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Differentiation of iPSCs with the NGN2 construct into cortical neurons protocol

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NGN2 Day 0 Differentiation

Overview: Replate cells and induce them today

1. Matrigel coat plates
2. Prepare Day 0 NGN2 Differentiation media
3. Dissociate iPSCs with Accutase, quenching with 1X DPBS at a volume 5 times that of Accutase used
4. Spin down cells at 800 rpm for 3 minutes
5. Resuspend cells in mTeSr+ with 10 mM ROCK inhibitor (1:1000 dilution)
6. Count cells: calculate desired volume
    a. (Number of cells you want (cells)) / (number of cells from countess (cells/mL))
    b. 4 million cells in a 10-cm dish
    c. 677.3k cells in a well of a 6-well plate
7. Aliquot the needed volume of cells depending on the number of cells needed into another conical vial
8. Spin down cells at 800 rpm for 3 minutes
9. Resuspend in Day 0 NGN2 Differentiation media
10. Plate induced cells (2 mL/well in a 6-well plate)

NGN2 NIM (induction) media:

50 ml KO DMEM/F12	
0.5 ml NEAA (non-essential amino acids)	1:100 dilution
0.5 ml N2	1:100 dilution

NGN2 Day 0 media:

NGN2 NIM	
ROCK inhibitor (10 mM)	1:1000 dilution
Dox	1:1000 dilution
BDNF	1:2000 dilution
NT3	1:2000 dilution
laminin (1mg/ml stock laminin)	1:1000 dilution

NGN2 Day 1 Differentiation

Overview: Feed the cells (2 mL/well)

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NGN2 Day 1 media

NGN2 NIM

Dox	1:1000 dilution
BDNF	1:2000 dilution
NT3	1:2000 dilution

laminin (1mg/ml stock laminin) 1:1000 dilution

1. Prepare Day 1 NGN2 Differentiation media
2. Replace d0 media with d1 media, using a full media change

NGN2 Day 2 Differentiation

1. Coat 1X PDL plates (D2) or ensure that you have a pre-PDL coated plates

NGN2 Day 3 Differentiation

Overview part 1: Replating the neurons

1. If using pre-PDL coated plates, skip to step 4
2. Aspirate PDL solution from the plate and wash 3x with PBS.
3. Let the plate dry completely in the hood (typically takes 30 min – 1 hr).
4. While plates are drying, make necessary media

NGN2 NMM

50 mL DMEM/F12

50 mL Neurobasal-A

1 mL NEAA	1:100 dilution
1 mL GlutaMAX	1:100 dilution
1 mL N2	1:100 dilution
2 mL B27	1:50 dilution

NGN2 D3 Media

NGN2 NMM

Dox	1:1000 dilution
BDNF	1:2000 dilution
NT3	1:2000 dilution

laminin (1mg/ml stock laminin) 1:1000 dilution

Overview part 2: Seeding NGN2 D3 neurons

1. Wash the differentiating cells with PBS
2. Treat the cells with Accutase
    a. Use 2 mL/dish for a 10 cm dish and 0.5 mL/well for a 6-well plate.
3. Incubate the cells at 37°C for 5 min.
4. Quench Accutase with PBS (2.5 times the volume of Accutase).
5. Gently triturate 4-5 times to break the cells apart. Transfer the PBS + Accutase + cell solutions to 15mL falcon tubes to spin.

6. Centrifuge the cells at 200 x g for 5 min.
7. Aspirate the supernatant and resuspend in 6 mL of NGN2 Day 3 media.
8. Count the cells using the Countess™.
    a. (Number of cells you want) / (number of cells from countess)
9. Add the appropriate number of cells into a tube. Then add the appropriate number of media into each tube to bring it up to the desired total volume.
    a. 96 well plates: 10,000 cells/well (recommended)
    b. 6 well plates: 500,000 cells/well (recommended)
10. If using pre-PDL coated plates only: rehydrate the receiving plate once with 1X PBS by washing it once with 1X PBS
11. Plate the cells into the appropriate plates, from the appropriate tubes.

NGN2 Day 7 Differentiation

NGN2 D7 Media

NGN2 NMM
BDNF 1:2000 dilution
NT3 1:2000 dilution
laminin (1mg/ml stock laminin) 1:1000 dilution

1. Prepare the Day 7 media
2. Perform the full media change (2 mL/well if using a 6-well plate)

NGN2 Day 10 Differentiation

NGN2 D10 Media

NGN2 NMM
BDNF 1:2000 dilution
NT3 1:2000 dilution
laminin (1mg/ml stock laminin) 1:1000 dilution

1. Prepare the Day 10 media
2. Perform the half media change (2 mL/well if using a 6-well plate)

Item	Manufacturer	Catalog Number
KnockOut DMEM/F-12	ThermoFisher Scientific	12660012
Growth Factor Reduced Matrigel	Corning	356231
mTeSR™ Plus and supplement	Stemcell Technologies	1000276
Gibco™ DPBS, no Ca, no Mg	ThermoFisher Scientific	14-190-235
Accutase	Stemcell Technologies	07920
DMEM/F-12, HEPES	ThermoFisher Scientific	11330032
N-2 Supplement	ThermoFisher Scientific	17502048
MEM Non-Essential Amino Acids Solution (NEAA)	ThermoFisher Scientific	11140050
GlutaMAX Supplement	ThermoFisher Scientific	35050061

B-27 Supplement, serum free	ThermoFisher Scientific	17504044
Poly-D-Lysine (PDL)	ThermoFisher Scientific	A3890401
Laminin Mouse Protein, Natural	ThermoFisher Scientific	23017015
Doxycycline Hyclate (reconstituted in water)	Millipore Sigma	D3447
ROCK1 Inhibitor (Y-27632 2HCl)	Selleckchem	S1049
Neurobasal-A	ThermoFisher Scientific	10888022
Recombinant Human BDNF	Peprotech	450-10
Recombinant Human NT3	Peprotech	450-03

Table 1: Items needed for the creation of the media and reagents used in the differentiation of NGN2 iPSCs into cortical neurons.

Notes on some items that need to be reconstituted

Doxycycline: diluted in cell culture grade water to 2 mg/mL and stored at -20°C (long-term storage) or 4°C (short-term storage), minimizing exposure to light.

BDNF and NT3: 50 µg reconstituted in filtered 1X DPBS with 0.1% BSA, then aliquoted and stored at -20°C for up to 3 months.

Equipment	**Manufacturer**	**Catalog Number**
Falcon® 96-well Black/Clear Flat Bottom TC-treated Imaging Microplate with Lid	Corning	353219
Corning™ BioCoat™ 96-Well, Poly-D Lysine-Treated, Flat-Bottom Microplate	Corning	354640
Eppendorf® Centrifuge 5810/5810R	Millipore Sigma	EP022628168
Invitrogen Countess™ II automated cell counter	ThermoFisher Scientific	AMQAX1000

Table 2: Equipment used in the differentiation of NGN2 iPSCs into cortical neurons.