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Version 2

Neurogenesis with iron supplementation and label-free nDIA proteomics V.2



Version 1 is forked from [Neural differentiation of AAVS1-TRE3G-NGN2 pluripotent stem cells](#)

Neural differentiation
of AAVS1-TRE3G-NGN2
pluripotent stem cells

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We use this protocol and it's working

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Abstract

This protocol describes the NGN2-driven differentiation of H9 stem cells into iNeurons ± FAC iron supplementation. The protocol also covers sample preparation of label free proteomics and analysis via nDIA on the Astral Orbitrap mass analyzer.

Attachments



Neural_differentiati...

74KB

Safety warnings

! For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Before start

Prepare ND1 Medium and ND2 Medium:

ND1 Medium

DMEM/F12

N2 (100x)	1x
BDNF	10 ng/ml
NT3	10 ng/ml
NEAA (100X)	1x
Laminin	0.2 µg/ml
Doxycycline	2 µg/ml

ND2 Medium

Neurobasal medium

B27 (50x)	1x
Glutamax (100x)	1x
BDNF	10 ng/ml
NT3	10 ng/ml
Doxycycline	2 µg/ml

Note

- * Doxycycline can be withdrawn on Day 10 (or even earlier-not tested yet).
- * Cells can be split from Day 5 to Day 7 (or even earlier-not tested yet).



Neuronal differentiation - Day 0:

- 1 Treat H9 AAVS1-TRE3G-NGN2 cells with Accutase and plate the dissociated cells in matrigel-coated 6-well plates (2×10^5 cells/well) in **ND1 Medium** supplemented with Y27632 ([M] 10 micromolar (μM)).
- 2 Harvest samples for day 0. Wash wells twice in 1xPBS and harvest in ice-cold 1xPBS on ice. Snap freeze samples and store at -80°C .

Day 1:

- 3 Replace the medium with **ND1 Medium**.
For iron-addback samples, supplement media with $10\mu\text{M}$ FAC

Day 2:

- 4 Replace the medium with **ND2 Medium**.
For iron-addback samples, supplement media with $10\mu\text{M}$ FAC

Day 4:

- 5 Exchange 50% of the medium from each well.
For iron-addback samples, supplement media with $10\mu\text{M}$ FAC
- 6 Harvest samples for day 4. Wash wells twice in 1xPBS and harvest in ice-cold 1xPBS on ice. Snap freeze samples and store at -80°C .

Day 6:

- 7 Treat the cells with Accutase and replat the dissociated cells in matrigel-coated 6-well plates ($2-4 \times 10^5$ cells/well) in **ND2 Medium**.
For iron-addback samples, supplement media with $10\mu\text{M}$ FAC

Day 8

- 8 Exchange 50% of the medium from each well every other day.
For iron-addback samples, supplement media with $10\mu\text{M}$ FAC



Note

- * Doxycycline can be withdrawn on Day 10 (or even earlier-not tested yet).
- * Cells can be split from Day 5 to Day 7 (or even earlier-not tested yet).

- 9 Harvest samples for day 8. Wash wells twice in 1xPBS and harvest in ice-cold 1xPBS on ice. Snap freeze samples and store at -80°C.
- 10 **Day 10:** Exchange 50% of the medium from each well.
For iron-addback samples, supplement media with 10µM FAC
- 11 **Day 12:** Exchange 50% of the medium from each well.
For iron-addback samples, supplement media with 10µM FAC
- 12 **Day 14:** Exchange 50% of the medium from each well.
For iron-addback samples, supplement media with 10µM FAC

Day 16

- 13 **Day 16:** Exchange 50% of the medium from each well.
For iron-addback samples, supplement media with 10µM FAC
- 14 Harvest samples for day 16. Wash wells twice in 1xPBS and harvest in ice-cold 1xPBS on ice. Snap freeze samples and store at -80°C.
- 15 **Day 18:** Exchange 50% of the medium from each well.
For iron-addback samples, supplement media with 10µM FAC
- 16 **Day 20:** Exchange 50% of the medium from each well.
For iron-addback samples, supplement media with 10µM FAC

Day 21

- 17 Harvest samples for day 21. Wash wells twice in 1xPBS and harvest in ice-cold 1xPBS on ice. Snap freeze samples and store at -80°C.

Sample Prep for LFQ



- 18 Cell pellets are lysed in 140 μ L of 8 M Urea and 100 mM Tris (pH 8.0) using a syringe equipped with a 21G needle.
- 19 The protein concentration is measured using a protein BCA assay.
- 20 10 mM TCEP is added to the lysate for protein reduction.
- 21 40 mM chloroacetamide is added to the lysate for protein alkylation.
- 22 LysC (from FUJIFILM Wako) is added to the lysate at a protein-to-LysC ratio of 50:1.
- 23 The sample is incubated on a rocker for 4 hours at room temperature.
- 24 The urea concentration in the sample is diluted to 2 M by adding 100 mM Tris (pH 8.0).
- 25 Trypsin is added to the sample at a protein-to-trypsin ratio of 50:1.
- 26 The sample is incubated on a rocker overnight at room temperature to facilitate digestion.
- 27 Digested peptides are desalted using Waters 25 mg Sep-Pak tC18 96-well plates (Cat# 186002319).
- 28 The concentration of the desalted peptides is measured using a peptide BCA assay.

label-free nDIA proteomics

- 29 Separation is performed on a C18 capillary column (30 cm length $\times$ 100 μ m inner diameter) packed with Accucore C18 resin (2.6 μ m, 150 Å, Thermo Fisher Scientific) at a temperature of 55 °C using an EASY-nLC 1200 system (Thermo Scientific).
- 30 The flow rate is set to 450 nL/min.

- 31 Mobile phase A is prepared as 0.125% formic acid in ACN/water (5:95, v/v).
- 32 Mobile phase B is prepared as 0.125% formic acid in ACN/water (95:5, v/v).
- 33 Peptides are loaded onto the column, and the gradient of mobile phase B is increased from 5% to 23% over 22 minutes.
- 34 The gradient is further increased to 100% over 2 minutes and maintained at 100% for 6 minutes.
- 35 Eluting peptides are analyzed using an Orbitrap Astral mass spectrometer (Thermo Scientific).
- 36 Spray voltage is set to 2.2 kV.
- 37 The ion transfer tube temperature is maintained at 290 °C.
- 38 The FAIMS compensation voltage (CV) is set to -55 V.
- 39 The MS1 scan range is set from 380 to 980 m/z.
- 40 The MS1 resolution is set to 240,000 (at 200 m/z).
- 41 The source RF is configured at 50%.
- 42 The MS1 AGC target is set to 500%.
- 43 The MS1 injection time is set to 50 ms.



- 44 Precursors are isolated in the quadrupole using an isolation width of 2 m/z.
- 45 Fragmentation is performed by higher-energy collisional dissociation (HCD) with a normalized collision energy (NCE) of 27%.
- 46 MS2 mass spectra are acquired in data-independent mode using the Astral analyzer.
- 47 The MS2 scan range is set from 110 to 2,000 m/z.
- 48 The MS2 AGC target is configured at 500%.
- 49 The MS2 maximum injection time is set to 3 ms.
- 50 Raw files were converted to mzML format using MSConvert and analyzed using FragPipe (v22.0) with DIA-NN (v1.8.2). All settings were kept default.