Generation of induced neurons from human induced pluripotent stem cells.

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We use this protocol and it's working

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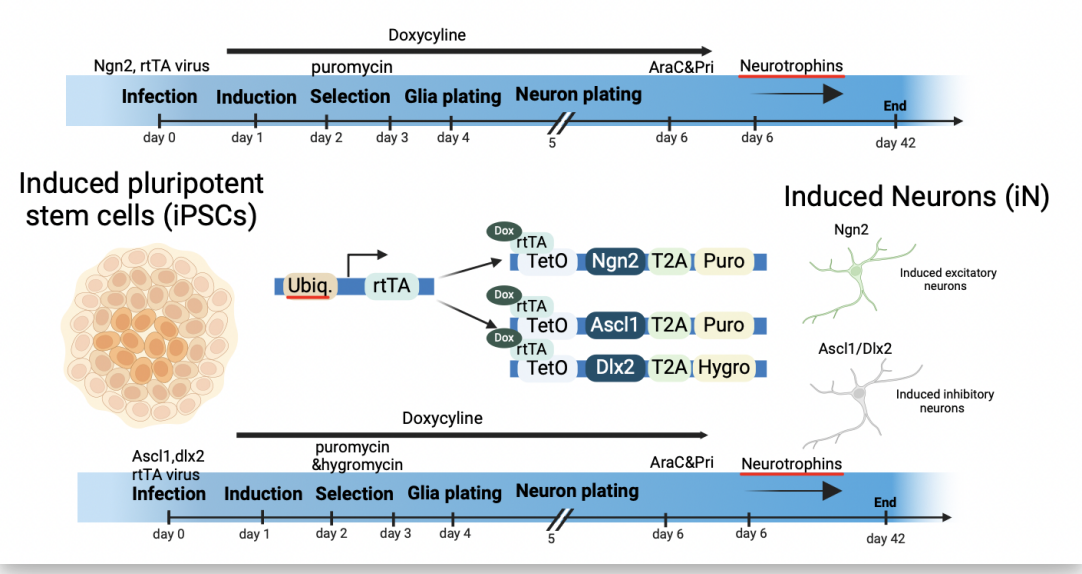
ABSTRACT



Overview of induced neuron protocol for the generation of excitatory and inhibitory neurons.

Protocol for the generation of Ngn2 (Excitatory) and Ascl1/Dlx2 (Inhibitory) induced neurons from human induced pluripotent stem cells (iPSCs) and co-culture of excitatory and inhibitory neurons on mouse glia (Protocols modified from Wang et al., 2022; Yang et al., 2017; Zhang et al., 2013).

iPSCs are maintained in 35mm plates or 6 well plates in mTESR+ media (https://dx.doi.org/10.17504/protocols.io.ewov1qd5ogr2/v1).

Lentiviral vectors were generated by transfecting HEK293T cells with lentivirus packaging plasmids (pMDLg/pRRE, VsVG and pRSV-REV) with the desired vectors as previously described (Pang et al., 2011) using lipofectamine 3000. The following plasmids were used: pMDLg/pRRE (Addgene 12251), pRSV-Rev (Addgene #12253), pCMV-VSV-G (Addgene #8454), FUW-M2rtTA (Addgene #20342), FUW-TetO-Ngn2-P2A-puromycin (Addgene #52047), FUW-TetO-Ascl1-T2A-puromycin (Addgene #97329), FUW-TetO-Dlx2-IRES-hygromycin (Addgene #97330). Lentiviral particles were collected in mTESR+ media and stored at -80°C until further use.

Induced neurons are generated by transducing iPSCs with the necessary genes using the lentiviral vectors to induce the expression of different transcription factors with doxycycline: rtTA + Ngn2 for excitatory neurons, rtTA + Ascl1 + Dlx2 for inhibitory neurons. Induced neurons are plated into 96 well plates (18×10^3 cells) or in 12 well plates (1×10^6 cells) with mouse glia after 5 days of induction. Primary mouse glia was obtained from postnatal day 0-2 mice cortex kept in DMEM 10% FBS 1% pen/strep, glia is used for plating after the second or third passage (P2-P3). CEPT cocktail was used during iPSC and neuronal plating to enhance cell viability (Chen et al., 2021). Induced neurons are cultured for 30-35 days before analysis.

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MATERIALS

	Reagent	Vendor	Catalog Number	Stock concentration	Working concentration
	Accutase	innovative cell technologies.inc	AT-104	1x	1x
	AraC	C1768	C1768	8mM	2-4 µM
	B27	Gibco	17504044	50x	1x
	BDNF	pepro tech	10781-164	250µg	10ng/mL
	CET/P	medchem express, selleck chem, R&D systems sigma-aldrich	HY-15392, S7775, 5284, P8483	C (50µM), E (5000µM), P(1x), T (0.7mM)	C (50nM), E (5uM), P(1x), T (0.7µM)
	DMEM	Gibco	11995-065	1x	1X
	Doxycycline	MP biomedicals	198955	2mg/mL	2µg/mL
	dPBS	SAFC	D8537	1x	1x
	FBS	r&d systems	S11550	100%	1x
	GDNF	Pepro tech	10781-226	200µg	10ng/mL
	glutamax	gibco	35050061	100x	1x
	hygromycin	sigma-aldrich	H9773	50mg/mL	100µg/mL
	matrigel	Cornig	354234		
	MEM	gibco	51200-038	1x	1x
	mTESR+	Stem cell technology	100-0275	1x	1x + supplement
	neurobasal	gibco	21103-049	1x	1x
	NT3	pepro tech	10781-174	250µg	10ng/mL
	penicilin/streptomycin	thermo-fisher	15070-63	100x	1x
	puromycin	sigma-aldrich	P8833	1mg/mL	1-2µg/mL
	Primocin	InvivoGen	ant-pm-1	50mg/mL	100µg/mL
	0.05% trypsin EDTA	gibco	25300-054	1x	1x
	12 well plate	falcon	353043	1mL/well	4cm^2

	Reagent	Vendor	Catalog Number	Stock concentration	Working concentration
	6 well plate	biofil	2304117-074-F	2mL/well	9cm^2
	96well plate	greiner	655090	200uL/well	0.32cm^2
	24well plate	biofil	230606-076-F	1mL/well	2cm^2

Media preparation

Neurobasal (neuronal for induction, selection , recovery)- 500mL neurobasal, 10mL B27 (0.5mM), 5mL glutamax supplement
Neurobasal + FBS (neuronal media for maintenance) - 500mL neurobasal, 10mL B27 (0.5mM), 5mL glutamax supplement, 25mL FBS (5%)
mTeSR+ (iPSC maintenance and infection) - 400uL mTeSR, 100mL mTeSR supplement, 1mL Primocin
DMEM - 500mL DMEM, 50mL FBS (10%), 5mL penecillin/streptomycin (1%)

**all media filtered during preparation.*

Infection of iPSCs - day 0

3h

1 Follow the below protocols for excitatory and inhibitory neuron generation.

STEP CASE

Excitatory neurons 46 steps

2 Warm up 1.5 mL of mTESR+ per cell line
Warm up 2.5 mL MEM per cell line
Warm up 1 mL of accutase per cell line
Thaw required volumes of NGN2 and RTTA virus on ice
Coat and incubate 12 well plates (1 well per line) with 500 µL of Matrigel per well for at least 01:00:00

3 Aspirate old media from confluent iPSC plate. Always use different tips to avoid contamination between the lines.

4 Wash with 500 µL MEM

5 Add 500 µL accutase, incubate at 37 °C and 5% CO_2 for 00:06:00 6m

6 Make sure all the cell colonies are suspended, transfer all cell from each well to 2 mL of MEM and centrifuge for 00:05:00 at 1000rpm at 23 °C . 5m

7 Add 1:1000 of CET/P (1 µL of CET and 1 µL of P per mL of media) to mTESR+, mix well

8 Add NGN2 and RTTA virus (1:1) to the 500 µL mTESR+ with CET/P per line, mix well (discard all tips and tubes in 10% bleach)

9 Aspirate excess Matrigel from well

10 Add 500 µL of virus+mTESR+ with CET/P to a labelled well.

11 Aspirate out the supernatant, and resuspend each line of cells will 1 mL mTESR+, make sure cells are resuspended well to ensure cells do not form large colonies for better infection.

12 Use 1:1 dilution of trypan blue to cell to count cells using an automatic cell counter. For a 12 well plate seed ~ 1x10^5 - 1.25x10^5 cells. For a 6 well plate seed ~ 2x10^5 - 2.5x10^5 cells. Add an appropriate volume of cell suspension to the labelled well(s), incubate at 37 °C and 5% CO_2

Induction - day 1

13 Warm up 1 mL of Neurobasal+B27+Glutamax per well of a 12 well plate.

14 Prepare induction media

Add 1:1000 of Doxycycline (1 µL). *2µg/mL stock solution, 2µg/mL working solution*

Add 1:1000 CET/P (1 µL of each). *2µg/mL stock solution, 2µg/mL working solution*

15 Remove media from the 12 well plate into 10% bleach (including all tips and tubes)

16 Add 1 mL of induction media (NB+Glut+B27+dox+CET/P) to each well. Incubate at 37 °C and 5% CO_2

Selection - day2&3

17 Prepare selection media (NB+Glut+B27+puro+dox). Warm up 1 mL neurobasal+glutamax+B27 per well of a 12 well plate

18 Add 1:1000 of Doxycycline(1 µL)
Add 1:1000 - 1:500 of puromycin (1 µL - 2 µL) - depending on the number of iPSCs

19 Aspirate out old media from the 12 well plate

20 Add 1 mL of selection media (NB+Glut+B27+puro+dox) to each well, Incubate at 37 °C and 5% CO_2

Recovery- day 4

21 Take out 1 mL of Neurobasal+glutamax+B27 per well of a 12 well plate

22 Add 1:1000 of Doxycycline (12 µL)

23 Aspirate out old media from 12 well plate

24 Add 1 mL of recovery media (NB+Glut+b27+dox) to each well, Incubate at 37^{O}C and 5% CO_2

Glia plating - day 4

25 Coat 96 well plates for sensor (5 wells per line) or high content imaging (8 wells per line) experiments with 1h 100 µL matrigel, 12 well plate for RNAseq experiment (1 well per line) with 500 µL materiel for 01:00:00

26 Warm up 5 mL trypsin, 10 mL of NB+B27+Glutmax+5% FBS (plating media), 3 mL of NB+B27+Glutmax+5% FBS (resuspension), and 5 mL DMEM+10%FBS+1%penecillin-streptomycin

500mL neurobasal, 10mL B27 (0.5mM), 5mL glutamax supplement, 25mL FBS

27 Select a confluent plate of P2/P3 mouse glia

28 Aspirate out old media from the glia, wash with 5 mL dPBS

29 Add 5 mL of trypsin, incubate for 00:05:00 at 37 °C and 5% CO_2 5m

30 Make sure all the cells are lifted and transfer the trypsin suspended cell to 5 mL of DMEM+10%FBS+1%p/s, centrifuge for 00:05:00 at 1000rpm at 23 °C . 5m

31 Aspirate out supernatant, and resuspend cells with 3 mL NB+B27+Glutmax+5% FBS

32 Count cell in 3 mL suspension, calculate volume for 8x10^3 - 1.2x10^3 cells per well of a 96well plate or 2.5x10^3 - 4x10^3 cells per well of a 12well plate.
(*number of glia seeded depends on if the cultures are P2 or P3 and how old the cultures are at the time of plating)*

33 Aspirate excess Matrigel from the wells.

34 Add the calculated volume of cell suspension required for plating in 100uL per well of 96well plate and 750 µL per well of a 12 well plate. Add cell volume to plating media. Mix well, add to well. Incubate at 37 °C and 5% CO_2

iN plating - day5

5m

35 Warm up 10 mL of Neurobasal+Glutamax+B27+5%FBS (plating media) per 96well plate (100 µL per well) or 12 well plate 750 µL per well).
Warm up 500 µL of Neurobasal+Glutamax+B27+5%FBS (plating media) per cell line for resuspension.
Warm up 500 µL of accutase per well
Warm up 3 mL of MEM for each well

36 Aspirate out old media from excitatory iNs well

37 Wash with 500 µL of MEM

38 Add 500 µL of accutase to each well, Incubate at 37^{O}C and 5% CO_2 for 00:06:00 6m

39 Transfer suspended cell into 2 mL of MEM in 15mL falcon tube, centrifuge for 00:05:00 at 1000rpm at 23 °C 5m

40 Resuspend each line in 500 µL

41 To count cells, mix 10 µL trypan blue with 10 µL cell suspension, add 10µl of diluted cells to one sides of the cell counter.

42 Calculate volume for 12x10^3 cells per well of a 96 well plate or 8.4x10^5 cells per well of a 12 well plate, add calculated volume to 500 µL plating media for each well. Make sure to label each well with the condition. Mix well. Incubate at 37 °C and 5% CO_2

43 Excitatory and inhibitory cells are co-cultured for all experiments mentioned above, check inhibitory neuron protocol for more details.

Day6

44 Change media to neuronal media - 100 µL for a 96 well plate or 750 µL of NB+Glut+B27+5%FBS + Factors per well of a 12 well plate

Factors: 1:1000 Doxycyline

1:1000 *100ug/mL* GDNF *10ng/mL working solution (1:10 dilution in dPBS)*

1:1000 *100ug/mL*BDNF. *10ng/mL working solution*

1:1000 *100ug/mL* NT3. *10ng/mL working solution*

1:2000 or 1:4000 8mM AraC 2uM-4u*M working solution* (based on glia density, only first feeding- either day6 or day8)

1:500uL of Primocin. 100u*g/mL working solution* (only first feeding - day6)

Incubate at 37 °C and 5% CO_2

Day8

45 Add 100 µL or 750 µL of neuronal media with factors (except for doxycycline and primocin) per well of a 96 well plate and a 12well plate, respectively.

NB+Glut+B27+5%FBS + Factors

Factors: 1:1000 GDNF

1:1000 BDNF

1:1000 NT3

1:2000 or 1:4000 AraC (based on glia density, only first feeding- either day6 or day8)

Incubate at 37 °C and 5% CO_2

Day 13, Day18, Day23, Day28, Day 33....

46 Discard half of the old media, replace with 100 µL or 750 µL media every 5 days (make sure outside wells are not evaporating media faster) per well of a 96well plate and 12 well plate, respectively.

1 mL NB+Glut+B27+5%FBS + Factors

Factors: 1:1000 GDFN

1:1000 BDFN

1:1000 NT3

Incubate at 37 °C and 5% CO_2

47 Maintain cultures for 30-35days