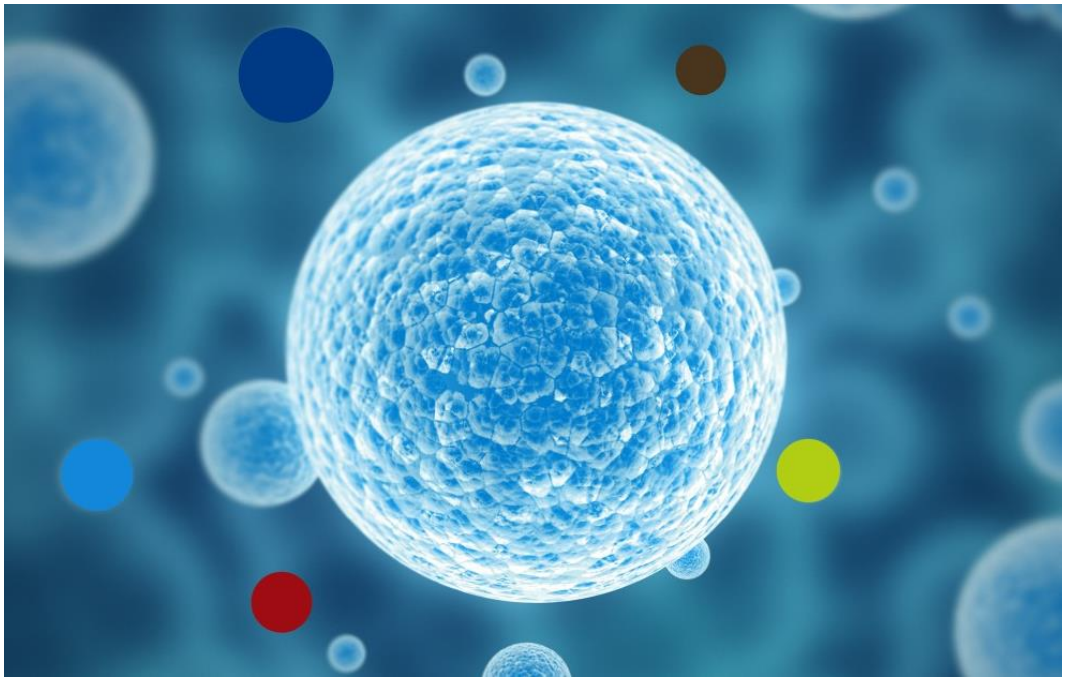
PROTOCOL FOR THE USE OF
EBiSC iNGN2 neuronal cell products



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This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (JU) under grant agreement No 821362. The JU receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA.

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1. Important information: Read prior to commencing any work

Human induced pluripotent stem cell (iPSC) lines and their derivatives are different to any other established cell line. If you are not familiar with culturing iPSCs make sure you read these instructions carefully.

Key points for success

- Read these instructions carefully, including the sections on required reagents, thawing and culturing
- Make sure all necessary reagents are available prior to thawing the cells. iPSC lines and their derivatives do not grow in standard media such as DMEM.
- Use the correct media and matrix combination for your cells (information found on Certificate of Analysis).
- Make sure your equipment is calibrated regularly and no reagents have expired.

2. General guidelines for handling human iNGN2 neurons

This document provides guidance on how to resuscitate and culture human iNGN2 neurons supplied by the European Bank of induced pluripotent Stem Cells (EBiSC). All recommendations refer to the culture of iNGN2 neurons in one well of a 6-well plate.

All cell manipulations, tissue culture vessel preparations and medium preparations should be performed under aseptic conditions within a Class II Microbiology Safety Cabinet (MSC). The cabinet should be cleaned thoroughly before use and after processing each cell line by wiping all base surfaces with 70% alcohol.

For recommendations on culture systems refer to the Certificate of Analysis (CoA) specific for the cell line and lot number.

3. Materials and reagents

The following materials are not provided with the EBiSC-iNGN2 product, but are necessary to perform neuronal differentiation experiments

- culture matrix and diluents
  - **recommended:** Poly-L-ornithine / Laminin (Sigma-Aldrich, Cat. No. P4957 / L2020)
  - Matrigel (BD, Cat. No. 354230)
- Neurobasal culture medium
  - DMEM/F-12, GlutaMAX Supplement (Thermo Fisher Sci, Cat. No. 31331-028)
  - Neurobasal Medium (Thermo Fisher Sci, Cat. No. 21103-049)
  - B-27 Supplement, w/o vitamin A (Thermo Fisher Sci, Cat. No. 11500446), optional with vitamin A (Thermo Fisher Sci, Cat. No. 11530536)
  - N-2 Supplement (Thermo Fisher Sci, Cat. No. 17502048)
  - GlutaMAX Supplement (Thermo Fisher Sci, Cat. No. 35050038)
  - MEM Non-Essential Amino Acids Solution (Thermo Fisher Sci, Cat. No. 11140-035)
  - Sodium Pyruvate (Thermo Fisher Sci, Cat. No. 11360-070)
  - 2-Mercaptoethanol (Thermo Fisher Sci, Cat. No. 31350010)
  - Insulin solution human (Sigma-Aldrich, Cat. No. I9278)
  - Optional: Penicillin-Streptomycin (Sigma-Aldrich, Cat. No. P4333)
- tissue culture treated polystyrene plates of desired format (f.e. 6-well plates, VWR, Cat. No. 700-1425)
- PBS (-/-) (f. e. Thermo Fisher Sci, Cat. No. 12559069)
- Rho-associated kinase (ROCK) inhibitor (f.e. Abcam, Cat. No ab120129)
- Doxycyclin (f.e. Sigma-Aldrich, Cat. No. D3447)
- DMEM/F12 -/- (f.e. Thermo Fisher Sci, Cat. No. 21331020)

4. Matrix preparation

Preparation of Poly-L-ornithine / Laminin Coating (recommended)

1. Upon receipt, store Poly-L-ornithine solution at 4°C and Laminin at -20°C.
2. Dilute Poly-L-ornithine solution 1:10 in PBS -/-.
3. Add 1 mL of the diluted Poly-L-ornithine solution per well of a 6-well plate (adjust volume to other plate formats accordingly).
4. Incubate the coated plates overnight at 4°C or for 4 hours at 37°C / 5% CO_2
5. Aspirate Poly-L-ornithine solution and wash plates once with PBS -/-.
6. Thaw the required volume of laminin stock solution slowly at 2 - 8°C.
7. Dilute Laminin solution 1:100 in PBS -/-.
8. Add 1 mL of the diluted Laminin solution per well of a 6-well plate (adjust volume to other plate formats accordingly).
9. Incubate the coated plates overnight at 4°C or for 4 hours at 37°C / 5% CO_2.
10. Coated laminin plates can be sealed with Parafilm™ and stored for up to two weeks at 2-8°C.
11. Prior to use, aspirate laminin solution from the vessel using a stripette™ or similar.

Preparation of Matrigel

Stock vials of Matrigel should be thawed overnight on ice or within a refrigerator (5°C) prior to use. Culture vessels, tubes and stripettes™ should be pre-chilled prior to making aliquots or coating. All manipulations of Matrigel must be carried out on ice to avoid premature gelling. Do not repeatedly freeze thaw stock or working vials of Matrigel.

1. Upon receipt, store Matrigel at -20°C.
2. Protein concentration within the Matrigel stock vial is batch dependent and must be obtained from its accompanying certificate of analysis. Use this concentration to prepare a working solution of 1 mg / mL by using DMEM/F12 -/- as dilution reagent.
3. Transfer 1 mL with 1 mg of protein into pre-chilled 15 mL tubes. These tubes are working aliquots and should be stored at -20°C until required.
4. When required, thaw the working Matrigel aliquot in the fridge (5°C) overnight. Add 11 mL of cold (5°C) DMEM/F12 -/- to the vial and mix by pipetting up and down thoroughly. This is enough for two whole 6-well plates as 1 mL of the Matrigel/DMEM mix is required for one well of a 6-well plate. Chilled, diluted Matrigel must be used immediately to coat tissue culture vessels. Then the vessel should be incubated at 37°C / 5% CO_2 for 1 hour. Alternatively, vessels can be sealed with Parafilm™ after the incubation at 37°C / 5% CO_2 and stored at 5°C for a maximum of one week.
5. Prior to use aspirate Matrigel from the vessel using a stripette™ or similar and replace with an appropriate volume of culture medium (2 mL of medium per well of a 6-well plate). Vessels are now ready for cell culture use.

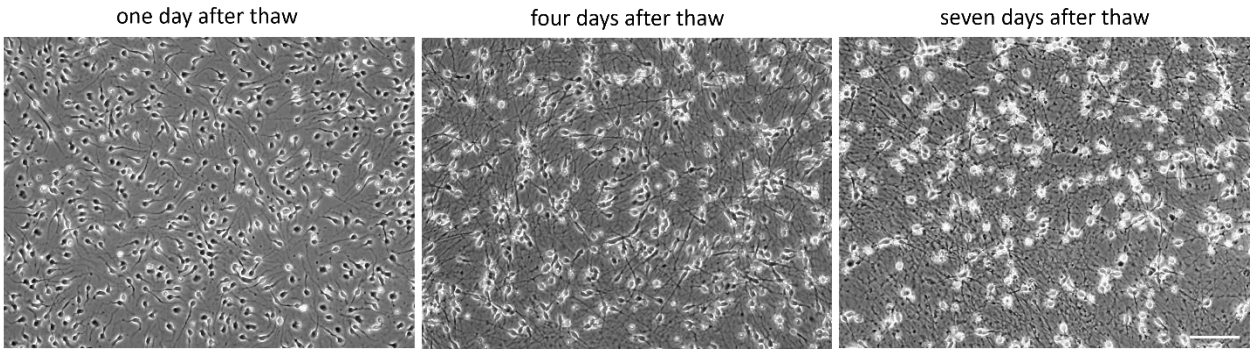
5. Medium preparation

Neurobasal-medium

1. Prepare Neurobasal-medium (NBM)
    - 50 % DMEM/F-12, GlutaMAX Supplement
    - 50 % Neurobasal Medium
    - 0.5 X B-27 Supplement (50X), w/o vit A
    - 0.5 X N-2 Supplement (100X)
    - 0.5 X GlutaMAX Supplement
    - 0.5 X MEM Non-Essential Amino Acids Solution (100X)
    - 500 nM Sodium Pyruvate (100 mM)
    - 50 nM 2-Mercaptoethanol (50 mM)
    - 0.025 % Insulin solution human (Sigma-Aldrich, I9278)
    - Optional: 5 U/ml Penicillin-Streptomycin
2. Complete Neurobasal-medium may be stored at 4°C for 2 weeks

6. Thawing and culturing of human iNGN2 neurons

1. Cells should be thawed rapidly by placing the cryovial in a water bath set to maintain 37°C. Swirl the cryovial gently in the water bath to ensure rapid thaw but do not submerge the cap of the cryovial. Disinfect the cryovial with 70% alcohol or an equivalent disinfectant before opening.
2. Using a 5 mL sterile stripette™ or similar, transfer the cryoprotectant/cells mix slowly from the cryovial into a 15 mL centrifuge tube prepared with 10 mL of prewarmed NBM. Care should be taken not to physically damage cells.
3. Rinse the cryovial with 1 mL of NBM and gently resuspend cell suspension in the 15 mL tube.
4. Centrifuge the cells at 300 x *g* for 5 minutes. Aspirate the supernatant and add 1-2 mL NBM supplemented with 2 µg / mL Doxycycline and 10 µM Rock inhibitor.
5. Prepare culture vessels by adding an appropriate amount of NMB supplemented with 2 µg / mL Doxycycline and 10 µM Rock inhibitor (f.e. 2 mL per one well of a 6-well plate, adjust if using other culture formats).
6. Resuspend the cell pellet carefully, determine cell numbers and seed cells into coated culture plates at the recommended seeding density of 100.000 cells / cm^2.
7. Gently rock plate side to side, back and forth to spread the cells evenly across the well.
8. It is advisable to record images of cells after 24 hours (see exemplary images below).
9. Switch medium after 24 hours to NBM supplemented with 2 µg / mL Doxycycline.
10. Daily medium changes with NBM / Dox is recommended for 4 days.
11. After this initial phase of NGN2 induction, Doxycyline can be omitted and cells can be cultured in NBM with half-media changes twice a week.



Exemplary brightfield images of iNGN2 neurons after thaw. Scale bar: 200 µm.

Co-cultures with astrocytes are recommended for long-term culture, as iNGN2 neurons may start to detach after 16 days without astrocytic support.

Passaging and expanding of iNGN2 neurons is not recommended.

Further Batch-specific information is provided in the corresponding Certificate of Analyses.

7. Contact details

In case of queries, please get in touch:

Email: Contact@EBiSC.org