[Protocol 3.02] iNGN protocol

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Protocol 3.02 iNGN protocol
Version: 1.0 (22.02.2023)
According to the Busskamp paper

Media and Reagents:
TrypLE™ Select Enzyme (1X), no phenol red (12563011, Thermo Fisher) ***or***
StemPro™ Accutase™ Cell Dissociation Reagent (A1110501, Thermo Fisher)
DMEM/F-12, HEPES (11330-032, Thermo Fisher)
DPBS, no calcium, no magnesium (14190250, Thermo Fisher)
doxycycline (D3447-500MG, Sigma-Aldrich)
Antibiotic-Antimycotic (100x) (15240-062, Life Technologies (GIBCO))
puromycin (4089, tocris)
hygromycin (10687010, Thermo Fisher)
Neurobasal Medium (21103-049, Thermo Fisher)
N2 supplement (100x) (17502-048, Thermo Fisher)
MEM Non-Essential Amino Acids Solution (100X) (11140-035, Thermo Fisher)
B27 supplement (50x) (17504-044, Thermo Fisher)
GlutaMAX Supplement (35050061, Thermo Fisher)
hBDNF (450-02, peprotech)
hNT-3 (267-N3-025, R&D systems)

Virus information is added below

Materials and Equipment:
6/12/24 well plates
10ul/100ul/200ul/100ul pipettes & pipette tips
5ml/10ml pipettes
Cell counting equipment
15/50 ml Falcon tube
1,5 ml sterile Eppis

1. Introduction and Purpose
This protocol describes the protocol using two Neurogenin (NGN1/NGN2) induction to drive iPS cells rapidly and homogeneously to bipolar neurons (4 days).

Note: Every species acquire different media compositions, but while running the iNGN protocol, mTeSR1 media will be used independent of species
Note: Prior to running this protocol, running at least two tests on each individual is recommanded
- Virus titer test: Estimate how many virus units are needed
- Antibiotic concentration test: Estimate what concentration of puromycin and hygromycin is needed for the most optimal selection

2. Protocol

Day -2: seed cells for neuronal differentiation
- Aspirate medium
- Add 1ml of TrypLE/Accutase per 1x6 well
- Incubate 6-8 min at 37°C
- Resuspend and add 2 ml of E8 home/mTeSR1/StemMACS+UPPS depending to the species
- Spin at 1000 rpm for 3-5 min
- Aspirate the supernatant
- Resuspend in desired media + ROCK inhibitor
- Count
- Dilute cell suspension
- Aspirate GelTrex from the new coated wells
- Seed cells with desired media + ROCK inhibitor, e.g. 1x6 well: seed 200.000 cells/well (next day around **40 - 50 %** confluent)

Day -1: virus infection
- Prepare 2 ml of mTesR1 + viruses per 1x6 well
- Change media, preferably in the morning. So, media without viruses can be changed in the afternoon in case the viral infection is too cruel.
- Standard amount of viruses: x µl Ngn1/2, x µl rtTA per 1x6 well (Define the optimal virus titer if necessary)

Day 0: medium change and dox activation

- Aspirate media and replace with mTesR1 + Dox (0.5 µg/ml)
- Add dox always fresh to the media and keep protected from light! (The dox aliquot)
- The thawed dox aliquot can be stored at 4°C for one week

Day 1: medium change
- Aspirate medium and replace with mTesR1

Day 2: medium change
- Aspirate medium and replace with mTesR1

Day 3: medium change
- Aspirate medium and replace with mTesR1

Day 4: adding the mouse astrocytes
- Detach mouse astrocytes using trypsin, spin, resuspend in NB-B27, 1x T75 for 72 x 24 wells / 14x 6wells-suck up medium, add medium containing glias enhancing synapse formation
- **OR** use the conditioned media from mouse glia

Day 5 - : medium change
- Aspirate medium and replace with NB-B27

3. Media preparation

F12-N2 media:

Component	Stock concentration	Final concentration	50 ml
DMEM/F12			48.9 ml
N2 supplement	100 x	1 x	0.5 ml
NEAA	100 x	1 x	0.5 ml
hBDNF	10 µg/ml	10 ng/ml	50 µl
hNT-3	50 µg/ml	10 ng/ml	10 µl
laminin	1 mg/ml	0.2 µg/ml	10 µl
doxycycline	2 mg/ml	3 µg/ml	add directly before use

NB-B27 media:

Component	Stock concentration	Final concentration	50 ml
Neurobasal medium			48.5 ml
B-27 supplement	50 x	1 x	1 ml
GlutaMAX	100 x	1 x	0.5 ml
hBDNF	10 µg/ml	10 ng/ml	50 µl
hNT-3	50 µg/ml	10 ng/ml	10 µl
laminin	1 mg/ml	0.2 µg/ml	10 µl
~~AraC~~	~~4mM~~	~~2µM~~	~~Add only at day 4 (1:2000)~~
doxycycline	2 mg/ml	3 µg/ml	add directly before use

4. Confirmation of neuronal differentiation
1. Immunofluorescence imaging - MAP2 & Tuj1 (pan neuronal markers)
2. ddPCR with target gens, e.g. SOX1, PAX6, MAP2, TUBB3, NGN2, ZEB2

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