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Cortical neuron differentiation using forced NGN2 expression

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Protocol status: Working
We use this protocol and it's working

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Abstract

This is a modified protocol based on (Zhang et al.2013 and Meijer et al., 2019) to generate cryopreserved NGN2 neurons, using hPSCs that carry a doxycline-inducible NGN2 cassette.

The voluming are calculated for 10cm TC dishes and should be adapted accordingly

Materials

Media:

KSR medium

	500ml	250ml	50ml	Reagent
	415ml	207.5ml	41.5ml	KO DEMEM
	75ml	37.5ml	7.5ml	KO SRM
	5ml	2.5ml	0.5ml	GlutaMax, NEAA, P/S
	0.5ml	0.25ml	0.05ml	ß-mercapto ethanol

N2B medium

	500ml	250ml	50ml	Reagent
	480ml	240ml	48ml	DMEM F12 + HEPES
	5ml	2.5ml	0.5ml	GlutaMax, N2, P/S
	7.5ml	3.75ml	0.75ml	20% Dextrose

D2 N2B w/o B27 supplement
D3 N2B 1:100 B27 supplement

NBM medium

	500ml	250ml	50ml	Reagent
	485ml	242.5ml	48.5ml	Neurobasal
	5ml	2.5ml	0.5ml	GlutaMax, P/S
	7.5ml	3.75ml	0.75ml	20% Dextrose
	10ml	5ml	1ml	B27 supplement
	2.5ml	1.25ml	0.25ml	NEAA

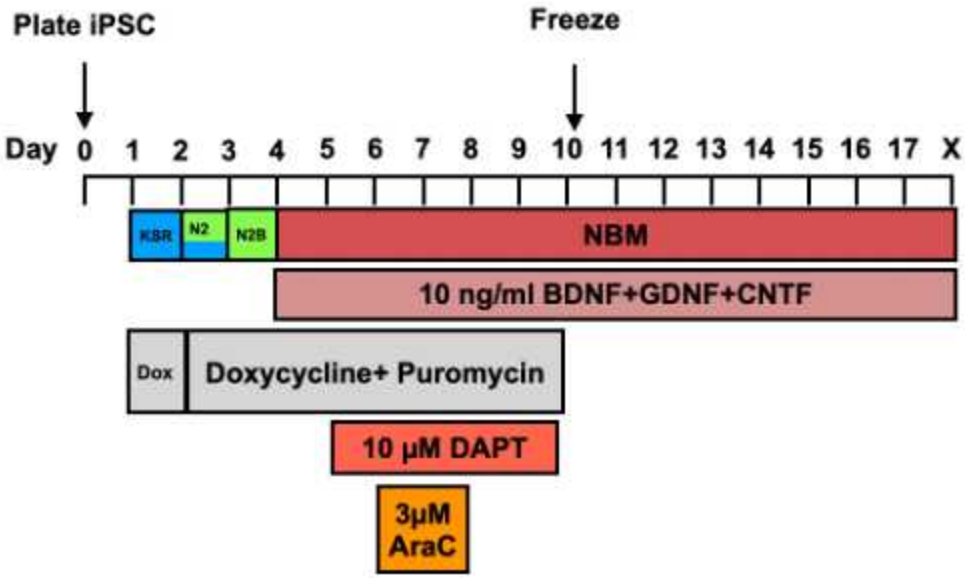
Consumables: **For preparation of Growth factors check Cell culture recipes sheet**

	A	B	C	D	E	F
	Reagent	Vendor	Cat no.	Stock con.	Working conc.	Dilution
	Neurobasal medium	Thermofisher Scientific	#21103049	n.a.	n.a.	n.a.
	Knock DMEM	Gibco	#10829-018	n.a.	n.a.	n.a.
	KnockOut Serum Replacement	Invitrogen	10828-028	n.a.	n.a.	n.a.
	MEM non-essential amino acids	Invitrogen	11140-050	n.a.	n.a.	n.a.
	Beta-mercaptoethanol	Invitrogen	21985-023	n.a.	n.a.	n.a.
	GlutaMax	Invitrogen	35050061	n.a.	n.a.	n.a.
	DMEM F12 HEPES	Gibco	11330057	n.a.	n.a.	n.a.
	N2 supplement	Invitrogen	17502048	n.a.	n.a.	n.a.
	20% Dextrose			20%	n.a.	n.a.
	Penicillin/Streptomycin	Invitrogen	15140122	100x	1x	1:100
	Trehalose	Sigma	#T9531-25G	n.a.	n.a.	n.a.
	DMSO	Sigma	#D8418-250M	n.a.	n.a.	n.a.
	mTeSR plus	Stem cell Technologies	# 100-0276	n.a.	n.a.	n.a.
	Matrigel (Differentiation)	Corning	354234	n.a.	n.a.	n.a.
	Dnase	NEB	#M030	n.a.	n.a.	n.a.
	BDNF	Peprotech	#450-02	10µg/ml	10ng/ml	

	A	B	C	D	E	F
	GDNF	Peprotech	#450-10	10µg/ml	10ng/ml	
	CNTF	Peprotech	# 450-13	10µg/ml	10ng/ml	
	DAPT	Fisher Scientific	#26-341-0	10mM	10µM	
	Doxycyclin Hyclate	Sigma	D9891-5g	2mg/ml	2µg/ml	
	Puromycin	Life technology	A11138-03	10mg/ml		
	Accutase	Gibco	#A11105			
	Y27632 ROCK Inh.	Stem cell technologies	#72304	10mM	10µM	
	Cell strainer (40µm)	Falcon/Corning	#352340	n.a.	n.a.	n.a.
	B27 Supplement	Invitrogen	17504044	n.a.	n.a.	n.a.

Overview

1



Differentiation overview

D0

2 Seed 4×10^6 single cells onto 1×10 cm dish (coating MG-GFR or with GF 2mg) in iPSC medium supplemented with 10 µM RI in 7ml medium

D1

3 Add 8ml KSR with 2µg/ml DOX

D2

4 9ml 1:1 KSR/N2B w/o B27 2µg/ml DOX and 5µg/ml Puro

Ensure 24h window between this media change and the previous one

D3

5 10ml N2B containing 2µg/ml DOX) and 5µg/ml Puro

D4

6 10 ml NBM containing 2µg/ml DOX, 5µg/ml Puro, BDNF 10µg/ml , GDNF 10µg/ml , CNTF 10µg/ml

D5

7 10 ml NBM containing 2µg/ml DOX, 5µg/ml Puro, BDNF 10µg/ml , GDNF 10µg/ml , CNTF 10µg/ml and 10µM DAPT

D6 AraC treatment

8 10 ml NBM 2µg/ml DOX, 5µg/ml Puro, BDNF 10µg/ml, GDNF 10µg/ml, CNTF 10µg/ml, 10 µM DAPT
add 3 µM AraC varies between cell lines

! Discard AraC trash according to environmental health guidelines!

D7 AraC treatment

9 10 ml NBM 2µg/ml DOX, 5µg/ml Puro, BDNF 10µg/ml, GDNF 10µg/ml, CNTF 10µg/ml, 10 µM DAPT
add 3 µM AraC varies between cell lines

! Discard AraC trash and used medium according to environmental health guidelines!

D8

10 2x wash with 5ml NBM w/o GF to remove AraC completely
add 8-10 ml NBM 2µg/ml DOX, 5µg/ml Puro, BDNF 10µg/ml, GDNF 10µg/ml, CNTF 10µg/ml and
10 µM DAPT
! Discard AraC trash and used medium according to environmental health guidelines!

D9

11 **add 8-10 ml** NBM 2µg/ml DOX, 5µg/ml Puro, BDNF 10µg/ml, GDNF 10µg/ml, CNTF 10µg/ml and
10 µM DAP

D10 Cryopreservation

12 remove medium

12.1 1x wash with 4 mL l PBS

12.2 add 4 mL Accutase supplemented with 10µM RI for 90-120min at 37 °C

12.3 After incubation time; If needed add 200 µL DNAse I

12.4 Add 3 mL NBM and dissociate using 10 ml serological pipette

12.5 Pool 3×10cm dishes and collect cells in 1×50 ml falcon after pipetting through a 40µm cell strainer on Ice

!collect all cells in a T175 TC falsk on ice!

12.6 Rinse dishes with 3 ml NBM

12.7 Take a sample from the flask to determine cell number
!Always store cells suspension on ice from now on!

12.8 Aliquot pooled cells into 15 ml falcons and centrifuge at 300g for 5min at 4˚C

12.9 Freeze cells in 1.5M or 3M aliquots and store at -80˚C overnight and **transfer the next day** into the liquid nitrogen tank

Freezing medium:
70% KOSRM
20% 1M Trehalose
10% DMSO