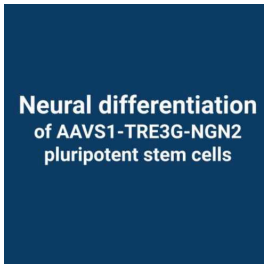
Oct 31, 2024 Version 1

Neurogenesis with iron supplementation and label-free nDIA proteomics V.1



Forked from Neural differentiation of AAVS1-TRE3G-NGN2 pluripotent stem cells

In 1 collection

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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* Doxycycline can be withdrawn on Day 10 (or even earlier-not tested yet).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 10µM FAC

Day 2:

4 Replace the medium with **ND2 Medium**.
For iron-addback samples, supplement media with 10µM FAC

Day 4:

5 Exchange 50% of the medium from each well.
For iron-addback samples, supplement media with 10µM 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 replate the dissociated cells in matrigel-coated 6-well plates (2-4×10^5 cells/well) in **ND2 Medium**.
For iron-addback samples, supplement media with 10µM FAC

Day 8

8 Exchange 50% of the medium from each well every other day.
For iron-addback samples, supplement media with 10µM 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 µL 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 × 100 µm inner diameter) packed with Accucore C18 resin (2.6 µm, 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 MSConvert60 and analyzed using FragPipe (v22.0) with DIA-NN (v1.8.2). All settings were kept default.