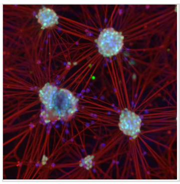

May 16, 2022

Differentiation of iPSCs with the hNIL construct into motor neurons protocol

Maria Sckaff[1], Kenneth Wu[2], Hana Ghanim[2], Aradhana Sachdev[2], Gokul N Ramadoss[2], Carissa M. Feliciano[2], Luke M. Judge[2], Bruce Conklin[2], Claire D Clelland[3]

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2

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This protocol describes the differentiation of induced pluripotent stem cells (iPSCs) into motor neurons using the hNIL transgenic factors in a CLYBL safe harbor site.

Differentiation of iPSCs with the hNIL construct into motor neurons_ClellandLab.pdf

DOI

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hNIL differentiation, Maintaining iPSCs, Passaging iPSCs, motor neurons, iPSC, stem cells

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Mar 30, 2022 Maria Sckaff University of California, San Francisco

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This protocol is based on hNIL differentiation protocol by Fernandopulle et al. from Michael Ward's lab[1].

References

[1] Michael S Fernandopulle, Ryan Prestil, Christopher Grunseich, Chao Wang, Li Gan, Michael E Ward. Transcription Factor-Mediated Differentiation of Human iPSCs into Neurons. Curr Protoc Cell Biol. 2018 Jun;79(1): e51. doi: 10.1002/cpcb.51. Epub 2018 May 18. PMID: 29924488; PMCID: PMC6993937.

[2] Erika Lara Flores (2021). Maintenance protocol of iPSCs. Protocols.io

[3] Merissa Chen*, Nina Draeger*, Martin Kampmann*, Kun Leng*, Emmy Li*, Connor Ludwig*, Greg Mohl*, Avi Samelson*, Syd Sattler*, Ruilin Tian* (2019). Kampmann Lab iNeuron pre-differentiation & differentiation protocol. Protocols.io

Items needed for the creation of the media and reagents used in the differentiation of hNIL iPSCs into motor neurons.

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A	B	C
Item	**Manufacturer**	**Catalog Number**
KnockOut DMEM	ThermoFisher Scientific	10829018
Growth Factor Reduced Matrigel	Corning	356231
mTeSR™ Plus and supplement	Stemcell Technologies	1000276
ReLeSR™	Stemcell Technologies	05872
Gibco™ DPBS, no Ca, no Mg	ThermoFisher Scientific	14-190-235
Gibco™ DPBS, Ca, Mg	ThermoFisher Scientific	14-040-117
Accutase	Stemcell Technologies	07920
DMEM/F-12, HEPES	ThermoFisher Scientific	11330032
N-2 Supplement	ThermoFisher Scientific	17502048
MEM Non-Essential Amino Acids Solution (NEAA)	ThermoFisher Scientific	11140050
GlutaMAX Supplement	ThermoFisher Scientific	35050061
Culture One Supplement	ThermoFisher Scientific	A3320201
B-27 Supplement, serum free	ThermoFisher Scientific	17504044
y-Secretase Inhibitor XXI, Compound E	Millipore Sigma	565790
Poly-D-Lysine (PDL)	ThermoFisher Scientific	A3890401
Laminin Mouse Protein, Natural	ThermoFisher Scientific	23017015
Doxycycline Hyclate (reconstituted in water)	Millipore Sigma	D3447
5-Bromo-2′-deoxyuridine (BrdU)	Millipore Sigma	B9285
ROCK1 Inhibitor (Y-27632 2HCl)	Selleckchem	S1049
Neurobasal	Life Technologies	21103-049
HyClone Characterized Fetal Bovine Serum (FBS)	Cytiva	SH30071.03HI
Recombinant Human BDNF	Peprotech	450-10
Recombinant Human GDNF	Peprotech	450-02
Recombinant Human NT3	Peprotech	450-03
Aphidicolin	Millipore Sigma	89458

Notes on some items that need to be reconstituted

Compound E: **1 mg** is reconstituted in **255 µL** of ethanol and **255 µL** of DMSO to make a 10,000 stock, then aliquoted and stored at **-20 °C** for up to 6 months, minimizing exposure to light. Doxycycline: diluted in cell culture grade water to **2 mg/mL** and stored at **-20 °C** (long-term storage) or **4 °C** (short-term storage), minimizing exposure to light.

BrdU: reconstituted in water to a stock of [M] **40 millimolar (mM)** (**12.284 mg/mL**).

BDNF, GDNF, and NT3: **50 µg** reconstituted in filtered 1X DPBS with 0.1% BSA, then aliquoted and stored at **-20 °C** for up to 3 months.

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KnockOut™ DMEM **Thermo Fisher Scientific Catalog #10829018**

Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix **Corning Catalog #356231**

mTeSR™ Plus **Stemcell Technologies Catalog #1000276**

ReLeSR™ 100 mL **Stemcell Technologies Catalog #5872**

Gibco™ DPBS no calcium no magnesium **Thermo Fisher Scientific Catalog #14190235**

Gibco™ DPBS calcium magnesium **Thermo Fisher Scientific Catalog #14040117**

ACCUTASE™ **Stemcell Technologies Catalog #07920**

DMEM/F-12, HEPES **Thermo Fisher Scientific Catalog #11330032**

N2 supplement (100x supplement) **Gibco, ThermoFisher Catalog #17502048**

MEAA (MEM Non-Essential Amino Acids) **Gibco - Thermo Fisher Catalog #11140050**

GlutaMAX™ Supplement **Thermo Fisher Scientific Catalog #35050061**

CultureOne™ Supplement (100X) **Thermo Fisher Catalog #A3320201**

B-27™ Supplement (50X), serum free **Gibco - Thermo Fisher Catalog #17504044**

γ-Secretase Inhibitor XXI Compound E **Merck Millipore Catalog #565790**

Poly-D-Lysine **Thermo Fisher Scientific Catalog #A3890401**

Laminin Mouse Protein, Natural **Thermo Fisher Catalog #23017015**

5-Bromo-2'-deoxyuridine **Millipore Sigma Catalog #B9285**

Y-27632 **Selleckchem Catalog #S1049**

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Gibco™ Neurobasal™ Medium **Thermo Fisher Scientific Catalog #21103049**

HyClone Characterized Fetal Bovine Serum (FBS) **Cytiva Catalog #SH30071.03HI**

Recombinant human GDNF **peprotech Catalog #450-10**

Recombinant human BDNF **peprotech Catalog #450-02**

Recombinant Human NT-3 **peprotech Catalog #450-03**

Equipment usedin the differentiationof hNIL iPSCs intomotor neurons.

A	B	C
Equipment	**Manufacturer**	**Catalog Number**
Falcon® 96-well Black/Clear Flat Bottom TC-treated Imaging Microplate with Lid	Corning	353219
Eppendorf® Centrifuge 5810/5810R	Millipore Sigma	EP022628168
Invitrogen Countess™ II automated cell counter	ThermoFisher Scientific	AMQAX1000

6-well Black/Clear Flat Bottom TC-treated Imaging Microplate with Lid
Microplate
Falcon® 353219

Centrifuge 5810/5810R
Centrifuge
Eppendorf® EP022628168-1EA

LIFE TECHNOLOGIES COUNTESS II
Automated Cell Counter
Invitrogen Countess™ AMQAX1000

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Maintenance and Preparation of the iPSCs for the hNIL differentiation into motor neurons: Maintaining iPSCs

20m

1 Culture iPSCs for at least 2-3 passages after initial hNIL transfection or from frozen stocks before starting a motor neuron differentiation.

iPSCs that have been recently thawed or are otherwise stressed (e.g., recently nucleofected) can result in phenotypically abnormal motor neurons and poor differentiation. See Flores et al. for complete iPSC culture methods[2].

2 Briefly, we maintain iPSCs on Matrigel-coated plastic culture dishes (Growth Factor Reduced Matrigel diluted in **50 mL** of KnockOut DMEM to a concentration of **80 µg/ml**) with mTeSR™ Plus medium.

3 Change media every other day, and passage every 4-5 days with ReLeSR™ (see below).

4

Add ROCK1 inhibitor at **[M]10 micromolar (µM)** in media to freshly passaged iPSCs for 1 day to limit spontaneous differentiation.

Maintenance and Preparation of the iPSCs for the hNIL differentiation into motor neurons: Passaging iPSCs

20m

5 Passage iPSCs as clumps (for routine expansion of iPSC cultures) or single cells (for differentiation).

6 1m

For routine expansion, wash the well with DPBS -Ca/-Mg (1-2 mL/well*) then add **1 mL** per well* of ReLeSR™ for **00:01:00** .

7 4m

Aspirate most of the ReLeSR™, but do not overdry, and incubate the wells at **Room temperature** for 3-4 minutes.

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8

Add fresh mTeSR™ Plus media to the well (1 mL /well*) and pipette gently to detach the iPSCs and break colonies into small clumps.

Clumps should be visible by eye.

9 A confluent well can be split 1:6 to 1:20 depending on the desired confluency and rate of growth of the iPSC line.

10 15s

If desired, before replating, cells can be centrifuged at 800 rpm, 00:00:15 to pellet clumps and remove any single cells (which remain in suspension).

ROCK1 inhibitor is not necessary when clump passaging, but can be added at [M]10 micromolar (µM) (final concentration in well solution) for 1 day to limit spontaneous differentiation.

11

For differentiation (or anytime cell counting is needed), wash the well with DPBS -Ca/-Mg (1-2 mL/well*) then add 0.5 mL * of Accutase®.

* These volumes are based on experiments run on a 6-well plate and are scalable to other cell culture vessels.

12 5m

Incubate the plate for 00:05:00 at 37 °C .

13 After the incubation, tap the plate to fully detach the iPSCs, then add 0.5 mL * of KnockOut DMEM with 20% FBS (alternately, 2.5 mL/well* of DPBS +Ca/+Mg can be used in place of DMEM/FBS).

* These volumes are based on experiments run on a 6-well plate and are scalable to other cell culture

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vessels.

14

3m

Transfer the cells and solution to a conical vial and centrifuge for **800 rpm, 00:03:00** to pellet the cells.

15 Aspirate the supernatant carefully as to not disturb the cell pellet, and resuspend the cells in mTeSR™ Plus with ROCK1 inhibitor at **[M]10 micromolar (µM)** .

ROCK1 inhibitor is highly recommended when passaging iPSCs as single cells to prevent spontaneous differentiation.

hNIL differentiation into motor neurons: Day 0 1h

16

30m

Coat the receiving vessel with Matrigel at least **00:30:00** before starting Day 0 (but no more than 24-36 hours prior to starting) and incubate at **37 °C** .

17 Bring the mTeSR™ Plus with ROCK1 inhibitor (final concentration of **[M]10 micromolar (µM)** in well, 1000X dilution from **[M]10 millimolar (mM)** stock) and the Accutase® to **Room temperature** .

18

Aspirate the spent media and wash the wells with cells once with 1X DPBS -Ca/-Mg.

19

Add appropriate volume of Accutase® per well.

20

5m

Incubate the plate for **00:05:00** at **37 °C** .

21 Remove plate from the incubator and tap the plate to release the cells.

22 Quench the Accutase® using five times the volume DPBS +Ca/+Mg (if you used **0.5 mL** of Accutase®,

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quench with 2.5 mL of DPBS).

23 Transfer the cells to a conical vial.

24 3m

Centrifuge the vial at 800 rpm, 00:03:00 .

25 Aspirate the Accutase® with DPBS, being careful not to disturb the cell pellet at the bottom of the vial.

26 Resuspend the cells with an appropriate volume of mTeSR™ Plus with ROCK1 inhibitor (final concentration of [M]10 micromolar (µM) in well, 1000X dilution from [M]10 millimolar (mM) stock). Aim for roughly 1 mL of mTeSR™ Plus with ROCK1 inhibitor per 1 million cells.

27

Pipette up and down to mix well and produce an evenly distributed solution.

28 Count the number of cells using a Countess II (any preferred equivalent way of counting cells is also appropriate). 5m

Countess II count parameters:
1. Size 0 to 30
2. Brightness 0 to 255
3. Circularity 80
4. Auto Lighting selected

29 Aspirate the Matrigel from the receiving vessel.

30

Add appropriate volume of media (the mTeSR™ Plus with ROCK1 inhibitor at [M]10 millimolar (mM)) to the receiving vessel.

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31

Add volume of cell mixture appropriate for the number of cells desired in the well. For a 10cm dish, we have found 1-2 million iPSCs at day 0 produces 3-5 million cells at day 3.

32 5m

Place the receiving vessels (which now has your cells) back in the incubator at 37 °C .

33 Shake the plate in all four directions to ensure the cells are evenly distributed in the well.

hNIL differentiation into motor neurons: Day 1 30m

34 Prepare the neural induction medium (NIM) as follows:

A	B
DMEM/F12	97 mL
N-2	1 mL
NEAA	1 mL
GlutaMAX	1 mL

35 Calculate the volume of freshly prepared NIM needed to perform a full media change (see Table 2) and aliquot in a vial of appropriate size.

A	B	C	D	E
Cell culture vessel	**Surface area (cm2)**	**Media volume per well**	**Day 3 Seeding density for ICC applications**	**Day 3 Seeding density for protein, RNA, DNA extraction**
96-well plate	0.32	150 µL	20-25k cells	-
24-well plate	1.9	500 µL	80-100k cells	-
12-well plate	3.5	1 mL	2 x 105 cells	-
6-well plate	9.6	2 mL	0.5 x 106 cells	1 x 106 cells
10-cm dish	56.7	10 mL	-	5 x 106 cells
15-cm dish	145	30 mL	-	15 x 106 cells

Table 2: Number of cells and volume of media matrix needed on the day-3 replating in the differentiation of hNIL iPSCs into motor neurons based on the size and format of the cell culture vessel used, as well as the application. Modification from the Kampmann's Lab protocol[3].

36

Add the appropriate factors for day 1 to the NIM aliquot you aliquoted in step 35:

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A	B	C	D
Factor	**Final Concentration**	**Stock**	**Dilution**
ROCK1 inhibitor	10 µM	10 mM	1:1,000
Doxycycline	2 µg/mL	2 mg/mL	1:1,000
Compound E	0.2 µM	2 mM	1:10,000

37 Aspirate the spent media from the wells with cells.

38

Add the appropriate volume of media to each well.

39 Place the plate with cells in fresh media back in the incubator at **37 °C** .

hNIL differentiation into motor neurons: Day 2 10m

40 Select the receiving cell-culture vessel. This is the plate on which your neurons will grow permanently (see recommendations below).

Recommendations for protein or RNA extraction: A minimum surface area equivalent to three 6-wells is recommended per RNA or protein sample to be extracted. Regular cell-culture plates (plastic, flat bottom) are well-suited for this application.

Recommendations for imaging purposes: We recommend high quality plastic plates, such as the Falcon® 96-well Black/Clear Flat Bottom TC-treated Imaging Microplate (see Table 4) due to improved long-term neuron attachment. Glass-bottom plates or coverslips may also be used, but in our hands, about 20-50% of the wells experience lifting as the neurons age past day 14 in culture. Glass-bottom plates are recommended when the neurons will be fixed for imaging at an earlier stage, such as between day 7 and day 14 in culture. Premature detachment can be minimized by very gentle handling, slow pipetting, and never completely removing the media from the wells (including when PFA fixing, see below).

Note on PDL pre-coated plates: If using a PDL pre-coated plate, you will only need to rehydrate it on day 3 in DPBS once prior to coating receiving wells with laminin and the seeding of the cells.

41 Coat the wells which will be receiving cells on day 3 with the appropriate volume of 1X PDL at **0.1 mg/mL** (see Table 1).

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A	B	C	D
Cell culture vessel	**Matrigel, PDL, and laminin in KO DMEM volume per well**	**DPBS volume for washes per well**	**Accutase® volume per well**
96-well plate	120 µL	150 µL	50 µL
24-well plate	250 µL	400 µL	125 µL
12-well plate	500 µL	1 mL	250 µL
6-well plate	1 mL	2 mL	500 µL
10-cm dish	4 mL	6 mL	3 mL
15-cm dish	12 mL	18 mL	9 mL

Table 1: Volumes of coating solutions (Matrigel, PDL, and laminin in KO DMEM), DPBS, and Accutase® for each cell culture vessel.

42

Place the coated plate back in the incubator at 37 °C .

hNIL differentiation into motor neurons: Day 3; Part 1 40m

43

If using PDL pre-coated plates, rinse once with DPBS and skip to step 46.

Aspirate out the 1X PDL from the receiving cell-culture vessel.

44

Wash the receiving wells.

44.1 Wash the receiving wells with 1X DPBS. (1/2)

44.2 Wash the receiving wells with 1X DPBS. (2/2)

45 Let receiving wells dry in the laminar flow hood for 20-30 minutes. 30m

46 Coat receiving cell-culture vessel with the appropriate volume of NIM with laminin mouse protein (at a concentration of 15 µg/ml) for 2-5 hours at 37 °C (see Table 1 for volume)

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96-well plate	120 µL	150 µL	50 µL
24-well plate	250 µL	400 µL	125 µL
12-well plate	500 µL	1 mL	250 µL
6-well plate	1 mL	2 mL	500 µL
10-cm dish	4 mL	6 mL	3 mL
15-cm dish	12 mL	18 mL	9 mL

hNIL differentiation into motor neurons: Day 3; Part 2 1h 30m

47 Prepare quenching media DMEM/F12 with 20% FBS at an appropriate volume. Per well in a 6- well plate, **0.5 mL** of quenching media will be needed.

48 Calculating the volume of day 3 NIM media needed

1. When calculating the volume of day 3 NIM media needed, consider that you will need the volumes in Table 2, as well as **1 mL** per 1 million cells resuspended before seeding.
2. That is, if you have about 3 million cells that you will seed on 2 wells of a 6-well plate and 72 wells of a 96-well plate, you will need roughly **3 mL** + **4 mL** + **10.8 mL** = **17.8 mL** of day 3 media. However, you should always prepare about 10% more to account for loss and round up, which in this case would be **20 mL** of day 3 NIM media.

A	B	C	D	E
Cell culture vessel	**Surface area (cm2)**	**Media volume per well**	**Day 3 Seeding density for ICC applications**	**Day 3 Seeding density for protein, RNA, DNA extraction**
96-well plate	0.32	150 µL	20-25k cells	-
24-well plate	1.9	500 µL	80-100k cells	-
12-well plate	3.5	1 mL	2 x 105 cells	-
6-well plate	9.6	2 mL	0.5 x 106 cells	1 x 106 cells
10-cm dish	56.7	10 mL	-	5 x 106 cells
15-cm dish	145	30 mL	-	15 x 106 cells

Table 2: Number of cells and volume of media matrix needed on the day-3 replating in the differentiation of hNIL iPSCs into motor neurons based on the size and format of the cell culture vessel used, as well as the application. Modification from the Kampmann's Lab protocol[3].

49 Preparing day 3 NIM media:

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A	B	C	D
Factor	**Final Concentration**	**Stock**	**Dilution**
ROCK1 inhibitor	10 µM	10 mM	1:1,000
Doxycycline	2 µg/mL	2 mg/mL	1:1,000
Compound E	0.2 µM	2 mM	1:10,000
Laminin	1 µg/mL	1 mg/mL	1:1,000
BrdU	40 µM	40 mM	1:1,000

Recommendation to minimize the chances of mitotically-active cells overwhelming the culture: Include Aphidicolin (a reversible polB inhibitor) at a final concentration of 5 µM in the well at every media change starting at day 3. This is particularly useful if maintaining the motor neurons for more than four weeks and for imaging purposes.

50

Aspirate the spent media and wash the wells with cells once with 1X DPBS -Ca/-Mg.

51

Add appropriate volume of Accutase® per well (**0.5 mL** /well in a 6-well plate).

52 5m

Incubate the plate for **00:05:00** at **37 °C** .

53 Remove plate from the incubator and tap the plate to release the cells.

54 Quench the Accutase® with DMEM/F12 + 20% FBS, at a volume equal to the volume of Accutase® per well (if you used **0.5 mL** of Accutase®, quench Accutase® with **0.5 mL** of DMEM/F12 + 20% FBS). DPBS +Ca/+Mg is also suitable.

55

Pipette the solution gently around the well thoroughly to ensure cells have dissociated from the well bottom.

56 Move the cells from their wells to a 15-mL vial (or vial of appropriate volume depending on how much volume of cells you have).

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57 3m

Centrifuge the vial at **800 rpm, 00:03:00** .

58 Aspirate the Accutase® with DMEM/F12 + 20% FBS, being careful not to disturb the cell pellet at the bottom of the vial.

59 Resuspend the cells with an appropriate volume of day 3 NIM media (about 1 mL per 1 million cells in the vial).

60 Count the cells using a Countess™ (or preferred counting method) using the same parameters from Day 0.

61 Aspirate the NIM with **15 µg/ml** laminin from each receiving well.

62 Plate appropriate number of cells per well using the appropriate media volume per well depending on your receiving cell culture vessel.

63 Sample calculation and coating advice for uniform seeding of cells onto receiving vessel:

63.1 Calculate the volume of day 3 NIM media needed per well.

63.2 Multiply that volume by the desired concentration of cells per well (e.g., 20k cells/well or 1 x 10^6 cells/well).

63.3 Divide that number by the concentration found by counting using the Countess™ (e.g. 2 x 10^6 cells/well).

63.4 The resulting number is the volume you need from the cell solution you resuspended in day 3 NIM media in step 59.

63.5

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,

Add that volume to your final desired volume of day 3 NIM media (e.g., **4 mL** if seeding onto 2 wells of a 6-well plate).

63.6

Mix well to ensure a uniform distribution of cells.

63.7 Seed appropriate media volume (which now has cells as well) onto the receiving cell culture vessel.

This is particularly important when seeding cells onto small wells (e.g., 96-well plates), where a uniform distribution of cells can improve survival rates.

64 Carefully place the plate with cells back in the incubator at **37 °C**, rocking the plate briefly after placing the plate back, such that the cells are uniformly distributed onto the well.

hNIL differentiation into motor neurons: Day 4 30m

65 Prepare the day 4 **NIM** media for performing a **full-media change**

A	B	C	D
Factor	**Final Concentration**	**Stock**	**Dilution**
B-27 Supplement		50x	01:50
Culture One Supplement		100x	1:100
Laminin	1 µg/mL	1 mg/mL	1:1,000
BDNF	20 ng/mL	20 µg/mL	1:1,000
GDNF	20 ng/mL	20 µg/mL	1:1,000
NT3	20 ng/mL	20 µg/mL	1:1,000

In addition, consider including Aphidicolin (a reversible polB inhibitor) at a final concentration of 5 µM in the well, if appropriate for your application.

66 Aspirate the media slowly from the wells, using a pipette, discarding into a reservoir.

67

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Add the fresh media slowly at a **direction normal to the well side wall**.

Never add the fresh media directly onto the well bottom, which will produce a shear force capable of lifting (and consequently killing) the neurons.

68 Place the plate back in the incubator at 37 °C .

hNIL differentiation into motor neurons: Day 7 30m

69 The risk of lifting only increases as the motor neurons age and mature on the well bottom.

1. Proper care is needed to ensure the neurons neither dry out nor lift; consider increasing the volume of media per well, starting on day 7 by up to 50%.
2. That is, if you had **150 µL** per well in a 96-well plate, then consider maintaining **200 µL** per well from now on, by aspirating **50 µL** per well on day 7, and adding back **100 µL** per well of fresh day 7 media.

70 Prepare the day 7 **NIM** media for performing a **half-media change.**

Note on growth factors BDNF, GDNF, and NT3 concentrations: although this is a half-media feed, we provide the full amount of growth factors, so their **dilution doubled**. **This doubling of the growth factor dilution is maintained during all half-media changes from here**.

A	B	C	D
Factor	**Final Concentration**	**Stock**	**Dilution**
B-27 Supplement		50x	1:50
Culture One Supplement		100x	1:100
Laminin	500 ng/mL	1 mg/mL	1:1,000
BDNF	20 ng/mL	20 µg/mL	**1:500**
GDNF	20 ng/mL	20 µg/mL	**1:500**
NT3	20 ng/mL	20 µg/mL	**1:500**

In addition, consider including Aphidicolin (a reversible polB inhibitor) at a final concentration of 5 µM in the well, if appropriate for your application.

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71 Aspirate the media slowly from the wells, using a pipette, discarding into a reservoir.

72

Add the fresh media slowly in at a direction normal to the well side wall (which is easier by tilting the plate about 60-degrees towards you).

Never add the fresh media directly onto the well bottom, which will produce a shear force capable of lifting (and consequently killing) the motor neurons.

73 Place the plate of motor neurons back in the incubator at 37 °C .

hNIL differentiation into motor neurons: Day 10 on 30m

74 Prepare Neurobasal Medium (**NMM**) as follows:

A	B
Neurobasal	97 mL
N-2	1 mL
NEAA	1 mL
GlutaMAX	1 mL

75 Prepare the day 10 NMM media for performing a **half-media change.**

A	B	C	D
Factor	**Final Concentration**	**Stock**	**Dilution**
B-27 Supplement		50x	1:50
Culture One Supplement		100x	1:100
Laminin	500 ng/mL	1 mg/mL	1:1,000
BDNF	20 ng/mL	20 µg/mL	**1:500**
GDNF	20 ng/mL	20 µg/mL	**1:500**
NT3	20 ng/mL	20 µg/mL	**1:500**

In addition, consider including Aphidicolin (a reversible polB inhibitor) at a final concentration of 5 µM in the well, if appropriate for your application.

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76 Aspirate the media slowly from the wells, using a pipette, discarding into a reservoir.

77

Add the fresh media slowly in at a direction normal to the well side wall (which is easier by tilting the plate about 60-degrees towards you).

78 Place the plate of motor neurons back in the incubator at **37 °C** .

79 Perform a half-media change with NMM and factors every 7 days for up to 9 weeks or more (if using the plate for imaging) or every 4 days (if using the plate for protein, RNA, or DNA extraction).

> The frequency of half-media changes was optimized for our genes of interest and applications and is recommended to be optimized for different genes and applications.

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80

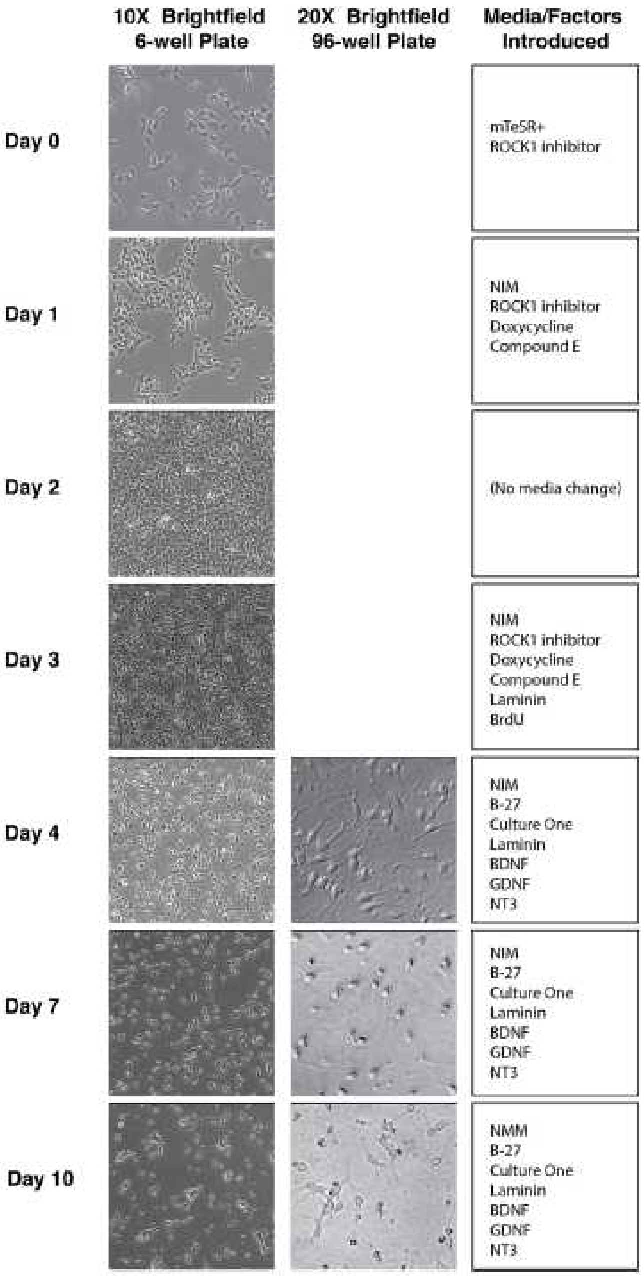

Figure: hNIL differentiation of iPSCs into motor neurons of WTC line initially on a 6-well plate (10X), then transferred to a 6-well plate and a 96-well plate (20X) after day-3 differentiation.

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