User Guide

Quick-Neuron™ Precursor - mRNA Kit (Small)

Catalog Number: NP-mRNA-S

Introduction

The Quick-Neuron™ Precursor - mRNA Kit (Small) facilitates rapid and efficient differentiation of human iPS or ES cells into a population of neural precursor cells (NPCs) in just 6 days. Our proprietary transcription factor-based stem cell differentiation method uses synthetic mRNAs to produce highly pure populations of NPCs without a genetic footprint. Quick-Neuron™ Precursor differentiated cell cultures continue to proliferate and express a variety of NPC markers such as nestin (NES), vimentin (VIM), Hes Family BHLH Transcription Factor 5 (HES5), SRY-Box Transcription Factor 1 (SOX1), and SRY-Box Transcription Factor 2 (SOX2). When handled and maintained according to the instructions in this user guide, NPCs are viable long-term and are suitable for further characterization and differentiation.

Scale: The Quick-Neuron™ Precursor - mRNA Kit (Small) contains a set of reagents for use with a total of 4 wells of a 24-well plate.

Related Products: Quick-Neuron™ Precursor - mRNA Kit (Large), Catalog Number: NP-mRNA-L
Quick-Neuron™ Precursor - Human iPSC-derived Neural Precursor Cells, Catalog Number: NP-mRNA-CW

Contents

Upon receipt, store the reagents at the temperatures indicated in the table below. All reagents are shipped on dry ice.

Contents	Volume	Storage	Thaw
QNP-mRNA	4 x 9 µl	-80°C	On ice
Coating Material A	15.7 µl	-20°C or -80°C	On ice or 4°C

This kit contains iMatrix-511 silk (Nippi, Inc.)

Condition of Use

This product is for research use only. It is not approved for use in humans or for therapeutic or diagnostic use.

Technical Support

For technical support please refer to the FAQ on our website.
You may also contact us at cs@elixirgensci.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

Required Consumables

Item	Vendor	Catalog Number
24-well tissue-culture-treated polystyrene plate (e.g., Corning Costar Flat Bottom Cell Culture Plates)	Fisher Scientific	07-200-740
Lipofectamine MessengerMAX	ThermoFisher	LMRNA001
Opti-MEM I Reduced Serum Medium	ThermoFisher	31985062
DMEM/F12	ThermoFisher	21331020
Neurobasal Medium	ThermoFisher	21103049
Gibco™ B-27™ Supplement (50X), minus vitamin A	Fisher Scientific	12-587-010
Gibco™ N-2 Supplement (100X)	Fisher Scientific	17-502-001
GlutaMAX	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
StemFit Basic04 Complete Type, or StemFit AK02N, or StemFlex Medium	Elixirgen Scientific TaKaRa ThermoFisher	ASB04-C, or AK02N, or A3349401
Recombinant Human FGF-basic (154 a.a.) (bFGF)	PeproTech	100-18B
Recombinant Human HB-EGF	PeproTech	100-47
LDN 193189 dihydrochloride	R&D Systems	6053
SB 431542	R&D Systems	1614
Bovine Serum Albumin solution, 30% in DPBS	Sigma-Aldrich	A9576-50ML
TrypLE Select Enzyme (1X)	ThermoFisher	12563011
0.02% EDTA in DPBS	Sigma-Aldrich	E8008-100ML
0.01% Poly-L-Ornithine	Sigma-Aldrich	P4957-50ML
Extracellular Matrix such as - Laminin Mouse Protein, Natural, or - Geltrex Basement Membrane Matrix	ThermoFisher	23017015 or A15696-01
Phosphate-buffered saline (without Ca^{++} Mg^{++})*	ThermoFisher	20012050
ROCK inhibitor Y27632	Selleckchem	S1049
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
(Optional) STEM-CELLBANKER**	AMSBIO	11890
(Optional) 100% Ethanol***	Multiple Vendors	
(Optional) 12-mm glass coverslips***	VWR	89167-106

*PBS should be used at room temperature unless otherwise specified.

**This is only required if you intend to cryopreserve the cells after differentiation.

***This is only required if plating on glass coverslips. Glass coverslips from different brands might have different effects on the growth of neural cells. We recommend using glass coverslips from Chemglass for best results.

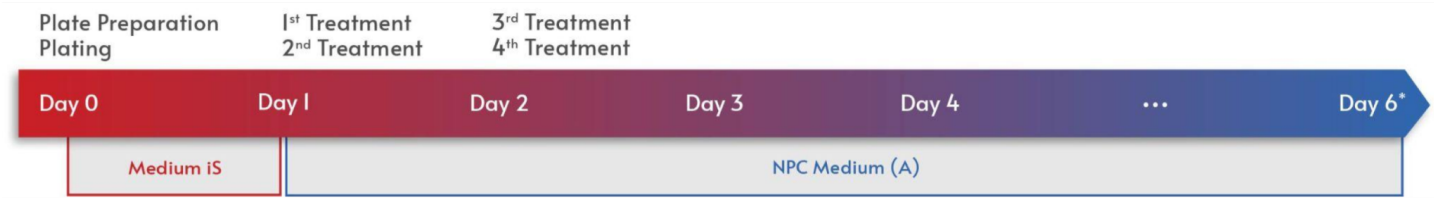
Source hPSC Culture Conditions

The Quick-Neuron™ Precursor - mRNA Kit (Small) gives the best differentiation results when source human pluripotent stem cells (hPSCs) have been maintained in StemFit® Basic04, StemFit® AK02N, StemFlex™ Medium, or other similar culture media which enable the maintenance of cultures by single-cell passaging. This protocol also assumes that the source hPSCs are cultured in a 35-mm culture dish or one well of a 6-well plate. If iMatrix-511 silk is routinely used as a coating substrate, prepare one culture dish or well precoated with 0.25 µg/cm^2 iMatrix-511 silk diluted in 2 ml chilled PBS for this kit.

- The protocols and reagents for StemFit® Basic04 and iMatrix-511 silk culture conditions are available at Elixirgen Scientific (Catalog Numbers: ASB04-C, NI511S).
- Differentiation should not be performed until the cells are at least 14 days post-thaw.
- We recommend preparing a minimum of 1.5 x 10^6 viable hPSC for use with this kit. This is usually obtained by using 1 well of a 6-well plate at 50-70% confluency.
- For optimal differentiation, hPSC confluency should be around 50% to 70%. Do not use wells more than 90% confluent.

Workflow

Note: This protocol assumes that Day 0 is a Tuesday.



* From Day 6, users may maintain differentiated NPCs in the medium best suited for their needs, though we recommend NPC Medium (A).

Preparation

Important Note! For the best possible delivery of QNP-mRNA into cells, we recommend Lipofectamine MessengerMax. If users prefer another transfection reagent, please make sure that the reagent provides a transfection efficiency of ≥80% prior to using this kit. QNP-mRNA mixed with Lipofectamine MessengerMax must be immediately applied to cultures and cannot be stored.

10 mM ROCK inhibitor Y27632 (iROCK)

1. Dissolve 10 mg ROCK inhibitor Y27632 in 3.12 ml DMSO.
2. Make aliquots of a convenient volume (e.g., 100 µl).
3. This solution, hereafter referred to as iROCK, can be stored at -20°C.

StemFit Basic04 Complete Type (Medium S)*

1. Thaw StemFit Basic04 Complete Type bottle overnight or multiple nights at 4°C.
2. Make aliquots of a convenient volume (e.g., 40 ml).
3. This solution, hereafter referred to as Medium S, can be stored at -80°C. Once thawed, Medium S should be stored at 4°C for up to 2 weeks.
    - After thawing users may choose to add Penicillin-Streptomycin at a 1:200 dilution (e.g., 200 µl in 40 ml of Medium S) before using Medium S.

*Medium S can be substituted with StemFit AK02N or StemFlex.

0.5X TrypLE Select with EDTA (Solution D1)

1. Mix 1 ml TrypLE Select Enzyme (1X) with 1 ml 0.02% EDTA in DPBS.
2. This mixture, hereafter referred to as Solution D1, can be stored at 4°C for 2 weeks.

0.002% Poly-L-Ornithine solution (ornithine)

1. Take 700 µl 0.01% Poly-L-Ornithine solution and mix it with 2.8 ml PBS.
2. The 0.002% Poly-L-Ornithine solution, hereafter referred to as ornithine, can be stored at 4°C for up to 2 weeks.

1 mg/ml laminin stock solution (laminin)

1. Thaw Laminin Mouse Protein, Natural and chill PBS at 4°C or on ice.
2. Mix the Laminin Mouse Protein, Natural and PBS to make the 1 mg/ml stock solution, hereafter referred to as laminin.
    - Laminin concentration varies by lot, so use the number specified on the vial or CoA when making your calculations.
3. Make aliquots of a convenient volume (e.g., 35 µl) and store at -20°C.

10 µg/ml bFGF stock solution

1. Prepare 1 mg/ml bFGF by following the vendor's instructions.
2. Further dilute with sterile 0.1% BSA prepared with PBS to 10 µg/ml bFGF stock solution.
3. Make aliquots of a convenient volume (e.g., 100 µl).
4. This solution, hereafter referred to as 10 µg/ml bFGF, can be stored at -80°C.

100 µg/ml HB-EGF stock solution

1. Prepare 1 mg/ml EGF by following the vendor's instructions.
2. Further dilute with sterile 0.1% BSA prepared with PBS to 100 µg/ml EGF stock solution.
3. Make aliquots of a convenient volume (e.g., 20 µl).
4. This solution, hereafter referred to as 100 µg/ml HB-EGF, can be stored at -80°C.

10 mM LDN-193189 stock solution

1. Dissolve 10 mg LDN-193189 in 1.99 ml DMSO.
2. Make aliquots of a convenient volume (e.g., 100 µl).
3. This solution, hereafter referred to as 10mM LDN-193189, can be stored at -20°C.

1 mM LDN-193189 stock solution

1. Dilute 10 µl 10mM LDN-193189 in 90 µl DMSO.
2. Make aliquots of a convenient volume (e.g., 10 µl).
3. This solution, hereafter referred to as 1 mM LDN-193189, can be stored at -20°C.

10 mM SB 431542 (1,000x) stock solution

1. Dissolve 10 mg SB 431542 in 2.38 ml DMSO.
2. Make aliquots of a convenient volume (e.g., 100 µl).
3. This solution, hereafter referred to as 10mM SB 431542, can be stored at -20°C.

NPC Medium (A)

1. Prepare NPC Medium (A) using the reagents listed in the table below.
    - Thaw N2 supplement (100x), B-27 Supplement (50x), 10 µg/ml bFGF, and 100 µg/ml HB-EGF on ice for 20-30 minutes.

- Warm all other reagents at room temperature for 20-30 minutes.
- Briefly spin down all tubes before use.
- Store NPC Medium (A) for up to 2 weeks at 4°C. The leftover reagents can be saved for other uses.

Reagents	Volume
DMEM/F12	10.5 ml
Neurobasal	10.5 ml
200 mM GlutaMAX	110 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	220 µl
N2 supplement (100x)	220 µl
B-27 Supplement (50X)	440 µl
10 µg/ml bFGF	44 µl
100 µg/ml HB-EGF	4.4 µl
1 mM LDN-193189	2.2 µl
10 mM SB431542 (1,000x)	22 µl

Day -1

~1 hour

Plate Preparation

1. Prepare diluted Coating Material A by mixing together the following components in a 15 ml conical tube.
   - Thaw Coating Material A for 20-30 minutes at the temperature indicated in the “Contents” table on page 1.
   - Keep Coating Material A on ice.
   - Make sure chilled PBS is used for this mixture.

Reagents	Volume
Coating Material A	11.6 µl
Chilled PBS	3.5 ml

2. Add 400 µl diluted Coating Material A to each new well of 8 wells.
3. Incubate the plate at 4°C.
   Note: For best results we recommend precoating the plate 1 day or up to a week before use and keeping at 4°C. Alternatively plates can be precoated on Day 0 and placed at 37°C for at least 2 hours before use.

Day 0

~8 hours

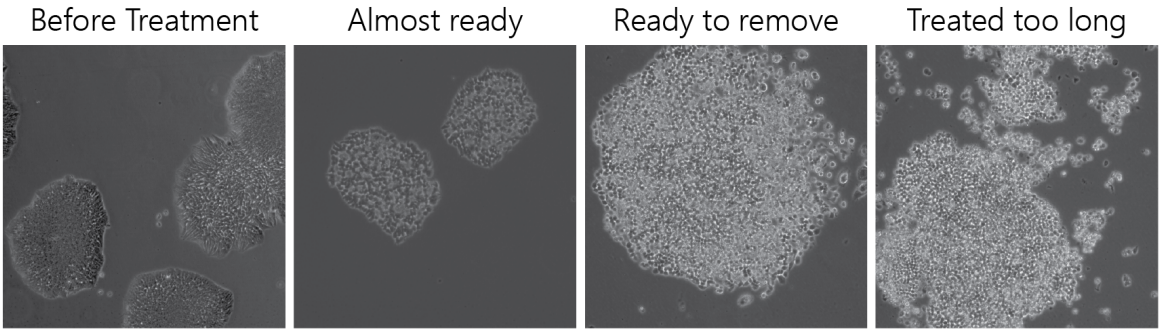
Note: This protocol assumes that Day 0 is a Tuesday.

Plating

1. Prepare Medium iS by mixing together the following components in a 15 ml conical tube.
   - Warm Medium S, iROCK, and Solution D1 at room temperature for at least 1 hour protected from light.
   - The rest of Medium S should be stored at 4°C for later use.

Reagents	Volume
Medium S	5.5 ml
iROCK	5.5 µl

2. Aspirate old medium from hPSC culture and add 2 ml PBS.
3. Rock the plate 3 times, aspirate PBS from the culture, and add 300 µl of the cell dissociation reagent Solution D1. Keep the rest of Solution D1 at 4°C for later use.
4. Incubate the culture plate at 37°C, 5% CO_2 for 5 minutes. If all the cells are not rounded under a microscope, continue to incubate at 37°C, 5% CO_2 in 1-2 minute increments (see images below).



5. Carefully pipet out Solution D1 from the culture and add 1 ml Medium iS to the well.
6. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
7. Using the same pipet tip, collect the cell suspension in a 1.5 ml tube.
8. Count cells and determine viability.

IMPORTANT! In this protocol, users will plate the hPSCs into 8 wells with 500 µl Medium iS per well. However, if users know the best plating density already, prepare a cell suspension enough to plate 5 wells (4 wells plus 1 extra to account for the pipetting/plating error). We recommend aiming for 50-70% initial cell confluency on Day 1, with 20-50 cells per colony. Our data indicate that cell counts ranging from 1.0 - 2.4 x 10^5 viable cells per well are suitable. Cell count may vary based on cell health and the method used for cell counting.

If the confluency on Day 1 is

- above the target range, the differentiation efficiency will decrease.
- below the target range, more cell death will be observed.
- For first time users, we recommend plating the following numbers of cells into each of 8 wells: 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2 and 2.4 x 10^5 cells. On Day 1, 4 wells showing 50-70% confluency should be selected for transfection.

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9. Take out the volume of the cell suspension needed for each cell density, according to the note above, and place each in a new tube labeled with the corresponding density.
10. Bring the volume of the cell suspension in each tube up to 500 µl with Medium iS.
    - If the volume in the tube exceeds 500 µl, centrifuge the required volume of cell suspension at 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet in 500 µl Medium iS.
11. Aspirate diluted Coating Material A from each coated well and add 500 µl cell suspension to each well.
    - Most of the diluted Coating Material A should be aspirated but not completely to prevent the coated well from drying before adding the cell suspension. The cell suspension should be added to the well immediately after diluted Coating Material A is removed. Handle one well after another.
12. Leave the plate flat at room temperature for 10 minutes.
13. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 1

~8 hours

IMPORTANT! Observe all wells under a microscope and select any 4 wells that show 50-70% confluency for transfection with QNP-mRNA. If none of the wells fall within the range of confluence, do not proceed.

First treatment

1. Thaw 1 vial of QNP-mRNA on ice for 30 minutes and warm Opti-MEM and Medium S at room temperature for 20-30 minutes.
2. Prepare QNP by the following steps:
    - Tap the QNP-mRNA tube 3 times and then briefly spin it down before use
    - Prepare a 15 ml tube and a 1.5 ml tube with 220 µl Opti-MEM each. Label the 15 ml tube "Mix 1" and the 1.5 ml tube "Mix 2".
    - Add 4.5 µl Lipofectamine MessengerMax (LMM) to the Mix 1 tube and mix by brief vortexing. Leave it at room temperature for 10 minutes (Mix 1). Keep the rest of LMM at 4°C for later treatments.
    - **IMPORTANT!** Immediately before 10 minutes pass (i.e., around 8 minutes), add 9 µl QNP-mRNA to the other 1.5 ml tube with Opti-MEM (Mix 2). Mix by tapping 5 times. Do not vortex.
    - 10 minutes after mixing LMM with Opti-MEM, add Mix 2 into Mix 1, and pipet up and down 8-10 times. This mixture is called QNP. Leave QNP at room temperature for 5 minutes and no longer.

Mix 1 Reagents	Volume
Opti-MEM	220 µl
LMM	4.5 µl

Mix 2 Reagents	Volume
Opti-MEM	220 µl
QNP-mRNA	~9 µl

3. Add 1.75 ml Medium S to QNP (for a final volume of 2.2 ml) and pipet up and down 2-3 times to mix.
4. Working with up to 2 wells at a time, aspirate the old medium out and add 500 µl of QNP mixture to each well. Repeat until QNP mixture has been added to all wells.
5. Incubate the culture plate at 37°C, 5% CO_2 for 2.5 hours.

Second treatment

1. Pipet out the medium from each well using and add 500 µl Medium S.
2. Incubate the culture plate at 37°C, 5% CO_2 for 2 hours.
    - Put 1 vial of QNP-mRNA on ice when the incubation above reaches 1.5 hours and leave it for 30 minutes.
3. Repeat Steps 2-5 of the previous "First Treatment" section.

Medium Change

1. Warm NPC Medium (A) at room temperature for 20-30 minutes.
2. Pipet out the medium from each well and add 500 µl NPC Medium (A).
3. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 2

~8 hours

IMPORTANT! Observe the QNP-treated cultures to make sure that they are reaching confluency (≥90%). If the cultures are <50% confluent and show signs of cell death (e.g., many floating cells), users should skip the third and fourth treatments on Day 2 and proceed directly to the "Medium Change" section. First time users, who have plated cells at different densities, should proceed to the third and fourth treatments for only two wells with higher plating densities. For the other two wells (with lower densities), skip the third and fourth treatments on Day 2 and proceed directly to the "Medium Change" section.

Third Treatment

1. Thaw 1 vial of QNP-mRNA on ice for 30 minutes and warm Opti-MEM and NPC Medium (A) at room temperature for 20-30 minutes.
2. Repeat Steps 2-5 of the previous “First Treatment” section using NPC Medium (A) in place of Medium S to prepare QNP mixture.

Fourth treatment

1. Pipet out the medium from each well and add 400 µl NPC Medium (A).
2. Incubate the culture plate at 37°C, 5% CO_2 for 2 hours.
    - Put 1 vial of QNP-mRNA on ice when the incubation above reaches 1.5 hours and leave it for 30 minutes.
3. Repeat Steps 2-5 of the previous “First Treatment” section using NPC Medium (A) in place of Medium S to prepare QNP mixture.

Medium Change

1. Pipet out the medium from each well and add 500 µl NPC Medium (A).
2. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 3

< 1 hour

Maintenance

IMPORTANT! It is optional, but recommended, to include the PBS wash if cell death/floating cells are observed.

1. Warm NPC Medium (A) at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well and* add 800 µl Medium NPC Medium (A).
    - **IMPORTANT!** For medium change, a small volume of the old medium (i.e., just enough to cover the surface of the well) should be left. The fresh medium should be applied along the wall of the well very slowly.
    - *(Optional) Slowly add 500 µl PBS alongside the wall of each well to avoid lifting attached cells. Gently pipet out PBS before adding NPC Medium (A).
3. Incubate the cultures at 37°C, 5% CO_2 for 3 days.

Day 6

Assay or Continuous Culture

- Differentiated neural precursor cells can be observed on Day 6. At this time point NPCs will have reached confluency and should be passaged into new plates or cryopreserved. Passaging at an earlier time point, e.g. at Day 4, can be performed without compromising NPC quality and is recommended if culture is confluent. See Appendix B for passaging instructions and Appendix C for instructions for cryopreservation.
- Differentiation into neural precursor cells after using the Quick-Neuron™ Precursor - mRNA Kit can be confirmed with the markers NES, VIM, HES5, SOX1, and SOX2.

Appendix A

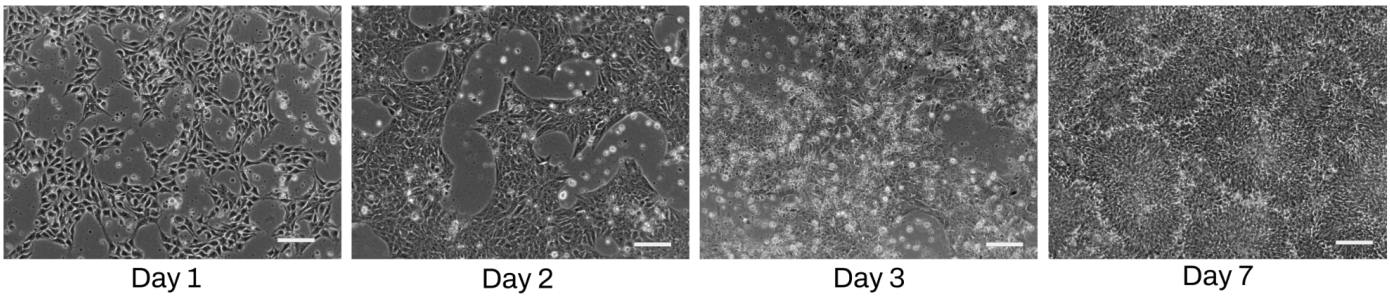


Figure 1. Representative phase contrast images of Quick-Neuron™ Precursor - mRNA Kit cell cultures on days 1, 2, 3 and 7 days post-differentiation (scale bar = 100 µm). Cells are ready for passaging at Day 7.

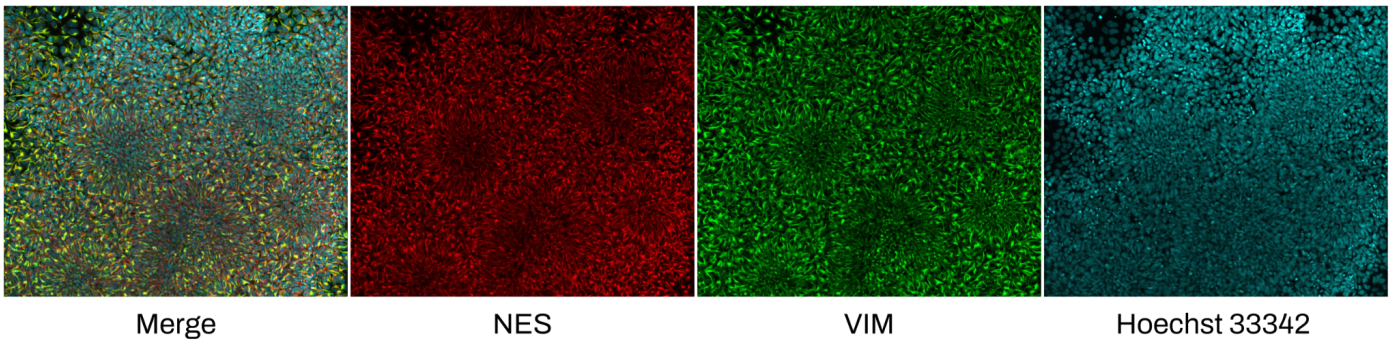


Figure 2. Immunofluorescent staining of Quick-Neuron™ Precursor - mRNA Kit cell cultures shows expression of NES and VIM on day 7 post-differentiation (scale bar = 100 µm). Staining conditions: Anti-Nestin Antibody, clone 10C2 (Millipore Sigma, Catalog Number: MAB5326, 1:500 dilution) was used in combination with a secondary antibody (Invitrogen, Catalog Number: A11032 Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, 1:500 dilution). Recombinant Anti-Vimentin antibody [EPR3776] - Cytoskeleton Marker (abcam, Catalog Number: ab92547, 1:500 dilution) was used in combination with a secondary antibody (Invitrogen, Catalog Number: A32731 Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488, 1:500 dilution). Nuclei were counterstained with Hoechst 33342.

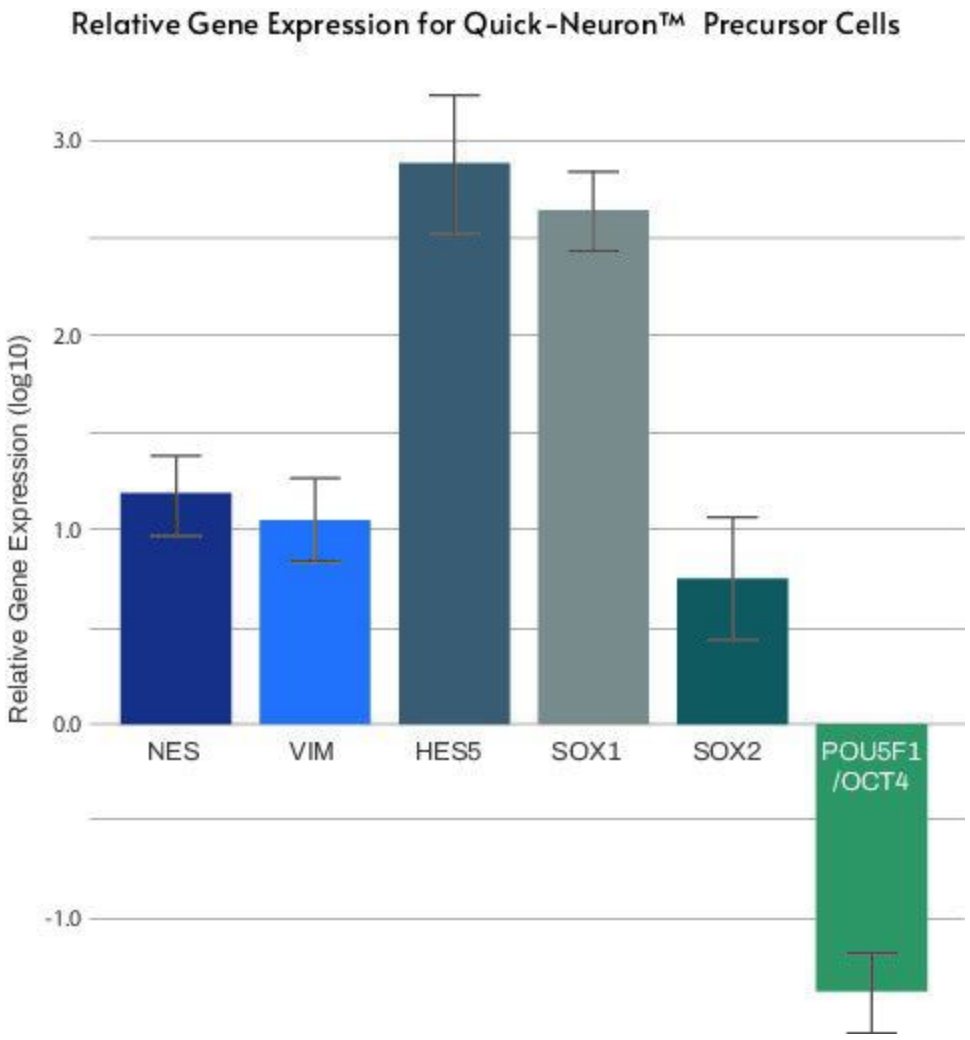


Figure 3. Real-time quantitative PCR analysis of expression levels of neuronal precursor-associated genes NES, VIM, HES5, SOX1 and SOX2, as well as the stem cell pluripotency marker POU Class 5 Homeobox 1 (POU5F1/OCT4), were examined. Graph shows gene expression in Quick-Neuron™ Precursor Culture on day 6. The relative gene expression is normalized to phosphoglycerate kinase 1 (PGK1), and then calculated as a fold induction relative to undifferentiated hiPSCs as a control. Error bars show standard deviation. Primers used are listed in Table 1.

Table 1. List of PCR primers used in Figure 3

Gene	Forward Primer	Reverse Primer	Primer Concentration
NES	CAGCGTTGGAACAGAGGTTGG	TGGCACAGGTGTCTCAAGGGTAG	250 nM
VIM	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC	250 nM
HES5	TGCTCAGCCCCAAAAGAGAAA	GAAGGCTTTGCTGTGCTTCA	250 nM
SOX1	CAGTACAGCCCCATCTCCAAC	GCGGGCAAGTACATGCTGA	250 nM
SOX2	ATGCACCGCTACGACGTGA	CTTTTGCACCCCTCCCATTT	250 nM
POU5F1/OCT4	GTGAAGCTGGAGAAGGAGAAGC	CATTGTTGTCAGCTTCCTCCACC	250 nM
PGK1	GTATGCTGAGGCTGTCACTCG	CCTTCCAGGAGCTCCAAACTGG	250 nM

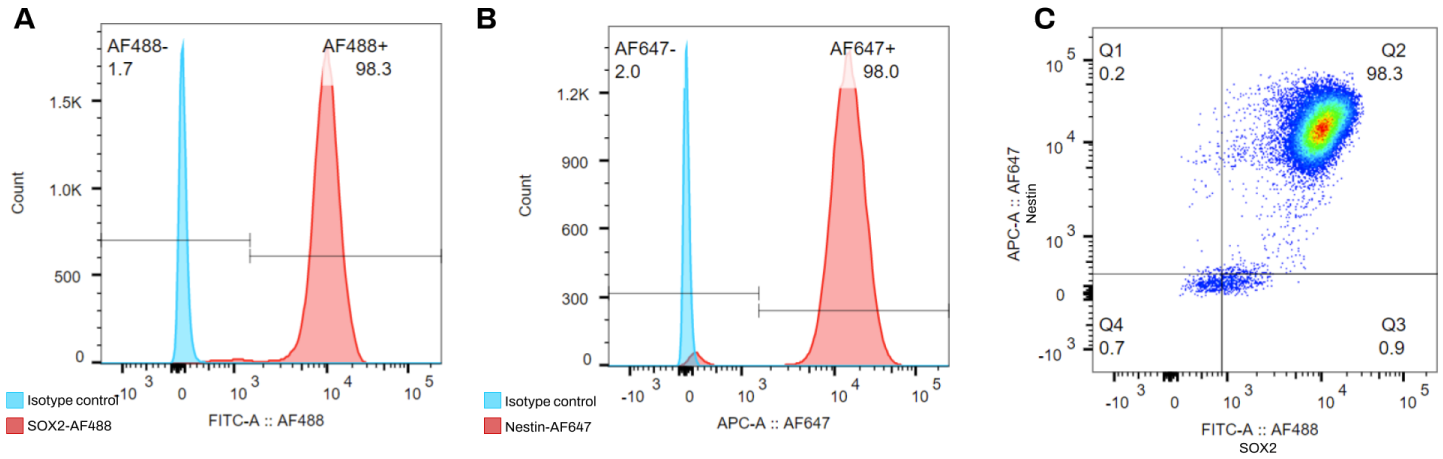


Figure 4. Flow cytometry analysis of NPC markers SOX2 and Nestin in Quick-Neuron™ Precursor cells at day 8 of differentiation. Cells were fixed in 4% PFA, permeabilized with BD Phosflow™ Perm Buffer III (Catalog Number: 558050) and then stained with (A) Alexa Fluor 488 Mouse-anti SOX2 (Catalog Number: 561593) or Alexa Fluor 488 Mouse IgG1 κ Isotype Control (Catalog Number: 557721) (B) Cells were stained with either Alexa Fluor 647 Mouse-anti Nestin (cat# 560393) or Alexa Fluor 647 Mouse IgG1 κ Isotype Control (Catalog Number: 557732). Plots were derived from gated events on forward and side light scatter from an unstained NPC sample. Dot plot (C) demonstrates that >98% of the Quick-Neuron™ Precursor cells in culture are positive for SOX2 and Nestin. All antibodies were purchased from BD Biosciences.

Appendix B

Planning

Cells should be passaged when they approach confluency. Cells can be plated on a variety of plate formats including glass coverslips and chamber slides. When using glass coverslips be sure to sterilize with ethanol and allow to dry before proceeding with coating. Here each of the following steps assumes the use of 6 wells of a 6-well plate with a surface area of 9.5 cm^2/well. When first transferring cells to laminin conditions we recommend plating cells at a density of between 5.6 x 10^4 - 1.0 x 10^5 cells/cm^2 (5.0 x 10^5 - 1.0 x 10^6 cells/well of a 6-well plate). During continuous culture, we recommend plating between 1.3 x 10^4 - 5.6 x 10^4 cells/cm^2 (1.2 x 10^5 - 5.0 x 10^5 cells/well of a 6-well plate) for a weekly passaging cycle. Other well formats can be used by adjusting the cell numbers and medium volume proportionally to the surface area size. Calculate the number of wells to be used in advance.

Plate Preparation

1. Warm NPC Medium (A) at room temperature for 20-30 minutes.
2. Vortex ornithine briefly and centrifuge it at a maximum speed for 1 minute.
3. Add 1.5 ml ornithine to the surface of each well.
4. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours (or at 4°C overnight one day before plating).
5. Prepare diluted laminin according to the table below.
    - Thaw laminin and chill PBS on ice for 20-30 minutes.
    - All PBS washes should be done dropwise and with room temperature PBS. Chilled PBS is only for the coating step.

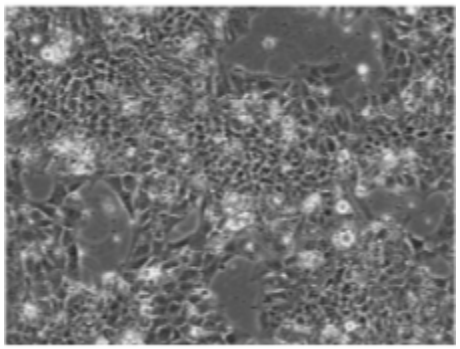
Reagents	Recommended Volume per well of a 6-well plate	Total Volume for 6-well plate
Chilled PBS	1.65 ml	9.9 ml
Laminin	1.65 µl	9.9 µl

6. Aspirate the supernatant from each well and add 2 ml PBS.
7. Repeat Step 6.
8. Aspirate PBS from each well and add 1.5 ml diluted laminin.
9. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours.
10. After the laminin incubation, pipet out most of the supernatant, but not all, from each well.
11. Rinse with 2 ml PBS.
12. Pipet out most of the PBS, but not all, from each well.
13. Repeat Steps 11-12.
14. Add 500 µl NPC Medium (A) to each well.
15. Incubate the plate at 37°C, 5% CO_2 until cells are ready for plating.

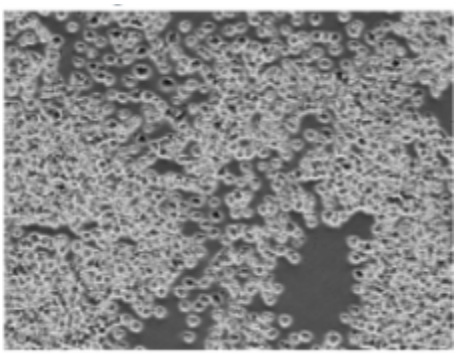
Passaging Cells

IMPORTANT! For the following steps, gently pipet and add solutions. Differentiating cells are delicate and should be handled with great care. Steps 2-10 below are critical. **Perform these steps one well at a time.** Refer to the images below to successfully manage cell treatment and dissociation.

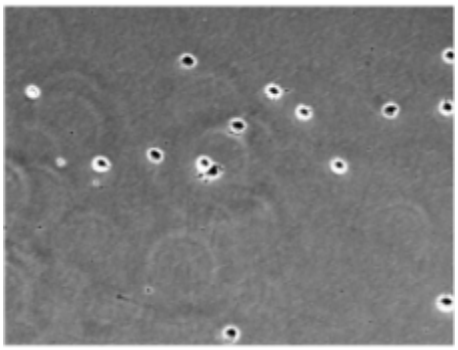
Before Solution D1 treatment



During Solution D1 treatment



After dissociation



1. Make sure that Solution D1 is at room temperature for at least 1 hour before use.
2. Pipet out the old medium from the well and add 1 ml PBS.
3. Pipet out the PBS from the well and add 300 µl Solution D1.
4. Rock the plate 3 times to spread the Solution D1 evenly.
5. Incubate the cultures at 37°C, 5% CO_2 for 3 minutes.
6. Carefully pipet out Solution D1 from the well using a P1000 pipettor and add 750 µl NPC Medium (A) to the well along the wall of the well.
7. Disperse the medium quickly over the bottom surface of the well by pipetting 6-8 times to detach cells using a P1000 pipettor.
8. Observe cells and/or cell aggregates floating in the well under a microscope. It is normal that 10-20% of cells remain attached to the well bottom after pipetting. The clusters of cells are not supposed to be lifted. Do not attempt to detach all of the cells remaining on the well bottom.
9. Gently pipet the cell suspension up and down in the well up to 5 times to break the cell aggregates using a P1000 pipettor. Excessive pipetting can damage the already-suspended neuronal cells.
10. Collect all of the cell suspension from each well in a tube using the same P1000 pipette tip.
11. Repeat Steps 2-10 for the rest of the wells.

Plating Cells

1. Count cells to determine the volume of cell suspension needed for the prepared number of wells and include an extra 10% for cell number and volume (1.2×10^5 cells multiplied by the number of prepared wells and add an additional 10%). Adjust the volume with NPC Medium (A) to 1.65 ml multiplied by the number of prepared wells with an additional 10%.
    - If the cell suspension density is less than 8×10^4 cells per ml, centrifuge the required volume of cell suspension at 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet into the appropriate volume of NPC Medium (A).
2. Add 1.5 ml cell suspension to each well.
3. Incubate the cultures at 37°C, 5% CO_2 overnight.

1 Day Post-Passaging Maintenance

1. Warm NPC Medium (A) at room temperature for 20-30 min.
2. Pipet out the old medium from each well and add 2 ml PBS to each well along its wall.
    - **IMPORTANT!** To avoid lifting cells, leave a small volume of the old medium (i.e., just enough to cover the surface of the well). PBS should be applied along the wall of the well very slowly. This procedure should be followed during all subsequent medium changes (i.e., on Day 2 and Day 4).
3. Pipet out the PBS and add 1.5 ml NPC Medium (A) to each well along its wall.
4. Incubate the culture at 37°C, 5% CO_2 overnight.

2 Day Post-Passaging and Continued Maintenance

1. Warm NPC Medium (A) at room temperature for 20-30 min.
2. Pipet out the old medium from each well and add 2 ml PBS to each well along its wall.
3. Pipet out the PBS and add 2 ml NPC Medium (A) to each well along its wall.
4. Incubate the culture at 37°C, 5% CO_2.
5. Repeat steps 1-4 every 2-3 days until cultures reach confluency.

Note: Before the weekend (i.e. Friday), we recommend feeding with 2.5 ml NPC Medium (A) instead of 2 ml.

Appendix C

Cryopreservation

Note: After thawing frozen cells, approximately 50% of the cryopreserved cells will be recovered as viable cells.

1. Determine the volume of the cell suspension and number of cryovials needed to freeze 0.2 ~ 2 x 10^6 cells per cryovial.
2. Centrifuge at 200 x g for 4 min.
3. While waiting for the centrifugation, label each cryovial. We recommend writing the name of the iPSC line used, the type of cells, harvesting day and date, and the number of cells in the vial.
4. Aspirate the supernatant and resuspend the pellet with 0.5 ml / vial STEM-CELLBANKER.
5. Distribute 0.5 ml of the suspension to each cryovial.
6. Make sure that the caps are closed tightly and transfer the cryovials into a Mr. Frosty Freezing Container. Make sure that Mr. Frosty contains 250 ml isopropanol.
7. Loosely close the lid of Mr. Frosty with cryovials, put it into a -80°C freezer and leave it overnight or up to a few days.
8. Transfer the cryovials into a liquid nitrogen storage tank.
9. Follow the thawing process in the user guide of Quick-Neuron™ Precursor - Human iPSC-derived Neural Precursor Cells, Catalog Number: NP-mRNA-CW.