StemXVivo®

Neural Progenitor Differentiation Kit

Catalog Number SC035

Reagents for the directed differentiation of human pluripotent stem cells into neural progenitor cells.

This package insert must be read in its entirety before using this product.
For laboratory research use only. Not for diagnostic use.
The safety and efficacy of this product in diagnostic or
other clinical uses has not been established.

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INTRODUCTION

Pluripotent stem cells, including both embryonic and induced pluripotent stem cells, provide much promise for the generation of sufficient quantities of specialized cells for use in regenerative medicine. Additionally, these cells are an important tool for understanding developmental and disease mechanisms.

The ability of pluripotent stem cells to differentiate into neural progenitor cells (NPCs) holds tremendous promise for applications in therapeutics, pharmaceuticals, and developmental research. NPCs generated from pluripotent cell sources can provide a potentially unlimited supply of cells for further downstream differentiation into neuronal or glial cell populations. Pluripotent stem cell-derived NPCs provide a platform for disease modeling and drug candidate screening. Fully characterized, differentiated NPCs or their derivative cells may eventually be transplanted as a therapeutic for a variety of neural-based diseases. Additionally, using pluripotent stem cells to study the processes involved in the generation of NPCs and their downstream derivative cell types will increase our understanding of the mechanisms involved in embryonic development and organogenesis (1-3).

PRINCIPLE OF THE ASSAY

The StemXVivo® Neural Progenitor Differentiation Kit contains specially formulated media supplements and factors for the differentiation of human pluripotent stem cells into neural progenitors. An antibody against SOX1 is included to verify NPC differentiation. The quantity of each component is sufficient to make 260 mL of media, which is enough media for the differentiation of six 60 mm plates, two 24-well plates, or an equivalent cell culture surface area of pluripotent stem cells.

TECHNICAL HINTS & LIMITATIONS

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix or substitute reagents with those from other lots or sources.
- The safety and efficacy of this product in diagnostic or other clinical uses has not been established.
- The quality and differentiation potential of human pluripotent stem cells at the onset of the differentiation protocol is of paramount importance to the efficiency of differentiation.
- If little to none of the cells are positive for SOX1 at day 7, it is recommended to test your pluripotent stem cell starting population for their ability to differentiate into ectoderm. This can be done using the StemXVivo® Ectoderm Kit (R&D Systems®, Catalog # SC031B).
- If differentiation is observed, but with low efficiency, the quality of the starting population should be verified prior to differentiation. Cell quality can be determined morphologically by staining with pluripotency markers such as Oct-4A (R&D Systems®, Catalog # MAB17591), Nanog (R&D Systems®, Catalog # AF1997), SSEA-4 (R&D Systems®, Catalog # MAB1435), or others. Alternatively, use the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems®, Catalog # SC027B) to determine the ability of the starting cell population to functionally differentiate into each of the three germ layers.

PRECAUTION

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed and protective clothing should be worn when handling kit reagents.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at ≤ -20 °C in a manual defrost freezer. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
NPC Differentiation Base Media Supplement (100X)	390609	2.6 mL of a 100X concentrated solution.	Store at 2-8 °C for up to 2 weeks or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
NPC Differentiation Cocktail I (1000X)	968105	1 vial of lyophilized factors; enough to make 260 µL of a 1000X stock.	Store at 2-8 °C for up to 2 weeks or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
Anti-Human SOX1	968106	25 µg of lyophilized goat anti-human SOX1 polyclonal antibody. Enough to make 2.5 mL of staining solution when used at suggested concentration of 10 µg/mL.	

*Provided this is within the expiration of the kit.

OTHER SUPPLIES REQUIRED

Materials

- Human pluripotent stem cells
- 24-well culture plates (or other, as needed)
- 60 mm culture plates
- 12 mm coverslips (optional)
- 15 mL and 50 mL centrifuge tubes
- 0.2 µm syringe filter
- 10 mL syringe
- Pipettes and pipette tips
- Serological pipettes
- Glass slides
- Fine pointed curved forceps

Reagents

- DMEM/F12 (1X)
- BSA, very low endotoxin
- N-2 MAX Media Supplement (R&D Systems®, Catalog # AR009)
- GlutaMAX™ Supplement
- Penicillin-Streptomycin (optional)
- Phosphate Buffered Saline (PBS)
- Pluripotent Stem Cell Maintenance Media (MEF Conditioned Media; R&D Systems®, Catalog # AR005) or equivalent
- Cultrex® Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract, Pathclear® (R&D Systems®, Catalog # 3434-001-02)
- Recombinant Human FGF basic (R&D Systems®, Catalog # 4114-TC or 233-FB)
- Y-27632 (Tocris®, Catalog # 1254)
- Trypan Blue Solution
- Accutase® or Versene™
- 95% Ethanol
- 4% Paraformaldehyde in PBS
- 1% BSA in PBS
- 0.3% Triton™ X-100, 1% BSA, 10% normal donkey serum in PBS
- 1% BSA, 10% normal donkey serum in PBS
- Mounting Medium (R&D Systems®, Catalog # CTS011)
- Secondary developing reagents (R&D Systems®, Catalog # NL001)
- Deionized or distilled water
- CryoDefend™-Stem Cells (R&D Systems®, Catalog # CCM018)

Equipment

- 37 °C and 5% CO_2 incubator
- Centrifuge
- Hemocytometer
- Inverted microscope
- 37 °C water bath
- Fluorescence microscope

REAGENT & MATERIAL PREPARATION

0.1% BSA in PBS - Dissolve 10 mg of BSA in 10 mL of PBS. Sterile filter the solution by syringe filter and store at 2-8 °C for up to 3 months.

NPC Differentiation Cocktail (1000X) - Reconstitute with 260 µL of sterile 0.1% BSA in PBS. Mix gently.

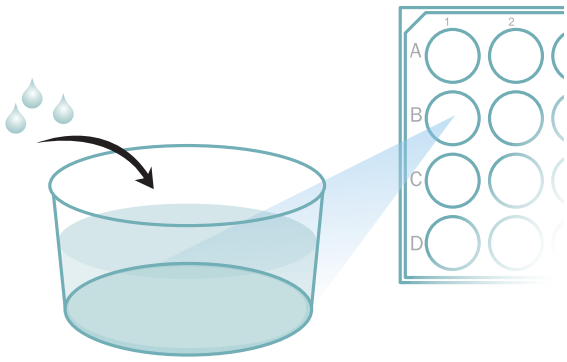
NPC Differentiation Base Media - Warm NPC Differentiation Base Media Supplement (100X) in a 37 °C water bath for 5 minutes. Vortex briefly to mix. Dilute warmed NPC Differentiation Base Media Supplement (100X) 1:100 with sterile DMEM/F12 containing 1X GlutaMAX and 1X Penicillin/Streptomycin (optional). Prepare fresh as needed.

Complete NPC Differentiation Media - Dilute NPC Differentiation Cocktail (1000X) 1:1000 in NPC Differentiation Base Media. Prepare fresh as needed.

NPC Maintenance Media - Dilute N-2 MAX Media Supplement 1:100 in sterile DMEM/F12 containing 1X GlutaMAX and 1X Penicillin/Streptomycin (optional). Add Recombinant Human FGF basic to a final concentration of 20 ng/mL. Keep the media cold, only warming the amount required.

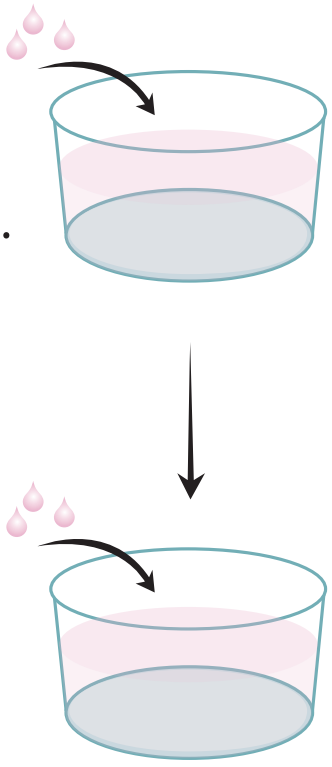
PROCEDURE OUTLINE

Coat wells with Stem Cell Qualified RGF BME.
Incubate at room temperature for 1-2 hours.



Day (-1) of Differentiation
Plate human pluripotent stem cells onto the coated plates at 1-2 x10^5 cells/cm^2 in pluripotent stem cell maintenance media.
Culture cells to 80-90% confluency.

Day (0) of Differentiation
Replace the pluripotent stem cell maintenance media with Complete NPC Differentiation Media.
Incubate at 37 ˚C and 5% CO_2 for 18-24 hours.



Days 1-7 of Differentiation
Replace the media with fresh Complete NPC Differentiation Media every 24 hours.
Incubate at 37 ˚C and 5% CO_2.

UNDIFFERENTIATED CELL PREPARATION

Note: *This protocol has been tested on cells cultured in pluripotent stem cell maintenance media (MEF Conditioned Media; R&D Systems®, Catalog # AR005) or equivalent.*

The quality and differentiation potential of human pluripotent stem cells at the onset of the differentiation protocol are of paramount importance to the efficiency of differentiation. Human pluripotent stem cells must be > 95% positive for Oct-3/4.

COATING PLATES WITH RGF BME

1. Thaw Cultrex® Stem Cell Qualified RGF BME, Pathclear® (RGF BME) on ice at 2-8 °C overnight.
2. Dilute the RGF BME 1:40 in ice cold DMEM/F-12. This can be stored at 2-8 °C for up to 2 weeks.
3. Coat the desired number of wells or plates with diluted RGF BME (0.5 mL/well for a 24-well plate) and incubate for 1-2 hours at room temperature.
4. RGF BME can be kept on ice at 2-8 °C for up to 2 weeks. Avoid repeated freeze/thaw cycles.

CELL DISSOCIATION

1. Warm the pluripotent stem cell maintenance media to room temperature.
2. Remove the existing media from the cells. Add 1 mL of Accutase or Versene solution to each 60 mm plate. Incubate at room temperature for 2-7 minutes or until the cells begin to slough off the plate. If using cells from several plates, work in small batches (1-2 plates at a time) so the cells are not exposed to the Accutase or Versene beyond the time it takes the cells to slough off the plate.
3. Pipette gently over the plate until the cells become detached.
4. Gently pipette the cell suspension up and down to break up large cell clumps.
5. Transfer the cell suspension to a 15 mL centrifuge tube containing 5 mL of pluripotent stem cell maintenance media and spin at 200 x g for 5 minutes.

CELL PLATING

1. Resuspend the pellet in pluripotent stem cell maintenance media and count the viable cells using Trypan Blue and a hemocytometer.
2. Plate the cells onto prepared RGF BME-coated plates at a concentration of 1-2 x 10^5 cells/cm^2. It is recommended to add the ROCK inhibitor (Y-27632) at 1 µM for the first 16-24 hours of culture.
3. Allow the cells to attach and grow at 37 °C and 5% CO_2 for 16-24 hours. The monolayer should reach approximately 80-90% confluence. Best results are obtained when differentiation is started within 24 hours after plating. If cells require longer than 48 hours to reach 80-90% confluence, plating density should be optimized.

NEURAL PROGENITOR CELL DIFFERENTIATION PROCEDURE

Neural Progenitor Cell (NPC) Differentiation Days 0 though 7

1. Warm the appropriate volume of Complete NPC Differentiation Media to room temperature. For 24-well plates, use 0.5 mL/well.
2. Remove and discard the pluripotent stem cell maintenance media and add the Complete NPC Differentiation Media to each well.
3. Culture the cells for seven days at 37 °C and 5% CO_2. Exchange the media daily with freshly prepared Complete NPC Differentiation Media.
4. Cells are now ready for continued passage as NPCs, immunocytochemical analysis, or other further downstream experimentation.

Differentiation Day 7 & Beyond

After 7 days in Complete NPC Differentiation Media, the cells are committed to a neural progenitor fate and express neural progenitor markers including SOX1, Pax6, Nestin, and N-Cadherin. At this point the NPCs can be analyzed for neural progenitor cell markers, passaged onto new plates and cultured as NPCs, differentiated into downstream lineages, or cryopreserved.

Differentiation Day 7 & Beyond: Neural Progenitor Cell Passaging & Continued Culture

1. Warm appropriate amount of 1X PBS and Accutase.
2. Remove and discard the spent media and add the pre-warmed PBS to each well. For 24-well plates, use 0.5 mL/well.
3. Remove and discard the PBS and add pre-warmed Accutase. For 24-well plates, use 0.5 mL/well.
4. Incubate at 37 °C and 5% CO_2 for 5-15 minutes. Monitor cells for detachment. Once cells are rounded and begin to detach from the cell culture surface, proceed to Step 5.
5. Gently pipette the cells up and down with a 1 mL pipette until cells are completely detached from culture surface and are suspended as single cells. Transfer into 15 ml tube.
6. Add 3 mL of DMEM/F12 to the 15 mL tube and spin at 200 x g for 5 minutes.
7. Remove supernatant and resuspend the pellet in Complete NPC Differentiation Media. Count the viable cells using Trypan Blue and a hemocytometer.
8. Plate cells onto prepared RGF BME-coated plates at a concentration of 0.5-1.0 x 10^5 cells/cm^2.

   Note: *Addition of the ROCK inhibitor (Y-27632) at 1 µM for the first 16-24 hours of culture is recommended to increase cell viability.*
9. Culture the cells until densely confluent (three to seven days) at 37 °C and 5% CO_2. Exchange media daily with freshly prepared Complete NPC Differentiation Media.
10. Repeat Steps 1-9.

    Note: *It is recommended to maintain cells in Complete NPC Differentiation Media for the first three passages. Following the third passage, the NPCs can be cultured in NPC Maintenance Media (see Reagents & Materials Preparation).*

NEURAL PROGENITOR CELL DIFFERENTIATION PROCEDURE *CONTINUED*

Differentiation Day 7 & Beyond: Neural Progenitor Cell Cryopreservation

Pluripotent stem cell-derived NPCs can be cryopreserved for use at a later time. We recommended using CryoDefend™-Stem Cells (R&D Systems®, Catalog # CCM018) for NPC cryopreservation. Cryopreserve at a density of 2 x 10^5 cells/vial with 500 µL/vial.

FIXING & STAINING PROCEDURE

Immunocytochemistry

1. Wash the cells twice with PBS (1 mL/well for a 24-well plate).
2. Fix the cells with 4% paraformaldehyde in PBS for 20 minutes at room temperature.
3. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well for a 24-well plate).
4. Permeabilize and block the cells with 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes (0.5 mL/well for a 24-well plate).
5. During the blocking, dilute the reconstituted Anti-Human SOX1 Primary Antibody to a final concentration of 10 µg/mL in PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum.

   Note: *A negative control should be performed using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in the absence of a primary antibody.*
6. After blocking, incubate the cells with diluted Anti-Human SOX1 Primary Antibody (300 µL/well of a 24-well plate) for 3 hours at room temperature or overnight at 2-8 °C.
7. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well of a 24-well plate).
8. Dilute the secondary antibody [*e.g.*, NL557-conjugated Donkey Anti-Goat Secondary Antibody (R&D Systems, Catalog #NL001)] at 1:200 in PBS containing 1% BSA.
9. Incubate the cells with diluted secondary antibody in the dark for 60 minutes at room temperature (300 µL/well of a 24-well plate).
10. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well of a 24-well plate).
11. Cover the cells with PBS (1 mL/well of a 24-well plate) and visualize with a fluorescence microscope. Alternatively, aspirate the PBS and add distilled or deionized water (0.5 mL/well of a 24-well plate). Carefully remove each coverslip with forceps and mount cell-side down onto a drop of mounting medium on a glass slide.
12. Slides are ready for microscopic observation.

DATA EXAMPLES

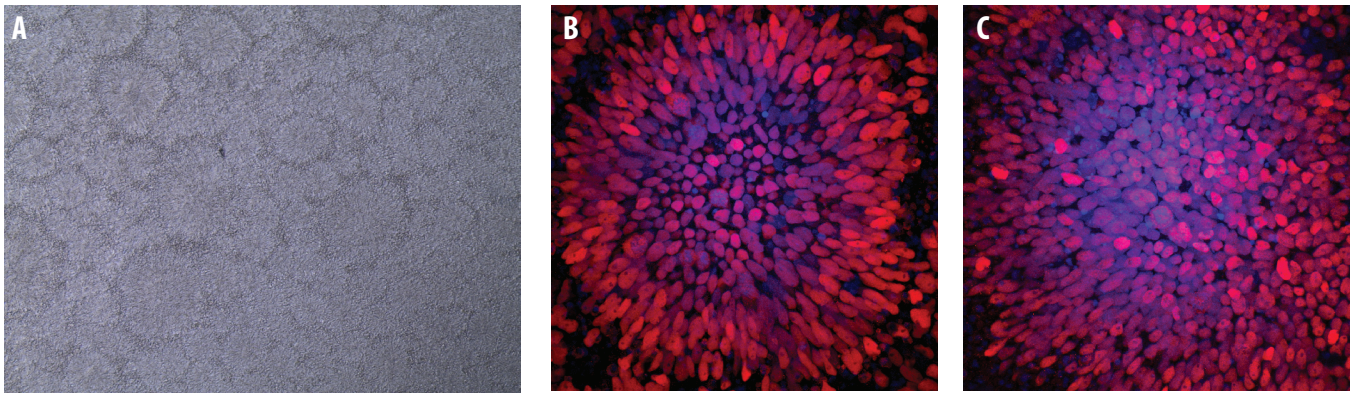


Figure 1: Differentiation of Pluripotent Stem Cells into Neural Progenitor Cells. iPSK3 human induced pluripotent stem cells were differentiated into neural progenitor cells using the media supplements included in this kit. **(A)** After 7 days of differentiation, cells were imaged using brightfield microscopy. Cells demonstrate rosette formation characteristic of neural progenitor cells in culture. **(B, C)** To evaluate lineage commitment, the cells were stained with the Anti-Human SOX1 Antibody **(B)** included in this kit or a Sheep Anti-Human Pax6 Polyclonal Antibody (R&D Systems®, Catalog # AF8150) **(C)** followed by NorthernLights™ (NL)557-Conjugated Donkey Anti-Goat or Donkey Anti-Sheep Secondary Antibodies, respectively (R&D Systems®, Catalog # NL001 and NL010).

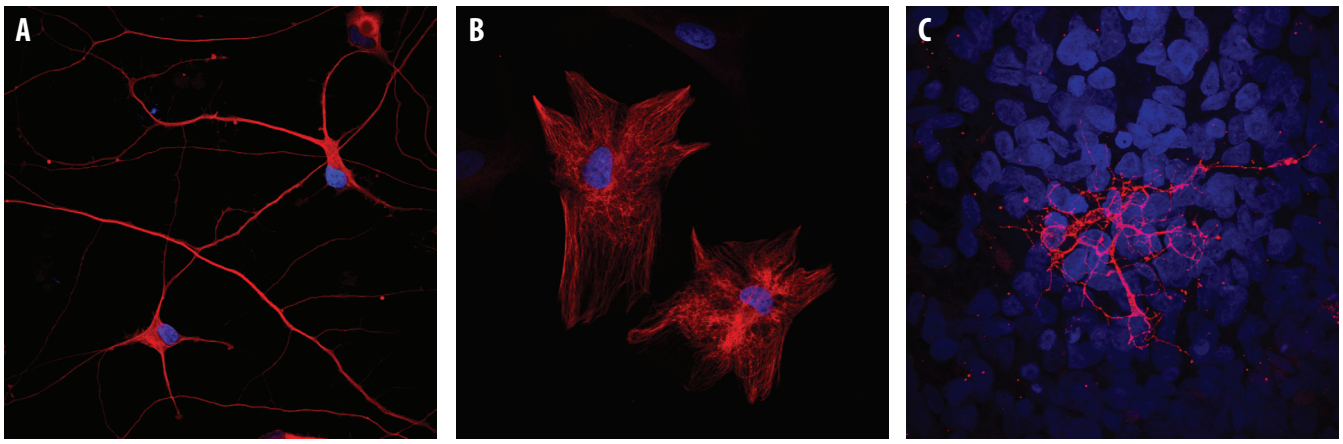


Figure 2: Pluripotent Stem Cell-derived NPCs Differentiate into Neurons, Astrocytes, and Oligodendrocytes. JOY6 human induced pluripotent stem cells were differentiated into neural progenitor cells using the media supplements included in this kit. Resulting cells were further differentiated into neurons **(A)**, astrocytes **(B)**, and oligodendrocytes **(C)** via growth factor withdrawal, resulting in random differentiation. To evaluate lineage commitment, cells were stained with the Mouse Anti-Neuron-Specific βIII Tubulin Monoclonal Antibody (Neurons; R&D Systems®, Catalog # MAB1195), Sheep Anti-Human GFAP Polyclonal Antibody (Astrocytes; R&D Systems®, Catalog # AF2594), and Mouse Anti-Human Oligodendrocyte Marker O4 Monoclonal Antibody (Oligodendrocytes; R&D Systems®, Catalog # MAB1326) followed by NorthernLights™ (NL)557-Conjugated Donkey Anti-Mouse, Donkey Anti-Sheep, or Goat Anti-Mouse Secondary Antibodies, respectively (R&D Systems®, Catalog # NL007, NL010, or NL019).

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3. Marchetto, M.C. *et al.* (2011) Human Molecular Genetics. **20**:R109.