4°C for up to 1 week.
5. After aspirating the DPBS, add 0.5 mL of blocking buffer (0.1% Triton® X-100, 1% BSA in DPBS) to each well and incubate for 30 minutes at room temperature.
6. Aspirate the blocking buffer, then add 0.2 mL of primary antibody against pluripotent or neural markers, diluted in blocking buffer, into each well. Incubate at 4°C overnight.
7. Rinse the cells 3 times with 0.5 mL DPBS per well, with 10 minutes per rinse (3 × 10 minutes) at room temperature.

IMPORTANT! Perform steps 8 through 11 under low light conditions.

8. After aspirating the DPBS, add 0.2 mL of Alexa Fluor® 488- or 594-conjugated donkey anti-mouse or goat IgG secondary antibody, diluted 1:1000 in blocking buffer, to each well. Incubate for 1 hour at room temperature.
9. Aspirate the secondary antibody, add 0.5 mL of DPBS per well, and incubate for 10 minutes at room temperature.
10. Aspirate the DPBS, add 0.2 mL of DAPI solution to each well, and incubate for 5 minutes at room temperature.
11. Aspirate the DAPI solution, and rinse the cells 3 times with 0.5 mL DPBS per well, with 5 minutes per rinse (3 × 5 minutes) at room temperature.
12. Aspirate the DPBS, apply a suitable quantity (about 1 drop) of ProLong® Gold Antifade Reagent to the space of each well, cover with a coverslip, and air dry the slide in the dark overnight before imaging.

Figure 6. NSCs derived from human PSCs were dissociated, plated and stained with antibodies against the pluripotent marker Oct4 **(A)**, the neural stem cell markers Sox1 **(B)**, Nestin **(C)**, and Sox2 **(D)**. Cell nuclei were counter stained with DAPI (blue).



Appendix B: Troubleshoot heterogeneous cell morphology

If you observe heterogeneous cell morphology (see **Fig. 4A**) with the contamination of non-neural cells during NSC expansion, the following steps can be used to diminish the number of non-neural cells (see **Fig. 4B**).

1. At day 3–4 of P0 or P1 NSC re-plating with 90–100% cell confluence, aspirate spent medium, wash once with DPBS, then add the appropriate amount pre-warmed StemPro® Accutase® Cell Dissociation Reagent into each culture vessel according to Table 5.

Table 5 Volume of StemPro® Accutase® Reagent Required

Culture Vessel	Surface Area (cm^2)	Volume of StemPro® Accutase® Reagent (mL)
6-well plate	9.6 cm^2/well	1 mL/well
35-mm dish	11.8 cm^2	1 mL
60-mm dish	20 cm^2	2 mL
100-mm dish	60 cm^2	5 mL

2. Mount the culture vessel under a phase contrast microscopy to observe the cells.
3. After the incubation in StemPro® Accutase® reagent for 3–4 minutes at room temperature, non-neural cells with flat morphology will detach from the culture vessel, while densely packed neural cells remain attached. The incubation time may be adjusted for different cell lines to avoid detaching all cells from the culture vessel.
4. Gently aspirate the StemPro® Accutase® reagent from the culture vessel.
5. Gently add the appropriate amount of DPBS toward the wall of the culture vessel to rinse off detached cells.
6. Aspirate the DPBS, add an appropriate amount of pre-warmed StemPro® Accutase® reagent into each culture vessel according to Table 5, then incubate at 37°C for 3–4 minutes until most of cells detach from the surface of the culture vessels.
7. Follow steps 5 through 18 in "Harvest and Expand P0 NSC" to further plate and expand NSCs.

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30 December 2013