1. OBJECTIVE

This document is aimed clarify the methods for convert hiPSCs to Motor Neurons.

2. MATERIALS

2.1. High quality Human iPSCs.

2.2. Versene® (EDTA) 0.02% (LONZA, Cat.: BE17 – 711E)

2.3. Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium (STEMCELL Technologies, Cat.:#37250)

2.4. TeSR™-E8™ Kit for hESC/hiPSC Maintenance (E8 media, STEMCELL Technologies.: 05940) or (mTeSR™1, STEMCELL Technologies.: Catalog #85850)

2.5. Corning® Matrigel® (Corning®, Cat.: 356230)

2.6. Cell lifter (Corning®, Cat.: 3008)

2.7. 6 well Plates TC-Treated Culture Dish

2.8. Rock inhibitor Y-27632 (Rocki Y-27632 dihydrochloride, Tocris, Cat. No. 1254)

2.9. Basal medium

2.10. B-27™ Supplement (50X), serum free (ThermoFisher, Cat.: 17504001 or 17504044)

2.11. N-2 Supplement (100X), (ThermoFisher, Cat.: 17502001 or 17502048)

2.12. KnockOut™ DMEM/F-12 (ThermoFisher, Cat.: 12660012)

2.13. Neurobasal™ Medium (ThermoFisher, Cat.: 12660012)

2.14. GlutaMAX™ Supplement (ThermoFisher, Cat.: 35050061)

2.15. Penicillin streptomycin (ThermoFisher, Cat.:)

2.16. CHIR99021 (CHIR, Tocris, Cat. No. 4423)

2.17. DMH1 (Tocris, Cat. No. 4423)

2.18. SB431542 (SB, Tocris, Cat. No. 1614)

2.19. All-Trans Retinoic Acid (STEMCELL Technologies, Cat.:#72262)

2.20. Valproic acid (VPA, Sigma, Cat.: PHR1061-1G)

2.21. BDNF Recombinant Human Protein (ThermoFisher, Cat.: PHC7074)

2.21. Purmorphamine (PMN, Tocris, Cat. No. 4551)

2.23. Compound E (Cpd E, Tocris, Cat. No. 6476)

2.24. CNTF Recombinant Human Protein (ThermoFisher, Cat.: PHC7015)

2.25. IGF1 Recombinant Human Protein (ThermoFisher, Cat.: PHG0078)

2.26. Vertical laminar flow hood certified for Level II handling of biological materials.

2.27. Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO2 in air.

2.28. StemPro™ Accutase™ Cell Dissociation Reagent (ThermoFisher, Cat.: A1110501)

2.29. Micropipettes and tips (2, 20, 200, 1000 µL)

2.30. Centrifuge

3. STANDARD OPERATING PROCEDURE

3.1. Preparing solutions

Basal Media

1. **To make 50mL**

	To achieve a final concentration of:	Volume to be added based on stock concentrations:
DMEM/F12		25 mL
Neurobasal medium		25 mL
N2 Supplement 100x*	0.5x	250 µL
B27 Supplement 50x*	0.5x	500 µL
Glutamax	1x	500 µL
Penicillin streptomycin	1%	500 µL

**Defrost overnight at 2-8ºC*

Store media at 2-8ºC for up to two weeks.

Media: Day 1 to 6

1. **To make 50mL**

	To achieve a final concentration of:	Volume to be added based on stock concentrations:	Stock solutions
Basal Media		50 mL	
CHIR	3µM	7.5 µL	20 mM
DMH1	2µM	5 µL	20 mM
SB	2µM	10 µL	10 mM

Media: Day 7 to 12

1. **To make 50mL**

	To achieve a final concentration of:	Volume to be added based on stock concentrations:	Stock solutions
Basal Media		50 mL	
CHIR	1 µM	2.5 µL	20 mM
DMH1	2 µM	5 µL	20 mM
SB	2 µM	10 µL	10 mM
RA	0.1 µM	5 µL	1 mM
PMN(Pur)	0.5 µM	5 µL	5 mM

Media: EXPANSION

1. **To make 50mL**

	To achieve a final concentration of:	Volume to be added based on stock concentrations:	Stock solutions
Basal Media		50 mL	
CHIR	3 µM	7.5 µL	20 mM
DMH1	2 µM	5 µL	20 mM
SB	2 µM	10 µL	10 mM
RA	0.1 µM	5 µL	1 mM
PMN(Pur)	0.5 µM	5 µL	5 mM
VPA	0.5 mM	83 µL	300 mM

Media: Day 13 to 18

1. **To make 50mL**

	To achieve a final concentration of:	Volume to be added based on stock concentrations:	Stock solutions
Basal Media		50 mL	
RA	0.5 µM	25µL	1mM
PMN(Pur)	0.1 µM	1 µL	5 mM

Media: Day 19 to 28

1. **To make 50mL**

	To achieve a final concentration of:	Volume to be added based on stock concentrations:	Stock solutions
Basal Media		50 mL	
RA	0.5 µM	25 µL	1mM
PMN(pur)	0.1 µM	1 µL	5mM
Compound-E	0.1 µM	4.9 µL	1.02 mM
BDNF	10ng/mL	50 µL	10 µg/mL
CNTF	10ng/mL	50 µL	10 µg/ml
IGF-1	10ng/mL	50 µL	10 µg/ml

Media: Day 28 to 40

1. **To make 50mL**

	To achieve a final concentration of:	Volume to be added based on stock concentrations:	Stock solutions
Neurobasal medium		50 mL	
B27 Supplement	1x	1 mL	50x
Penicillin streptomycin	1%	500 µL	
BDNF	10ng/mL	50 µL	10 µg/mL
CNTF	10ng/mL	50 µL	10 µg/ml
IGF-1	10ng/mL	50 µL	10 µg/ml

iPSC Passage

N.B. Plate iPSCs onto 6 well matrigel-coated plates, at a density that will ensure cells reach approaching 100% confluence within 24 hours of plating 24 hours prior to desired neuralisation start time in general 3:1.

1. Inspect iPSCs for quality. Remove any differentiating cells prior to passage.

2. Aspirate total volume of media from each well, and rinse with HBSS at room temperature (RT) (1mL/well) to remove calcium.

3. Add 1mL/well RT versene (EDTA) and incubate (37°C; 5% CO_2; 5% O_2) for 4-7 minutes. Periodically check plates for signs of colonies detaching.

4. Aspirate total volume of versene and replace with 1mL/well RT E8 media. Working rapidly, use a cell lifter to gently detach colonies from plate.

5. Using a 1000µL pipette, carefully pipette up and down (no more than 2-3 times) to achieve a homogenous suspension of iPSC clusters in suspension.

6. Transfer – dropwise, using a 10mL stripette – the correct amount of suspension onto a matrigel-coated plate containing the correct amount of RT E8 media/well supplemented with 10µM of Y27632 (ROCK inhibitor, ROCKi) such that a total volume of 3mL/well is achieved.

7. Rock plates to distribute cells evenly across wells, and incubate (37°C; 5% CO_2) for 24 hours.

Neuralisation

1. Following 24 hour incubation, aspirate total volume of iPSC culture medium from each well and replace with 3mL/well warm E8 medium. Incubate plate for 2 hours (37°C; 5% CO_2).

2. After incubation, aspirate total volume of iPSC culture medium from each well, and replace with 3mL/well (6 well plates) of warm Day 1 to 6 medium (supplemented with 2µM DMH1 + 2µM SB43, SMAD inhibitors + CHIR). Incubate plate for 24 hours (37°C; 5% CO_2). Continue replacing medium with fresh medium containing SMADi every 24 hours until Day 6, when a uniform neuroepithelial sheet is observed.

3. On Day 6 aspirate total volume of culture medium from each well and replace with 3mL/well warm Day 7 to 12 medium. Incubate plate for 24 hours (37°C; 5% CO_2). Continue replacing medium with fresh Day 7 to 12 medium every 24 hours until Day 12, when neural rosettes can be observed.

Cell Passage

1. Before you start coat the plates with Matrigel.

2. Remove 1mL/well of media (to leave a final volume of 2mL/well) and treat cells that are to be passaged with 10µM Y27632, add 2 µL/ well of ROCKi (ROCK inhibitor, ROCKi) 1 hour before desired start time.

   As plating density is a determinant of neural fate, it is recommended that wells be passaged 1:6.

3. Remove total volume of medium from each well, and rinse with 2mL/well warm HBSS, Modified (Without Ca++ and Mg++). Add 1mL/well room temperature (RT) Accutase and incubate plate for 5 – 7 minutes (37°C; 5% CO_2), until material can be lifted from plate with gentle pipetting.

n.b. 2 Avoid over-triturating cells; attempt to lift all cells by pipetting up and down as gently and as little as possible (no more than 5-10 times). Take care to avoid introducing air bubbles.

4. Using a 1000µL pipette collect Accutase + cells and transfer to 15mL tube for centrifugation. Add 2x the volume of Accutase of warm Day 7 to 12 medium to the tube prior to centrifugation, to mitigate further dissociation.
5. Centrifuge cell suspension (900 RPM for 5 minutes) and carefully remove total volume of Accutase + media from pelleted cells.
6. Resuspend in 1 mL warm Day 7 to 12 medium + 10µM of ROCKi by gently pipetting 1-2 times with a 1000µL pipette. Use a 10mL stripette to add remaining media, and slowly pipette up and down to mix.
7. Aliquot day 7 to 12 medium + 10µM of ROCKi across a six well matrigel-coated plate, and using a 10mL stripette add appropriate volume of cell suspension to achieve a total volume of 3mL/well. Rock plate to distribute cells, and incubate for 24 hours (37°C; 5% CO_2).

   Note: Although it's tempting to have a look, do not remove cells from incubator until the following day!

8. Cells should attach within 24 hours. Following incubation, remove total volume of medium and replace with 3mL/well day 13 to 18 medium. Incubate plate (37°C; 5% CO_2) for 24 hours. Continue replacing medium with fresh day 13 to 18 medium every 24 hours until Day 18.

Expansion

Note: Monitor cultures for the emergence of neuron/progenitor-like cells. Upon appearance of progenitors, begin expansion (around day 12). In order to expand the cells replace the medium for expansion medium (containing 3µM CHIR, 2µM DMH1, 2µM SB, 0.1µM RA, 0.5µM Pur and 0.5mM VPA and Split 1:6 once a week. MNPs can be freeze with regular frozen medium.

It is recommended to passage between Days 10-12 – *recommend Day 12 passage.*

9. On Day 19 aspirate total volume of culture medium from each well and replace with 3mL/well warm Day 19 to 28 medium. Incubate plate for 24 hours (37°C; 5% CO_2).

10. Continue replacing medium with fresh Day 19 to 28 medium every 24 hours until Day 28, when mature motor neurons can be observed.

It is recommended to do the final passage between Days 19-21, using the same protocol but using warm Day 19 to 28 medium – *recommend Day 20 passage.*

Monitor cultures for changes in cell doubling time. Slower growth/inability of cells to reach 100% confluence within a few days following passage indicates that their proliferative capacity may be reduced – plate cells onto matrigel with the intent to terminally differentiate to neurons, or cryopreserve progenitors.

3.12. REFERENCES

1. Du ZW[1], Chen H[2], Liu H[1], Lu J[1], Qian K[3], Huang CL[1], Zhong X[1], Fan F[4], Zhang SC[5]. Generation and expansion of highly pure motor neuron progenitors from human pluripotent stemcells. Nat Commun. 2015 Mar 25;6:6626. doi: 10.1038/ncomms7626.