# Product Specification Sheet

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| **Product Name** | **Human iPS Cells (Inducible NIL, ApoE3)** |
| **Description** | ALSTEM offers human iPS cell line with inducible expression of NGN2, ISL1 and LHX3 by doxycycline (Dox). This cell line is generated from pre-made footprint-free human iPS cells (cat# iPS26, ApoE3 isoform) with the inducible NGN2, ISL1, and LHX3 stably integrated into the human AAVS1 "Safe Harbor" site. It provides a convenient means to differentiate human iPS cells to lower motor neurons rapidly and efficiently. The integration at "Safe Harbor" sites ensures safe and reliable expression of NGN2, ISL1, and LHX3 when Dox is supplied. |
| **Catalog Number** | iP26MN |
| **Size** | $5x10^5$ cells/vial |
| **Shipping** | Dry ice |
| **Storage and Stability** | Store in the vapor phase of liquid nitrogen immediately upon receipt. This product is stable for 6 months when stored as directed. |
| **Quality Control** | Human iPS cells (inducible NGN2, ISL1 and LHX3) were grown in feeder-free conditions with mTeSR1 medium. Each lot of hiPSCs is tested for growth and viability following recovery from cryopreservation. In addition, each lot is tested for expression of TRA-1-60 and OCT4. |
| **Safety Precaution** | **ALSTEM highly recommends that protective gloves, a lab coat, and a full-face mask always be worn when handling frozen vials.** It is important to note that some liquid nitrogen can leak into the vials when submersed in liquid nitrogen. Upon thawing, the liquid nitrogen returns to the gas phase, resulting in excessive pressure within the vial that can cause the vial to explode or expel the cap with dangerous force. |
| **Restricted Use** | For Research Use Only. Not for use in diagnostic or therapeutic procedures. |

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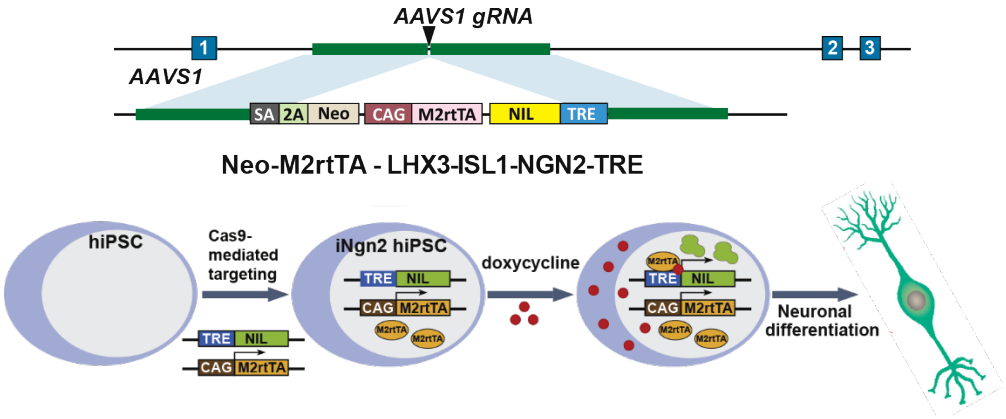
# Protocol

## Human iPS Cell Culture and Neuron Induction

### OVERVIEW

Overexpression of the transcription factors Islet-1 (ISL1) and LIM Homeobox 3 (LHX3) along with Neurogenin 2 (NGN2) promotes direct differentiation of pluripotent stem cells to functional lower motor neurons. AAVS1 (also known as PPP1R12C) locus supports robust and sustained transgene expression. Precise insertion of tetracycline inducible NGN2, ISL1, and LHX3 (NIL) into human iPS cells at AAVS1 site could generate a stable iPSC line for rapid lower motor neuron differentiation upon induction.

The highly responsive doxycycline inducible NIL-expressing human iPS cell line cat# iP26MN) is developed from pre-made footprint-free human iPS cells (cat# iPS26, ApoE3 isoform) by integrating the all-in-one inducible LHX3, ISL1, and NGN2 cassette into the human AAVS1 "Safe Harbor" site. This cell line is ideal for rapid *in vitro* neuronal differentiation.




An all-in-one cassette consisting of both inducible LHX3, ISL1, and NGN2 and also constitutive M2rtTA has been inserted into one of the AAVS1 alleles by gene editing through HDR pathway, while the other allele remains intact. Upon induction, NGN2, ISL1, and LHX3 are expressed and the iPS cells will be directly differentiated into lower motor neurons.

## PROCEDURE

## I. Feeder-free human iPSC culture conditions

### Preparation of feeder-free medium

1. Thaw mTeSR1 5X Supplement (Cat.no. 05850, STEMCELL Technologies) at room temperature or overnight at 4°C.
2. Add the 100 mL of thawed 5X Supplement to 400 mL Basal Medium for a total volume of 500 mL aseptically. Mix well and filter through a 0.22 µm, low-protein binding filter, if desired.

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3. Aliquot into appropriate volumes according to usage and store the aliquots at 4°C.

**Coating plates with Matrigel**

Matrigel (Cat.no. 354277, BD) should be aliquoted and stored at -80°C for long-term use.

1. Thaw Matrigel on ice until liquid. Dilute Matrigel 1:50 with pre-chilled KO DMEM/F12.
2. Immediately use the diluted Matrigel solution to coat tissue culture-treated plates. For a 6-well plate, use 1 mL of diluted Matrigel solution per well, and swirl the plate to spread the Matrigel solution evenly across the surface.
3. Let the coated plate stand for 1 h at 37°C or overnight at 4°C. If plate has been stored at 4°C, allow the plate to incubate at 37°C for at least 30 minutes before removing the Matrigel solution.

**Thawing cryopreserved human iPS cells (inducible NIL, APOE3)**

1. Quickly thaw the human iPS cells (inducible Ngn2) in a 37°C waterbath by gently shaking the cryovial continuously until half thawed. Remove the cryovial from the waterbath and spray with 70% ethanol to sterilize.
2. Transfer the contents of the cryovial to a 15 mL conical tube. Add 5 mL warm mTeSR1 dropwise to the tube, gently mixing as the medium is added.
3. Centrifuge cells at 200 *x g* for 5 minutes at room temperature.
4. After centrifugation, aspirate the medium from 15 mL tube. Gently resuspend the cell pellet in 2 mL mTeSR1 with 10 µM ROCK inhibitor, taking care to maintain the cells as small cell clumps.
5. Remove the Matrigel solution from a coated tissue culture 6-well plate. Transfer the medium containing the cell clumps to the Matrigel coated 6-well plate.
6. Place the plate into the 37°C incubator and move the plate in quick side to side, forward to back motions to evenly distribute the clumps within the wells. Culture the cells at 37°C, with 5% $CO_2$ and 95% humidity.
7. Change medium daily. Check for undifferentiated colonies that are ready to passage when colonies are big enough (approximately 7-10 days after thawing).

**Passaging human iPS cells (inducible NIL, APOE3) grown under feeder-free conditions**

1. Use a microscope to identify regions of differentiation. Mark the differentiated colonies using lens marker on the bottom of the plate.
2. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
3. Aspirate medium from the human iPS cell culture and rinse with DPBS (2 mL/well).
4. Add 1 mL per well of EZStem Enzyme-Free Stem Cell Dissociation Solution (cat. no. M100, ALSTEM), and incubate at 37°C for 2-3 minutes. Or add 0.5 mL per well of accutase (Cat.no. SCR005, Millipore, diluted 1:2 with DPBS before use), and incubate at 37°C for 1-2 minutes.
5. Remove EZStem Enzyme-Free Stem Cell Dissociation Solution or accutase, gently rinse each well 2 - 3 times with 2 mL of DMEM/F-12 per well and transfer the detached cell aggregates to a 15 mL conical tube.
6. Add 2 mL of mTeSR1 per well and scrape colonies off with a cell lifter. Transfer the detached cell aggregates to the above 15 mL conical tube.
7. Rinse the well with an additional 2 mL of mTeSR1 to collect any remaining aggregates. Add the rinse to the 15 mL tube.
8. Centrifuge the 15 mL tube containing the aggregates at 200 x *g* for 5 minutes at room temperature.

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9. Aspirate the supernatant. Resuspend pellet in mTeSR1 containing 5 µM ROCK inhibitor by gently pipetting and ensure that cells are maintained as aggregates.
10. Plate the human iPS cell aggregates with mTeSR1 in a new plate coated with Matrigel. (Remove Matrigel solution before plating). *If the colonies are at an optimal density, the cells can be split every 5 - 7 days using 1:3 to 1:6 ratios.*
11. Place the plate into the 37°C incubator and move the plate quickly back and forth and side to side to evenly distribute the clumps within the wells. Culture the cells at 37°C, with 5% $CO_2$ and 95% humidity.
12. Change medium daily, omitting the ROCK inhibitor after 24 hours.

**Cryopreserving human iPS cells (inducible NIL, APOE3)**

1. Prepare EZStem Freezing Medium (Cat.no. M050, ALSTEM) or mTeSR1 medium containing 10% DMSO and 10 µM ROCK inhibitor and keep on ice.
2. Perform steps 1-8 from **Passaging human iPS cells grown under feeder-free conditions.**
3. Gently aspirate the supernatant and loosen the cell pellet by tapping the bottom of the tube.
4. Gently resuspend the pellet in freezing medium, taking care to leave the clumps larger than what would normally be done for passaging.
5. Transfer 1 mL of clumps in freezing medium into each labeled cryogenic vial.
6. Place vials into a freezing container and place the container at -80°C overnight.
7. Transfer to a liquid nitrogen tank the next day.

## II. Motor neuron induction of human iPSCs (inducible NIL, APOE3)

### Cell culture media

**N2 medium**: To make 50 mL: 48 ml DMEM/F12, 0.5 ml N2 supplement (100X), 0.5 ml GlutaMAX (100X), 0.5 ml NEAA (100X) and 0.5 ml Pen/Strep (100X). Filter with a 0.22 µm filter and store at 4°C for up to one month.
**Motor neuron induction medium**: N2 medium with Doxycycline (2 µg/ml) and Compound E (0.2 µM).
**Motor neuron culture medium:** To make 50 mL: 47 ml Neurobasal medium, 0.5 ml N2 supplement (100X), 1 ml B27 supplement (50X), 0.5 ml GlutaMAX (100X), 0.5 ml NEAA (100X) and 0.5 ml Pen/Strep (100X). Filter with a 0.22 µm filter and store at 4°C for up to one month.
**Freezing medium**: To make 50 mL: 20 mL N2 medium, 25 mL HSA, and 5 ml DMSO. Store at 4°C for up to one month.
**Other components**: Poly-L-ornithine (PLO, 0.1 mg/ml), laminin (1 µg/ml), Y-27632 (5 µM), BrdU (40 µM)

**Neuron induction**

**(Day 0) Plating human iPS cells (inducible NIL, APOE3)**

1. When the human iPS cells (inducible NIL) in the 6-well plate reach ~80% confluence, use a microscope to identify regions of differentiation. Mark the differentiated colonies using lens marker on the bottom of the plate.
2. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
3. Aspirate medium from the human iPS cell culture and rinse with DPBS (2 ml/well).

4. Add 1 mL per well of EZStem Enzyme-Free Stem Cell Dissociation Solution (cat. no. M100, ALSTEM), and incubate at 37°C for 3 minutes.
5. Add 2 ml perwell of mTeSR1, and gently rinse each well 2-3 times to dislodge the cells.
6. Transfer the detached cell aggregates to a 15 ml conical tube and rinse the well with an additional 1 ml of mTeSR1 to collect any remaining aggregates. Add the rinse to the 15 ml tube.
7. Centrifuge the 15 ml tube containing the aggregates at 200x g for 5 min at room temperature.
8. Aspirate the supernatant. Resuspend pellet in 10 ml mTeSR1 containing 5 µM ROCK inhibitor by gently pipetting and ensure that cells are maintained as small aggregates.
9. Plate cell suspension (about 1.5 x $10^6$ cells) onto a 10-cm dish coated with Matrigel (remove Matrigel solution before plating).
10. Place the dish into the 37°C incubator and move the dish in quick side-to-side, forward-to-back motions to evenly distribute the clumps. Culture the cells at 37°C with 5% $CO_2$ and 95% humidity.

**(Day 1-3) DOX induction and passaging/freezing induced cells**

11. On Day 1, remove old medium from the culture. Add 10 ml of N2 media freshly supplemented with 5 µM Y-27632 ROCK inhibitor, 2 µg/ml Doxycycline, and 0.2 µM Compound E from stock.
12. On Day 2, observe differentiating cells under an inverted microscope. Cells should be beginning to spread out and form processes. If long-term culture is desired immediately, coat plates overnight with PLO.
13. On Day 3, freeze or passage induced cells:

a) Aspirate medium from the dish and rinse with 5 ml DPBS.
b) Aspirate DPBS, add 3 ml of Accutase (Cat.no. SCR005, Millipore), and incubate at 37°C for 3 minutes.
c) Gently tap the plate, add 3 ml of N2 medium, and gently rinse each well 2-3 times to dislodge the cells.
d) Transfer the cell suspension to a 15 ml conical tube, rinse the well with an additional 2 ml of N2 medium to collect any remaining cells, and add the rinse to the 15 ml tube.
e) Count the cells.
f) Centrifuge the 15 ml tube containing the cell suspension at 200x g for 5 min at room temperature.
g) Aspirate the supernatant, dislodge the cell pellet by tapping the bottom of the tube.
h) Cells may be re-plated immediately or frozen for future use.

To freeze the induced cells, resuspend the pellet in freezing medium at 1x$10^6$ cells/ml. Transfer 1 mL of cell suspension in freezing medium into each labeled cryogenic vial. Place the vials in a Mr. Frosty cell-freezing container and keep it at -80 °C overnight. Transfer the vials to the gas phase of liquid nitrogen tank for long-term storage.

i) Prepare wells of pre-coated plates with warm N2 medium supplemented with 5 µM ROCK inhibitor, 2 µg/ml doxycycline, 1:10,000 Compound E from stock, 40 µM BrdU* and 1 µg/ml laminin.

*Note: Typical cell counts and medium volumes are as follows:*

*a. 96-well plate (imaging): 1–5 × $10^4$ cells in 100 µl medium/well.*
*b. 48-well chamber slide (imaging): 0.5–1.5 × $10^5$ cells in 250 µl medium/well.*
*c. 6-well plate (biochemistry): 0.8–1.2 × $10^6$ cells in 2 ml medium/well.*
*d. 10-cm dish (biochemistry): 5–8 × $10^6$ cells in 10 ml medium.*

**Bromodeoxyuridine (BrdU) has proven effective at impairing mitosis without causing neural toxicity.*

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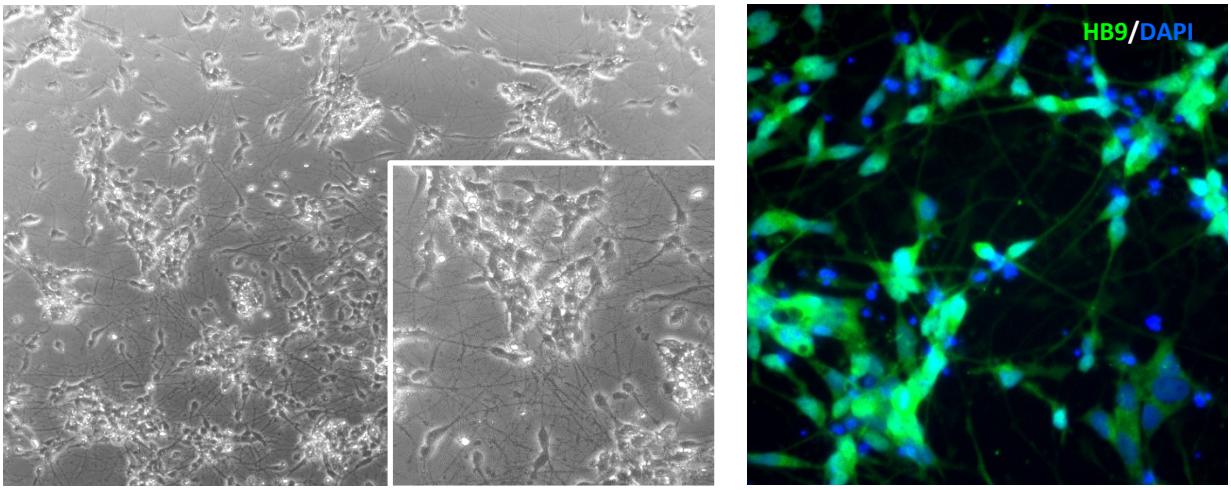
14. On Day 4, Aspirate the supernatant and replace with warm N2 medium supplemented with 5 µM ROCK inhibitor, 2 µg/ml doxycycline, 1:10,000 Compound E from stock, and 1 µg/ml laminin **without** BrdU.
15. For the first 4 days (Day 4 to Day 7), check cells daily under a phase-contrast microscope, paying particular attention to cell debris and morphological changes. Medium changes should be done every 2 to 3 days by replacing one-half of the medium with fresh warm N2 medium supplemented with 5 µM ROCK inhibitor, 2 µg/ml doxycycline, 1:10,000 Compound E from stock, and 1 µg/ml laminin.
16. After day 7, perform half-medium changes every 4 to 7 days with Motor neuron culture medium with 1 µg/ml laminin for long-term maintenance
17. *Optional:* Supplementation with astrocytes or astrocyte-conditioned medium has been shown to improve the overall health and electrophysiological activity of induced motor neurons in long-term cultures.

*Note: a. 1-day treatment of BrdU is recommended at the time of replating on Day 3 induction.*
*b. induced motor neurons may be used for experiment from Day 14.*




**Upon doxycycline treatment, inducible NIL iPS cells differentiate to motor neurons.** Nine days after Dox treatment, the majority of cells have converted to motor neurons (HB9 positive) with extensive growth of axons and dendrites.

## IMPORTANT NOTICE

Store the vials at vapor phase of liquid nitrogen immediately upon receipt.

## WARNING

**Do not use cryogenic vials for storage in the liquid phase of liquid nitrogen. Such use may cause entrapment of liquid nitrogen inside the vial and lead to pressure buildup resulting in possible explosion or biohazard release. Use appropriate safety procedures which are outlined by the ATCC when handling and disposing of vials. ALSTEM highly recommends that protective gloves and clothing always be used and a full-face mask always be worn when handling frozen vials.**

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