Version 1

Aug 19, 2022

iNDI Transcription Factor-NGN2 differentiation of human iPSC into cortical neurons Version 1 V.1

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Neurodegeneration Method Development Community | iNDI Protocol Development

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ABSTRACT

Induced pluripotent stem cell (iPSC)-derived neurons are an important tool for studying diverse types of neurodegenerative disorders, including Alzheimer's Disease, Parkinson's disease, and related dementias. Understanding the molecular and cellular mechanisms associated with these diseases is an important step in developing new therapeutic targets. Here we describe a robust differentiation protocol in which we expressed the human neurogenin 2 (NGN2) transcription factor under a tetracycline-inducible promoter as previously described (Fernandopulle et al. 2018), with several modifications and using a PiggyBac system for delivery. This differentiation protocol yields high percentages of cortical neuron markers.

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EXTERNAL LINK

https://www.jax.org/jax-mice-and-services/ipsc

PROTOCOL CITATION

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KEYWORDS

iNeurons, NGN2, Piggybac, cortical neuron differentiation, iNDI, Jackson Laboratory, CARD, NIH

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MATERIALS TEXT

Reagents

☒ Matrigel hESC-Qualified Matrix, LDEV-free **Corning Catalog #354277**

☒ KnockOut™ DMEM/F-12 **Thermo Fisher Catalog #12660012**

☒ N2 supplement (100x supplement) **Gibco, ThermoFisher Catalog #17502048**

☒ MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Catalog #11140050**

☒ Glutamax (100x) **Gibco - Thermo Fischer Catalog #35050-061**

☒ • Chroman I **MedChemExpress Catalog #HY-15392**

☒ Doxycycline hydrochloride **Sigma Aldrich Catalog #D9891**

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☒ PBS

1x **Lonza Catalog #BE17-516F**

☒ StemPro™ Accutase™ Cell Dissociation Reagent **Gibco - Thermo Fisher Catalog #A1110501**

☒ Poly-L-Ornithine (PLO) **Sigma Aldrich Catalog #P3655**

☒ Borate Buffered Saline **Sigma Aldrich Catalog #08059**

☒ BrainPhys™ Neuronal Medium **Stem Cell Technologies Catalog #05790**

☒ Recombinant human GDNF **peprotech Catalog #450-10**

☒ Recombinant Human/Murine/Rat BDNF **peprotech Catalog #450-02**

☒ Recombinant Human NT-3 **peprotech Catalog #450-03**

☒ Cultrex 3-D Culture Matrix Laminin I **R&D Systems Catalog #3446-005-01**

☒ Quality Bio Cell Culture Grade Water **Quality Biological Catalog #118-162-101CS**

☒ Uridine **Sigma Aldrich Catalog #U3003**

☒ 5-Fluoro-2'-deoxyuridine **Sigma Aldrich Catalog #F0503**

☒ N21-MAX Media Supplement (50X) **R&D Systems Catalog #AR008**

☒ Bovine Serum Albumin **Jackson Immunoresearch Catalog #001-000-173**

Medium Preparation

1 **Induction Medium:**
For day 0 to day 3

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A	B	C	D
Reagent	**Stock**	**Final concentration**	**Amount for 50mL of medium**
Knock out DMEM/F12	-------	------	48.5 mL
N2 supplement	100X	1X	0.5 mL
Non-essential amino acids (NEAA)	100X	1X	0.5 mL
Glutamax	100X	1X	0.5 mL
Doxycycline	2mg/mL	2µg/mL	0.05 mL
Chroman I	50 µM	50 nM	0.05 mL

Neuronal Maturation Medium:
For day 4 and 7

A	B	C	D
Reagent	**Stock**	**Final concentration**	**Amount for 50mL of medium**
Knockout DMEM/F12	-------	------	24mL
Brainphys	-------	-------	24mL
N21MAX	50X	1X	1mL
GDNF (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
BDNF (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
NT-3 (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
Laminin	6 mg/mL	1 µg/mL	0.01 mL
Doxycycline	2mg/mL	2µg/mL	0.05 mL

Neuronal Maturation Medium:
For day 10 to day 28

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A	B	C	D
Reagent	**Stock**	**Final concentration**	**Amount for 50mL of medium**
BrainPhys neuronal medium	----------	----------	49 mL
N21MAX	50X	1X	1 mL
GDNF (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
BDNF (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
NT-3 (in 0.1% BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
Laminin	6 mg/mL	1 µg/mL	0.01 mL
Doxycycline	2mg/mL	2µg/mL	0.05 mL

Differentiation Protocol 1h 45m

2 **Day 0**

The iPSCs with a stably integrated human NGN2 using PiggyBac system under a tetracycline-inducible promoter were exposed to doxycycline as follows:

2.1 Coat a well of 6 well plate or 10cm dish to be used for differentiation with 1h 30m **1 mL** or **4 mL** respectively of Matrigel solution, tilting to ensure coverage of entire surface area. Place in **37 °C** incubator for **00:30:00** to **01:00:00** .

Overnight *incubation gives better results*

2.2 Prepare Induction Medium and place in **37 °C** water or bead bath to warm during dissociation.

2.3 Observe iPSCs under a phase contrast microscope to assess confluency and presence of cell debris. Dish should be dissociated at ~70% to 80% confluency.

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2.4 Aspirate culture medium and wash with PBS 1X.

2.5 Aspirate PBS and add half of culture volume of Accutase

2.6 Transfer to **37 °C** incubator for **00:10:00** 10m

> *The time can vary by cell line and density (the optimal density is 70-80%) and the goal to use accutase is singularize as single cells.*

2.7 When Incubation is ready, tilt the plate and pipet the accutase solution two to three times up and down the culture surface to singularize as single cells.

2.8 Quench the Accutase adding half of the culture volume of PBS. Transfer to a new conical tube and rinse with more PBS the culture surface, combine with the cell solution in the tube.

2.9 Centrifuge **00:05:00** at 200 - 300 x g at **Room temperature** 5m

> *While centrifuge, aspirate Matrigel solution from plates and add Induction Medium.*

2.10 Aspirate supernatant and resuspend cell pellet with Induction Medium.

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2.11 Count cells, Gently transfer 0.5-1 x 10^6 iPSCs per one well of 6-well plate in 2-3 mL of Induction Medium or 4-6 × 10^6 per 10-cm dish in 10-12 mL to be differentiated.

2.12 Gently rock plate to evenly distribute cells and place in **37 °C** incubator.

3 Day 1

Check cells under the microscope, nascent neuritic extensions should begin to be evident after 24 h of doxyxycline exposure.

3.1 Prepare Induction Medium but without Chroman I and warm it.

3.2 Aspirate medium, wash once with PBS 1X and replace with warm induction medium.

4 Day 2

Check cells under the microscope, neuritic extensions should be more evident.

4.1 Repeat medium change with induction medium as on day 1.

5 Day 3

Check cells under a microscope. Neurites should be obvious by this time.

5.1 1. Repeat medium exchange with induction medium + ***Uridine and Fluorodeoxyuridine (FdU)*** *both at* **1 micromolar (µM)** .

When using PiggyBac system for hNGN2 the culture might have some mitotic cells, and to suppress them, we add to the neuronal maturation

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medium, Uridine and Fluorodeoxyuridine (FdU) both at 1 micromolar (µM) from day 3 to 28.

Differentiation Protocol 1h 45m

6 Day 4

Check cells under a microscope. Pre-differentiated neurons are ready to be re-plated.

6.1 Coating dishes 1h

Freshly prepared poly-L-ornithine (PLO), at final concentration at [M]**0.1 mg/mL** :

- Using Sodium Borate Buffer pH 8.2, make a [M]**1 mg/mL** stock PLO solution.
- To prepare working solution dilute to a [M]**0.1 mg/mL** with cell culture water then filter through a 0.22µm sterile filter and it is ready to use.
- Add half of the culture volume of PLO working solution to dishes and Place in **37 °C** incubator for **01:00:00** to **Overnight** .
- Aspirate PLO working solution from the dishes.
- Wash dishes with cell culture water three times.
- Let dry completely in a culture hood.
- Dishes are ready to use.

7 Plating pre-differentiated neurons day 4

Once cells are confirmed to be healthy, they should be dissociated with Accutase to re-plated onto final dishes for neuronal maturation and experimental manipulation

7.1

Prepare fresh Neuronal Maturation Medium + *Uridine and Fluorodeoxyuridine (FdU) both at* 1 micromolar (µM)

7.2

After dissociating cells with Accutase as step 2.4 to 2.9 resuspend cell pellet with Neuronal Maturation Medium *+ Uridine and Fluorodeoxyuridine (FdU) both at* 1 micromolar (µM) and count.

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7.3 Plate 2 x 10^6 pre-differentiated neurons onto a PLO-coated 6 well with **3-4 mL** of Neuronal Maturation Medium *+ Uridine and Fluorodeoxyuridine (FdU) both at* 1 micromolar (µM) .

The number of pre-differentiated neurons to be re-plated varies depending of the final assay but it can be as follows:

- *384 well plate (imaging) 10,000 in 100 µL medium/well.*
- *96 well plate (imaging) 6 x 10^4 in 300 µL medium/well.*
- *12 well plate (Biochemistry) 0.75 to 0.85 x 10^4 in 2 mL medium/well.*
- *6 well plate (Biochemistry) 2 x 10^6 in 3-4 mL medium/well*
- *10 cm dish 8 to 10 x 10^6 in 10- 12 mL medium.*

7.4 After day 4 do half of the medium change every 3-4 days with Neuronal Maturation Medium + *Uridine and Fluorodeoxyuridine (FdU) both at* 1 micromolar (µM) .

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