# [Protocol 2.02] NGN2 protocol

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Protocol 2.02 NGN2 protocol
Version: 1.0 (25.05.2022)
According to the Südhof paper

**Media and Reagents:**
DMEM/F-12, HEPES (11330-032, 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)
Laminin (L2020, Sigma-Aldrich)
doxycycline (D3447-500MG, Sigma-Aldrich)
puromycin (4089, tocris)
Virus
DPBS, no calcium, no magnesium (14190250, Thermo Fisher)
DPBS, calcium, magnesium (14040091, Thermo Fisher)

**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 converting human iPSCs into functional iN cells with nearly 100% yield and purity in less than 2 weeks by forced expression of a single transcription factor, NGN2. The resulting ES-iN or iPS-iN cells exhibit quantitatively reproducible properties independent of the cell line of origin, form mature pre- and postsynaptic specializations.

**2. Protocol**

**Day -2: seed cells for neuronal differentiation**
- Aspirate medium
- Add 1ml (6er well) /3ml (10 cm) Accutase
- Incubate 6-8 min at 37°C
- Resuspend and add 10 ml E8 home
- Spin: 1000 rpm for 3-5 min
- Resuspend in E8 home + ROCK inhibitor
- Count
- Dilute cell suspension
- Aspirate matrigel
- Seed cells, e.g. 6-well: seed 200.000 cells/well (next day around **40 %** confluent)

**Day -1: virus infection**
- Mix E8 and viruses
- Viruses: 4ul Ngn+GFP + 3ul rtTA per 6-well (Define the optimal virus titer if necessary)

**Day 0: medium change and dox activation**
- Aspirate medium and replace with F12-N2 medium + 3 µg/ml of dox
- Add dox always fresh to the media! (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 F12-N2 medium + 3 µg/ml of dox

**Day 2: puromycin selection**

- Aspirate medium and replace with F12-N2 medium + 3 µg/ml of dox
- add 0.8 ug/ml puromycin (stock: 10 mg/ml 1:12.500) to F12-N2 medium

**Day 3: adding the mouse glia**

- Detach glias 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

**Day 5 - 14: media change**

- change 50% medium NB-B27 every other day

**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. qPCR