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Differentiation of human induced pluripotent stem cells to neural stem cells

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2 Works for me dx.doi.org/10.17504/protocols.io.bgbxjspn

Cellular Generation and Phenotyping

Malin Andersson

ABSTRACT

This protocol outlines the steps of differentiating human induced pluripotent stem cells (iPSCs) into cortical lineage neural stem cells (NSCs) using a dual-SMAD and Wnt-inhibition method. The cells are cultured in 6 well plates over the course of 14 days, generating approximately 10 million NSCs per well. An immunocytochemistry quality control assay is used to assess the expression of NSC markers including PAX6, FOXG1 and NESTIN.

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KEYWORDS

iPSC, induced pluripotent stem cells, neural stem cells, iPSC differentiation, neural differentiation, ICC, immunocytochemistry

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36951

GUIDELINES

Unless otherwise noted, all steps should be performed under sterile conditions in a biological safety cabinet.

MATERIALS

NAME

CATALOG

VENDOR

NAME	CATALOG #	VENDOR
100 mM Sodium Pyruvate	S8636	Sigma
B-27 Supplement	17504044	Gibco - Thermo Fischer
DAPI	40043	Biotium
DPBS	14190	Invitrogen - Thermo Fisher
Essential 8™ Medium	A1517001	Gibco, ThermoFisher
Insulin solution human	I9278	Sigma Aldrich
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501	Thermo Fisher Scientific
DMEM/F-12, GlutaMAX® supplement	10565018	Thermo Fisher
MEM Non-Essential Amino Acids Solution (100X)	11140035	Thermo Fisher
DMEM/F-12, HEPES	31330038	Thermo Fisher
2-Mercaptoethanol (50 mM)	31350010	Thermo Fisher
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	A14700	Thermo Fisher
Adhesive PCR Plate Foils	AB0626	Thermo Fisher
UltraPure 0.5M EDTA pH 8.0	15575020	Invitrogen - Thermo Fisher
Falcon 50mL Conical Centrifuge Tubes	14-432-22	Fisher Scientific
DMSO	D2650	Sigma Aldrich
Knockout serum replacement (KSR)	10828028	Gibco - Thermo Fisher
Falcon 6 well plate	10110151	Fisher Scientific
Rock inhibitor (Y-27632)	Y0503	Sigma Aldrich
Falcon 15ml Conical Centrifuge Tubes	352097	Fisher Scientific
Falcon 50ml Conical Centrifuge Tubes	352098	Becton-Dickinson
Human FGF Basic (146AA)	100-18C	peprotech
L-Ascorbic Acid	A8960	Sigma Aldrich
Sodium Chloride	S5886	Sigma Aldrich
Sodium Bicarbonate	S5761	Sigma Aldrich
1L Filter Unit	567-0020	Nalgene
Corning Square Polycarbonate Storage Bottles 250ml	431431	Corning
Corning Square Polycarbonate Storage Bottles 150ml	431430	Corning
Insulin-Transferrin-Selenium (ITS -G) (100X)	41400045	Life Technologies
SB431542	ab120163	Abcam
XAV939	3748	Tocris
LDN193189	2092-5	Cambridge Bioscience
Cell Strainer 70um For 50ml Falcon Tube	352350	Corning
Cryogenic vials 1.8ml	375418	Thermo Scientific
200µl Yellow TipOne Pipette Tip (Sterile) Racked	S1111-0816	StarLab
Neurobasal -A Medium minus phenol red	12349015	Thermo Fisher Scientific
GlutaMAX Supplement	35050-061	Thermo Scientific
CTS N-2 Supplement-5 mL	A1370701	Gibco - Thermo Fischer
500ml PES Filter Unit 0.2µM	431097	Corning
DNase I from bovine pancreas	11284932001	Sigma
Greiner 96 well tissue culture treated black clear bottomed plate	655090	greiner bio-one
37% Formaldehyde	A0823.2500	AppliChem
Donkey Serum	C06SBZ	Bio-rad Laboratories
Rabbit Anti-Pax6	60433S	Cell Signaling Technology
Rabbit Anti-FoxG1	ab196868	Abcam
Mouse Anti-Oct3/4	sc-5279	Santa Cruz Biotechnology

NAME	CATALOG #	VENDOR
Donkey Anti-Rabbit AF488	A21206	Invitrogen - Thermo Fisher
Donkey Anti-mouse AF647	A31571	Invitrogen - Thermo Fisher
DPBS with MgCl ₂ and CaCl ₂	D8662	Sigma
50ml Reagent Reservoir	4870	Corning
Triton-X 100	X100-100ML	Sigma
1.5ml Eppendorf Tubes with Safe-lock	0030 120.086	Eppendorf
2ml Eppendorf Tubes with Safe-lock	0030 120.094	Eppendorf
Rabbit Anti-Nestin antibody	ab105389	Abcam

SAFETY WARNINGS

Please refer to the manufacturer's documentation and material safety data sheets (MSDS) for the products you are using when following this protocol.

2--Mercaptoethanol - may cause allergic skin reaction.

Rock inhibitor (Y-27632) - harmful if swallowed, in contact with skin or inhaled.

DNase I - may cause allergic skin reaction.

37% Formaldehyde - flammable liquid and vapour, toxic if swallowed/in contact with skin/inhaled, causes skin burns and eye damage, may cause allergic skin reaction, may cause respiratory irritation, suspected of causing genetic defects, may cause cancer, causes damage to organs (eyes).



Triton X-100 - harmful if swallowed, causes skin irritation, causes serious eye damage, very toxic to aquatic with long-lasting effects.

DAPI - causes skin irritation, causes serious eye irritation, may cause respiratory irritation.

BEFORE STARTING

Our iPSC are cultured on vitronectin-coated Falcon plates in E8 media using this protocol:

dx.doi.org/10.17504/protocols.io.bgbwjspe

Culture of established induced pluripotent stem cell lines

by **Malin Andersson,**
Wellcome Sanger Institute

PREVIEW

RUN

Ensure iPSCs are 70-80% confluent. For the neural induction to work effectively, the iPSCs morphology should be round, dense colonies of cells with minimal spontaneous differentiation present in the culture. It might be necessary to passage the iPSCs several times to achieve this morphology.

Passaging iPSC for Neural Induction

1 Preparation

Coat Falcon 6 well plates with Vitronectin diluted 1:50 in DPBS(-/-) and incubate for at least 2 hours at 37°C.


2 Aspirate media from iPSC wells and wash with 2ml DPBS(-/-) per well. Aspirate DPBS(-/-) and add 1ml accutase to each well. Incubate for 3-6 minutes at 37°C, checking after 3 minutes to see if cells have begun to detach.

3 Add 4ml E8 media per well, pipetting up and down using a stripette to dislodge the cells. Collect all wells into a 50ml Falcon tube.

4 Cell count

Determine the cell count using an appropriate cell counter. Calculate the volume of cell suspension required to seed 2×10^6 cells per well of a 6 well plate to be re-plated into (0.2×10^6 cell per cm^2).

5

Isolate required volume into a 15ml Falcon tube. Centrifuge all cells for  **200 x g 00:05:00**.

If you are planning RNA-Seq or qPCR analysis of the cell line, isolate the required suspension volume into separate 15ml Falcon tube(s) for making pellets. We recommend a minimum of 2 million cells per cell pellet.

6 Make up seeding media by supplementing complete E8 media with rock inhibitor (Y-27632) to a final concentration of 10 μ M. Make enough seeding media to give 2ml per well to be re-plated into.

7 Aspirate supernatant and re-suspend each cell line in 2ml E8 with rock inhibitor per 2×10^6 cells.

8 Quickly remove vitronectin from the newly coated plate(s) and transfer 2ml cell suspension into each well. Gently shake plate back and forth and side to side, ensuring that the cells become evenly distributed.


9

Transfer cells into a  **37 °C** 5% CO₂ tissue culture incubator overnight, gently agitating the plate once more.

10 To create cell pellets, aspirate supernatant media and re-suspend the cell pellet with DPBS(-/-) and divide between Eppendorf tubes. Centrifuge tubes for 30 seconds to pellet the cells, then aspirate supernatant and snap-freeze. Store snap-frozen cell pellets at **-80 °C**.

Neural Induction (Day 0 - Day 9)

11 Preparation:

Make up E6 base media. Excess media can be aliquotted into appropriate volume (e.g. 100ml) and stored  **-80 °C** for 12 months.

Neural Induction Media should be prepared on the day of its first use, then kept at  **4 °C**.

11.1 Preparation of E6 Base Media

Media Component	Volume	Final Concentration
DMEM/F12	1000ml	100%
L-Ascorbic Acid	64mg	64mg/L
Sodium Chloride	1g	1g/L
Sodium Bicarbonate	543mg	543mg/L

Components of E6 Base Media.

Weigh out 64mg of L-Ascorbic Acid, 1g sodium chloride, and 543mg sodium bicarbonate and add all to a 50ml Falcon tube.

Add mixed chemicals to 500ml of DMEM/F12. Use DMEM/F12 to wash all the residue out of the Falcon tube, ensuring all the powder is retrieved.

Add the 500ml DMEM/F12 plus chemical mixture to a 1L filter unit, and add a second 500ml of DMEM/F12 (without chemicals). Filter to create E6 base media.

Ensure E6 base media is fully mixed before aliquotting and freezing.

E6 base media can be stored at -80°C for up to 12 months.

11.2 Preparation of Neural Induction Media

Media Component	Volume	Final Concentration
E6 Base Media	100ml	100%
ITS (100X)	2ml	2X
SB431542 (10mM)	100 μl	10 μM
XAV939 (10mM)	20 μl	2 μM
LDN193189 (1mM)	10 μl	100nM

Components of Neural Induction Media.

Reconstitute SB431542 and XAV939 to 10mM using DMSO. Aliquots can be stored at -20°C for up to 6 months.

Reconstitute LDN193189 to 1 mM using DMSO. Aliquots can be stored at -20°C until the expiry date listed on the stock vial.

Thaw aliquot(s) of E6 Base Media.

Add 2ml of Insulin-Transferrin-Selenium (ITS) per 100ml of E6 Base Media.

Add 100 μl of SB431542, 20 μl XAV939 and 10 μl LDN193189 per 100ml E6 Base Media. **Note:** LDN193189 is light sensitive. If possible, work with cabinet lighting turned off and avoid leaving the media in the light for long periods of time.

Neural Induction Media should be stored at 4°C for up to 12 days. Bring to room temperature before use. Do not warm NIM in direct sunlight or under bright lighting; if possible keep it in the dark.

- 12 24 hours after plating, the cells should have attached in a uniform sheet across the well. From this point, the cells are prone to peeling. When aspirating, be sure not to touch the cell layer at any point. Media should be dispensed gently on the side of the well, never directly onto the cells.
- 13 Aspirate off the media from the well and gently wash cells with DPBS(-/-).
- 14 Aspirate the DPBS(-/-) and gently dispense 2ml of neural induction media onto the side of the well. Return the cells to the incubator. This is day 0 of neural induction.
- 15 Media change the cells every 24 hours with 2-3ml of neural induction media per well until day 10.

- 16 The cells should form a neuroepithelial sheet around day 3. Towards the end of the differentiation period the cell proliferation is high and the media may be very yellow when media changing. There is also an increased risk of the neuroepithelial sheet peeling off on later days. If there is excessive peeling it might be necessary to passage the cells ahead of day 10. Neural rosette structures might start to appear in the culture around day 10.

Accutase Passage of Neuroepithelial Sheet (Day 10)

17

Preparation

Coat Falcon 6 well plates with 1:100 vitronectin in DPBS(-/-) and incubate for at least 2 hours at 37°C. We recommend plating cells for the ICC QC assay at this passage.


17.1 Preparation of Neural Maintenance Media (NMM)

Media Component	Volume	Final Concentration
DMEM/F12+Glutamax	250ml	50%
Neurobasal-A	250ml	50%
CTS N-2 (100X)	2.5ml	0.5X
B27 (50X)	5ml	0.5X
GlutaMAX (100X)	2.5ml	0.5X
MEM NEAA (100X)	2.5ml	0.5X
Sodium Pyruvate (100X)	2.5ml	0.5X
2-mercaptoethanol (50mM)	0.5ml	50µM
Insulin (10mg/ml)	125µl	2.5µg/ml

Components of Neural Maintenance Media.

If making larger volumes of the media we recommend filtering it using a filter unit.

NMM should be stored at **4 °C** and expires after 14 days.

- 18 Supplement NMM with rock inhibitor (Y-27632) to a final concentration of 10µM. Make up enough NMM plus rock inhibitor to give 4ml per well to be re-plated into.
- 19 Aspirate NI media from cells and gently wash cells with 2ml DPBS (-/-) per well. Aspirate DPBS and add 1ml accutase to each well. Incubate for 3-6 minutes at 37°C, checking after 3 minutes to see if cells are detaching.
- While cells are incubating, prepare wash buffer containing DMEM/F12+Glutamax and DNase I at a final concentration of 0.03mg/ml. This should be prepared fresh for each cell line to ensure optimum enzyme activity of the DNase.
- 20 After incubation, add 4ml wash buffer to each well, pipetting up and down using a stripette to dislodge the cells. Collect all wells for each cell line into a 50ml Falcon tube. Mix the cell suspension thoroughly and aliquot into 15ml Falcon tubes of equal volume for centrifugation.
- 21  Centrifuge cells for **200 x g 00:05:00**.
- 22 Aspirate the supernatant and resuspend the cells in 4ml NMM per well to be re-plated into. Since this passage is 1:1, this should be the same number of wells per cell line as in the Neural Induction stage.

- 23 Quickly remove the vitronectin from the newly coated plate(s) and transfer 4ml cell suspension into each well. Agitate plate back and forth and side to side to ensure cells become evenly distributed.

24 

Transfer plate(s) to a  **37 °C** 5% CO₂ tissue culture incubator overnight, gently agitating plate once more.

Neural Cell Maintenance Culture (Day 11 to Day 13)

3d

- 25 Culture the cells for three days, media changing on day 11 and day 13 with 4ml neural maintenance media per well.



During this period, the cells should proliferate and reach a morphology of small, round/oval cells. Neural rosette structures should be visible when viewing the cells under the microscope with a 10x or 20x objective.

Cryopreservation of Neural Stem Cells (Day 14)

1m

- 26 **Preparation:**

Make up neural freeze media by combining NMM (90%) with DMSO (10%). 1ml is needed per 2.5×10^6 cells.

- 27 Aspirate the media from your plate(s). Wash gently with 2ml DPBS(-/-) per well. Aspirate DPBS and add 1ml accutase per well. Incubate for  **00:04:00** at  **37 °C**, checking after 2 minutes to see if cells are detaching.

- 28 Neutralise accutase by adding 4ml DMEM/F12+Glutamax to each well, pipetting up and down with a stripette to break up the cell layer.


- 29 Pass the cells through a 70µm filter, collecting all the cells from one cell line into a 50ml Falcon tube.


- 30 **Cell count**


Determine the cell count using an appropriate cell counter.

NSCs should be frozen at 2.5×10^6 cells per vial. Isolate the required number of cells, dividing the volume evenly between 15ml Falcon tubes.

If you are planning RNA-Seq or qPCR analysis of the cell line, isolate the required suspension volume into separate 15ml Falcon tube(s) for making pellets. We recommend a minimum of 2 million cells per cell pellet.

- 31 

Spin cells in a centrifuge for  **200 x g 00:05:00**.

- 32 Remove supernatant from the cells to be cryopreserved. Resuspend in 1ml Neural Freezing Media per 2.5×10^6 cells, then divide cell suspension between 1.8ml cryovials, adding 1ml to each cryovial. Place the cryovials into a CoolCell or other appropriate freezing container, then transfer to a  **-80 °C** freezer. Cells should be transferred to LN₂ storage after 24 hours.

- 33 To create cell pellets, aspirate supernatant media and re-suspend the cell pellet with DPBS(-/-) and divide between Eppendorf tubes. Centrifuge tubes for 30 seconds to pellet the cells, then aspirate supernatant and snap-freeze. Store snap-frozen cell pellets at -80°C .

Thawing NSCs

34 Preparation

Coat Falcon 6 well plates with 1:100 vitronectin in DPBS(-/-) and incubate for at least 2 hours at 37°C .

- 35 Partially thaw cells in 37°C water bath. Add 1ml DMEM/F12+Glutamax to the cryovial dropwise and transfer the partially thawed NSC to a fresh falcon tube containing a further 7ml DMEM/F12+Glutamax. Use another 1ml of DMEM/F12+Glutamax to wash the cryovial to ensure all cells are collected.

- 36 Centrifuge the cells at 200rcf for 5 minutes. $200 \times g$, Room temperature 00:05:00

- 37 Aspirate the supernatant and gently re-suspend cell pellet in 4ml of Neural Maintenance Media supplemented with rock inhibitor (final concentration $10\mu\text{M}$) and bFGF (final concentration 20ng/ml).

- 38 If cells were frozen at $>1.5 \times 10^6$ cells/vial then seed one vial to one well of a 6 well plate. If cell numbers in the frozen vial are unknown it is recommended to count before plating and aim to seed at a density of $0.15\text{--}0.25 \times 10^6$ cells/cm² ($1.5\text{--}2.5 \times 10^6$ cells/well of a 6 well plate).

- 39 Remove vitronectin from the prepared plate and replace with 4ml of cell suspension at the correct density.

- 40 Gently agitate plate back and forth and side to side to ensure cells do not centre in middle of the vessel and transfer for overnight incubation in humidified incubator at 37°C .

- 41 24h after plating, remove spent media and replace with 4ml fresh NMM supplemented with bFGF (final concentration 20ng/ml).

- 42 Media change cells every other day with NMM supplemented with bFGF. The cells should be confluent enough to passage after 4-6 days of culture.

Passaging NSCs

43 Preparation

Coat Falcon 6 well plates with 1:100 vitronectin in DPBS(-/-) and incubate for at least 2 hours at 37°C .

- 44 Dispense Neural Maintenance Media into a falcon tube and supplement with rock inhibitor (final concentration $10\mu\text{M}$) and bFGF (final concentration 20ng/ml).

- 45 Aspirate media and wash cells with DPBS(-/-).
- 46 Aspirate DPBS(-/-) and add 1ml Accutase per well and incubate for 2-4 min in humidified incubator at **37 °C**
- 47 Add 4ml DMEM/F12+Glutamax per well, gently pipette up and down to dislodge cells and collect into a 50ml falcon tube. NOTE: If cells remain very clumpy despite accutase treatment it is possible to filter them using a 70µm filter into a 50ml falcon tube.
- 48 Determine the cell count using an appropriate cell counter. Calculate the volume of cell suspension required to seed 2×10^6 cells per well of a 6 well plate (0.2×10^6 cell per cm^2).
- 49 Centrifuge the cells at 200rcf for 5 minutes. **200 x g, Room temperature 00:05:00**
- 50 Aspirate supernatant and gently re-suspend the pellet in 4ml of NMM supplemented with rock inhibitor and bFGF per well to be seeded.
- 51 Remove vitronectin from the prepared plate and replace with 4ml of the cell suspension.
- 52 Gently agitate plate back and forth and side to side to ensure cells do not centre in middle of the vessel and transfer for overnight incubation in humidified incubator at **37 °C**.
- 53 24h after plating, remove spent media and replace with 4ml fresh NMM.

Immunocytochemistry Quality Control Assay

5h 35m

- 54 The immunocytochemistry assay is used to determine the success of the neural differentiation by estimating the expression of NSC markers. We also use the stem cell marker OCT4 to highlight any non-differentiated cells. In this section, PBS with Calcium and Magnesium is used to prevent cell detachment. Be very gentle when aspirating and pipetting as differentiated cells are prone to peeling. The ICC staining can be done in a non-sterile environment, however formaldehyde should be handled in a fume hood.
- 55 **Fixing the cells using formaldehyde** 20m
Aspirate the media from the cells and replace it with 4% formaldehyde in PBS with Calcium and Magnesium. Incubate for 20 minutes at room temperature.
- 56 Aspirate the formaldehyde and wash the cells twice times with PBS with Calcium and Magnesium, each time for 5 ^{10m} minutes.

Replace the PBS from the second wash with new PBS. At this point the cells are fixed and it is possible to store the plate ^{5m}

57 at 4°C for up to 7 days before staining the cells. Make sure plates are wrapped in parafilm to prevent evaporation during storage.

58 **Staining the cells** 30m
Make up 0.1% Triton X-100 in PBS with Calcium and Magnesium. It can be stored for up to 1 month at 4°C .

59 Prepare 10% donkey serum (DS) using 0.1% Triton X-100. 5m

60 Blocking and permeabilisation step: 2h
Aspirate PBS from the cells and add 10% DS. Incubate at room temperature for 2 hours.

61 Dilute 10% DS to 1% DS using 0.1% Triton X-100 (for example 1ml 10% DS in 9ml 0.1% Triton X-100).

62 Prepare primary antibodies (Table 1): Towards the end of the blocking time, dilute primary antibodies in 1% DS and mix by using a pipette (keep on ice until needed). If using the antibodies suggested in table 1, the anti-OCT4 stain can be co-stained with any of the NSC markers.

Antibody	Localisation	Dilution	Catalogue Number	Supplier
Rabbit anti-PAX6	NSC nuclear	1:200	60433S	Cell Signalling Technology
Rabbit anti-FOXP1	NSC nuclear	1:2000	ab196868	Abcam
Rabbit anti-Nestin	NSC cytoskeletal (intermediate filaments)	1:100	ab105389	Abcam
Mouse anti-OCT4	stem cell nuclear	1:100	sc-5279	Santa Cruz

Table 1. Primary antibodies.

63 Aspirate blocking buffer and add the primary antibodies (50 μ l per well for a 96 well plate, or 1ml per well for a 6 well plate). Incubate for 1 hour at room temperature. 1h
We recommend having a no primary control sample which are only stained with the secondary antibody. Add 1% DS to these wells during this step.

64 Prepare secondary antibodies (Table 2) in 1% DS and add DAPI at a dilution of 1:1000. Pipette to mix. Keep on ice in the dark until needed.

Antibody	Dilution	Catalogue Number	Supplier
Donkey Anti-Rabbit AF488	1:1000	A21206	Thermo Fisher
Donkey Anti-Mouse AF647	1:1000	A31571	Thermo Fisher
DAPI	1:1000	40043	Biotium

Table 2. Secondary antibodies.

65 Aspirate the primary antibody and wash 3 times with PBS with Calcium and Magnesium, leaving it on for 5 minutes at room temperature each time. 15m

- 66 Aspirate PBS and add the secondary antibodies (50µl per well for a 96 well plate, or 1 ml per well for a 6 well plate). Incubate in the dark (wrapped in foil or in a dark box) for 1 hour at room temperature. ^{1h}
- 67 Aspirate the secondary antibody and wash twice times with PBS, leaving it on for 5 minutes at room temperature in the dark each time. ^{10m}
- 68 Replace the PBS from the second wash with new PBS. At this point, the plate can be stored at **4 °C** in the dark. The fluorescence from the secondary antibodies will decrease with time. Analyse the staining using fluorescence microscopy or a high-content image scanner.



A successful assay would show that the NSCs are expressing PAX6, FOXG1 and Nestin. A lack of NSC marker expression or presence of OCT4 expression in the NSC population would indicate that the differentiation from iPSCs to NSCs did not work as expected.

If an iPSC control sample has been stained, this sample should be positive for OCT4 and negative for all NSC markers.

No staining should be visible in the no primary control wells. If there is, it is likely there is non-specific binding from the secondary antibodies and the staining observed may be false.