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# 🌐 DIFFERENTIATION OF i³LMNS (Basic Protocol 7)

📄 In 1 collection

**iPSCs**

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**Protocol status:** Working
**We use this protocol and it's working**

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# Abstract

This protocol describes the rapid and robust differentiation of hiPSCs into lower motor neurons (i$^3$LMNs) via induced expression of the transcription factors NGN2, ISL1, and LHX3 (hNIL) (Mazzoni et al., 2013; Shi et al., 2017). In particular, a donor construct containing these factors under the tetracycline response element (TRE3G) (Gossen & Bujard, 1992), a CAG promoter driving constitutive expression of the reverse tetracycline transactivator (rtTA3G), and an EF-1α promoter driving constitutive expression of selection genes (mCherry for **Addgene, cat. no. 105841** and SBP-LNGFR and mApple for **Addgene, cat. no. 105842**) was stably integrated into the safe-harbor *CLYBL* locus via TALENs (Cerbini et al., 2015). Motor neuron differentiation efficiency can vary between iPSC lines, and homozygous insertion into both CLYBL alleles may result in improved efficiency. Reduced differentiation efficiency of mCherry positive clones can occur with off-target integration of the donor construct. Additionally, the mCherry reporter is flanked by loxP sites, permitting excision by transient transfection of Cre recombinase if desired.

# Attachments



fernandopulle2018.pd...
1.7MB

## Materials

- iPSCs with stably integrated doxycycline-inducible hNIL transgenes (Basic Protocols **2**, **3**, **4**)
- Induction Medium (see **Table 2**)
- Compound E (Gamma-Secretase Inhibitor XXI, Calbiochem, cat. no. 565790); 500 μg reconstituted in 255 μl ethanol and 255 μl DMSO (2 mM stock) (store light-protected at 🌡 -20 °C up to 6 months)

> ⊗ γ-Secretase Inhibitor XXI, Compound E - Calbiochem  **Merck Millipore (EMD Millipore) Catalog** #565790

- Inverted microscope
- Cell strainer, 40-μm pore diameter (Corning, cat. no. 352340)

| Equipment | |
|---|---|
| **Falcon® 40 μm Cell Strainer** | NAME |
| Cell Strainer | TYPE |
| Falcon | BRAND |
| 352340 | SKU |
| https://ecatalog.corning.com/life-sciences/b2c/US/en/Cell-Culture/Cell-Culture-Accessories/Cell-Strainers/Falcon%C2%AE-Cell-Strainers/p/352340?clear=true# | LINK |

- Additional reagents and equipment for general iPSC culture (**Basic Protocol 1**) and counting cells (Phelan & May, 2015)

## Troubleshooting

## Safety warnings

> ❗ Please see SDS (Safety Data Sheet) for hazards and safety warnings.

# Before start

NOTE: It is critical that the health of iPSCs be optimal prior to differentiation; poorly maintained, spontaneously differentiated, or overly confluent iPSC cultures tend to differentiate poorly or variably. Apart from observation under the microscope, counting, and centrifugation, all steps should be carried out in a sterile biological safety cabinet.

This protocol assumes differentiation of one 10-cm dish, which should be seeded with $1.5 \times 10^6$ cells. Each dish in turn provides 6 to $8 \times 10^6$ cells at Day 3. Medium volumes and cell numbers may be scaled by surface area (i.e., seed $2 \times 10^4$ iPSCs/cm$^2$, or $1.8 \times 10^5$ iPSCs/well of a 6-well plate).

## Day 0

1   Prepare a sufficient amount of hNIL–iPSC for differentiation.

> **Note**
>
> Approximately **two wells** of a 6-well dish at **80 % confluency** are sufficient to begin the differentiation of a 10-cm dish, and cell numbers may be scaled by well surface area. Typically, hNIL lines proliferate for longer than hNGN2 lines, and this protocol calls for plating in E8 before switching to IM on d1, so hNIL cells are seeded at lower density on d0.

2   Prepare cells as for EDTA split (see **Basic Protocol 1**).

3   Incubate at 🌡 Room temperature for ⏱ 00:10:00 to ⏱ 00:15:00 (until colonies begin to detach).

4   Without aspirating the EDTA, tilt the plate and pipet ⚗ 3 mL DMEM/F12 into each well with a 10-ml pipet and remove the cells from the bottom of the dish. Gently triturate the cells by pipetting the cells up and down two to three times in each well.

5   Count the cells (Phelan & May, 2015).

6   Transfer $1.5 \times 10^6$ iPSCs per 10 cm dish to be differentiated to a 15-ml conical tube.

7   Centrifuge ⏱ 00:05:00 at ⚙ 300 x g , 🌡 Room temperature .

8   Aspirate the supernatant.

9   Plate the resuspended cells in ⚗ 12 mL E8 medium supplemented with [M] 10 micromolar (µM) ROCK inhibitor on a 10-cm Matrigel-coated dish.

## Day 1

10   Gently rock and swirl the plate by hand to resuspend and collect debris.

11   Aspirate off the medium.

12   Wash once with PBS.

13   Add ⚗ 12 mL of IM freshly supplemented with [M] 10 micromolar (μM) Y-27632 ROCK inhibitor, [M] 2 μg/ml doxycycline, and 1:10,000 Compound E from stock.

> **Note**
>
> Doxycycline will induce the expression of the hNIL construct and promote the differentiation into motor neurons. Morphological changes should be evident after 24 hr of doxycycline treatment. The induction efficiency can be tested by comparing parallel wells treated with and without doxycycline, or by performing a separate dox-GFP experiment as described in **Support Protocol 6**.

## Day 2

14   Observe differentiating cells under an inverted microscope.

> **Note**
>
> Cells should be beginning to spread out and form processes.

15   If long-term culture is desired immediately, coat plates overnight with PLO, PDL, or PEI (see **Basic Protocol 8**).

## Day 3

16   Treat the differentiating cells with Accutase (see **Basic Protocol 1**). Use ⚗ 3 mL Accutase to digest each 10-cm dish.

**17**  Tilt the plate and add [🧪 6 mL] PBS to the Accutase and cell solution using a 10-ml pipet.  Gently triturate four to five times to break the cells apart.

> **Note**
>
> For best results, pass the cells and solution through a 40-µm cell strainer to ensure full dissociation. Add 1 ml IM after straining to improve pelleting.

**18**  Centrifuge the cells [🕐 00:05:00] at [⚙ 300 x g] , [🌡 Room temperature] .

**19**  Aspirate the supernatant.

**20**  Resuspend in [🧪 5 mL] IM.

**21**  Count the cells (Phelan & May, 2015).

**22**  Cells may be re-plated immediately (see **Basic Protocol 8**) or frozen for future use (see **Basic Protocol 1**).

> **Note**
>
> Cells to be frozen should be diluted to a standard cell density (i.e., $1 \times 10^6$ cells/ml) in IM containing a final concentration of 10 % DMSO.