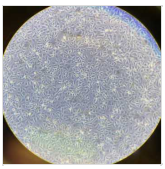

Jul 21, 2021

An Optimized Protocol for the Generation of Cortical Neurons from human iPSCs under Defined Conditions

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ABSTRACT

This protocol is about cortical differentiation.

ATTACHMENTS

Cortical Differentiation Protocol.pdf

DOI

dx.doi.org/10.17504/protocols.io.bu6znzf6

PROTOCOL CITATION

Clare L Parish, Lachlan L Thompson 2021. An Optimized Protocol for the Generation of Cortical Neurons from human iPSCs under Defined Conditions. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bu6znzf6

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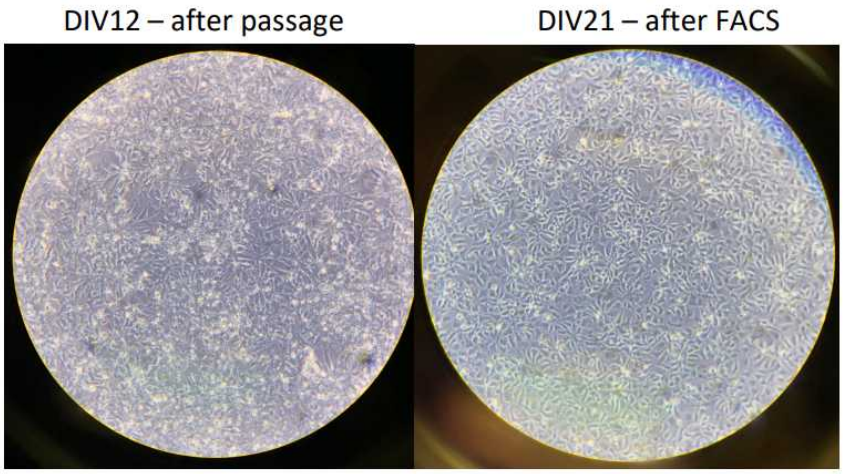

MATERIALS TEXT

Fix for staining:

A	B	C
d11, d16	OTX2 (rb) 1: 500 PAX6 M) 1:50 Fluorescent reporter (GFP/RFP)	Nestin (m) 1:800 SOX2 (g) 1:300 Fluorescent reporter (GFP/RFP)
d21, d28, d35	OTX2 (rb) 1: 500 PAX6 M) 1:50 Fluorescent reporter (GFP/RFP)	Nestin (m) 1:800 SOX2 (g) 1:300 Fluorescent reporter (GFP/RFP)
	Tbr1 (rb): Brn2 (g): Fluorescent reporter (GFP/RFP)	Tuj1 (m): Tbr1 (rb): Fluorescent reporter (GFP/RFP)

A	B	C
CTX Base media P/S 1X (100U/ml)		
DMEM/F12	48.2ml	241ml
NBM	48.2ml	241ml
B27 + VitA	1ml	5ml
N2	500ul	2.5ml
ITS-A	500ul	2.5ml
GMAX	500ul	2.5ml
P/S	1ml	5ml
B-mercap	90ul	450ul
TOTAL	100ml	500ml

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

Standard setup

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6x48 wells

Prepare CTX Base media:

A	B	C
CTX Base media P/S 1X (100 U/ml)		
DMEM/F12	48.2 ml	241 ml
NBM	48.2 ml	241 ml
B27 + VitA	1 ml	5 ml
N2	500 µl	2.5 ml
ITS-A	500 µl	2.5 ml
GMAX	500 µl	2.5 ml
P/S	1 ml	5 ml
B-mercap	90 µl	450 µl
TOTAL	100 ml	500 ml

Day -1: 6h

6h

1 Coat plastic cell culture plate (laminin 521 [M]**5 µg/ml**).

Make single cells of hPSCs using accutase for **06:00:00** - **07:00:00** @ **37 °C** .

Seed cells @ **375K/cm^2**.

d0:

2 mTESR1 media change ~1-2 hrs (up to 6 hrs) prior to differentiation initiation.

3

Wash cells with PBS-/- (**500 µl** for 48 wells, **200 µl** for 96 wells).

4 Add **500 µl d0 media** to initiate differentiation.

d0-11: Daily media change

5
- **500 µl CTX Base supplemented with SB (1:1000) LDN (1:2500)**

d11: Passage and fix 2h

2h

6
- 4 wells will be passaged 1:2.5 (10 in total) into 8 new hLaminin-coated wells.
- Remaining 2 wells will be fixed for staining
- hLaminin coat 8x 48 wells for passaging and allow > **02:00:00** for coating

d11: *Passage* 7m

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7

Aspirate media and wash wells with [M]**500 µl/well PBS-/-** .

8 4m

Add **500 µl EDTA** to each well and incubate for ~ **00:04:00** - **00:08:00** .

9 When clumps are ready to detach, collect into a 15 ml tube with **3 mL PBS-/-** .

Important note: in these stages of EDTA digestion, cells will be in clumps/rosettes, do not over-triturate suspension as this will break up cells resulting in poor rosette formation.

10

Wash each well again with 1x **500 µl EDTA** to ensure all cells have been collected.

11 3m

Centrifuge at **1100 rpm, 00:03:00** .

12 Aspirate supernatant and gently flick pellet.

13

Pipette **1 mL CTX base with SB/LDN + Ri (1:1000)** gently to get clumps/cells in suspension.

DO NOT vigorously pipette to single cell. Sometimes just the addition of the 1 ml is enough to distribute cells/clumps.

14 Add remaining CTX base with SB/LDN + Ri (1:1000) volume for [M]**600 µl/well** cell seeding.

15 Aspirate hLam from new plate and add **200 µl CTX base with SB/LDN + Ri (1:1000)** .

16

Pipette **600 µl cell suspspension** into each well.

17 Ensure even distribution by pipetting the peace sign gently.

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18

Allow clumps to attach, check on microscope and place gently into incubator.

d12: Media change every 2nd day 2h

19

- Aspirate the CTX media and refresh with **1 mL CTX base supplemented with FGF2 (1:5000)**
- From d14, perform media change every second day of CTX + FGF2.

d19: Passage and fix 2h

20 2h

- 5 wells will be passaged into 1:2.5; 10x 48 wells for maintenance
- Remaining 2.5 wells will be fixed for staining
- hLam coat 13x 48 wells for passaging and allow > **02:00:00** for coating

NB: At this point in the CTX differentiation protocol, a significant amount of progenitor expansion will have occurred in response to FGF2 exposure. Typically, a 5/6-fold increase in the cell numbers in achieved from Day 0 seeding. Cell numbers from 1 well of a 48-well plate can yield between 1.5-2.0 x 10^6 neural progenitors. Cells seeded at 4.5 x 10^5 cells/cm^2. Coat the required number of wells depending on specific application.

d19: *Passage* 7m

21

Aspirate media and wash wells with [M]**500 µl/well PBS-/-** .

22 4m

Add **500 µl Accutase** to each well and incubate for ~ **00:04:00** - **00:08:00** .

23 When cells are ready to detach, collect into a 15 ml tube with **3 mL PBS-/-** .

24

Wash each well again with 1x **500 µl PBS-/-** to ensure all cells have been collected.

25 3m

Centrifuge at **1400 rpm, 00:03:00** .

26 Aspirate supernatant and gently flick pellet.

27

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Pipette **1 mL CTX base with FGF2 + Ri (1:1000)** gently to get cells in suspension.

28 Perform cell count and transfer required volume of cell suspension for 4.5 x 10^5cell/cm^2 into a fresh centrifuge tube containing CTX base media supplemented with ROCKi (10 µM) and FGF2 (20 ng/ml).

29 Aspirate hLam from new plate and add **200 µl CTX base with FGF2 + Ri (1:1000)** .

30 

Pipette **300 µl cell suspspension** into each well.

31 Ensure even distribution by pipetting the peace sign gently.

32 

Allow cells to attach, check on microscope and place gently into incubator.

d20: Media change every 2nd day

33
- Media change with **1 mL CTX base (no factors or small molecules)** .
- Change media every 2nd day

d25: Passage and fix 2h

34 2h
- Cells are split at a 1:2 ratio. Coat enough plates in accordance with specific application.
- hLam coat wells for passaging and allow > **02:00:00** for coating

d25: *Passage* 7m

35 

Aspirate media and wash wells with **[M]500 µl/well PBS-/-** .

36 14m 

Add **500 µl Accutase** to each well and incubate for ~ **00:06:00** - **00:08:00** .

37 Add **300 µl CTX media** to each well and transfer cell suspension into a clean 15 ml centrifuge tube.

38 3m 

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Centrifuge at **1400 rpm, 00:03:00** .

39 Aspirate media and flick tube to dislodge pellet. Resuspend cells in (at least 5 ml) CTX base media supplemented with ROCKi.

40 Aspirate hLam from new plate and add
500 µl cell suspension in CTX base media supplemented with ROCKi .

41 Ensure even distribution by moving the plate in a 'figure 8' pattern.

42 

Allow cells to attach, check on microscope and place gently into incubator.

d26: Media change every 2nd day

43 - Media is replenished every other day.

d29: prepare coverslips for passaging

44 Remove sterile coverslips from Ethanol using sterile tweezers.

45 Place coverslips into 48-well plates and wash x 3 with 500 µL PBS-/-, with 5 min incubations, at Room temperature to remove all residual Ethanol.

46 Add 300 µL of Poly-L-Ornithine (PO) to each well and incubate overnight at 4˚C. Each well should contain a single coverslip.

d30: passage

47 Remove 48-well plate, containing PO-coated coverslips from the fridge and wash x 2 with 5-minute incubations at RT.

48 Add 190 µl of 5 µg/ml of L521 solution diluted in 1 x PBS +/+ to each well with a coverslip and incubate for 2 hours at 37˚C.

49 Dissociate cells as performed at D25 timepoint.

50 Add 300 µl of Accutase to each of the wells and place in the incubator for 10 minutes at 37˚C.

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51 Following incubation, add 300 µL of CTX media to each well, harvest cells and transfer cell suspension into a clean 15 ml centrifuge tube.

52 Centrifuge cell suspension at 1,400 rpm for 4 minutes.

53 Aspirate media and flick tube to dislodge pellet. Resuspend cells in (at least 5 ml) CTX base media supplemented with ROCKi, 10 µM.

d37-55: maturation

54 Media is aspirated and replaced with maturation media (NBB27 media) supplemented with BDNF (40 ng/ml), GDNF (40 ng/ml), dcAMP (0.05 mM), AA (200 nM) and laminin (1 µg/ml; Sigma-Aldrich).

55 Cortical neurons are maintained in this media with growth factors until Day 55, where a final immunocytochemical analysis for cortical patterning is performed.

Fix: 8m

56 

Wash wells with 200 µL PBS-/-.

57 Add **200 µl 4% PFA** for **00:08:00** . 8m

58 Aspirate PFA.

59 

Wash 3x with PBS-/-.

59.1 Wash with PBS-/-.

59.2 Wash with PBS-/-.

59.3 Wash with PBS-/-.

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60 Fill wells with PBS/Azide or begin staining.

Staining: 20m

61 Permeabilize cells with **200 µl** - **500 µl PADT** for **00:20:00** - **00:30:00** . 20m

62 

Aspirate PADT and pipette on primary antibodies to incubate **Overnight** at **4 °C** .

63 Aspirate primaries.

64 

Wash wells 2x with PBS-/-.

64.1 Wash wells with PBS-/-.

64.2 Wash wells with PBS-/-.

65 Block with **500 µl PADT** for **00:30:00** . 30m

66  1h

Aspirate PADT and pipette on secondaries to incubate for **01:00:00** - **01:30:00** at room temperature.

67 

Aspirate secondaries and wash 2x with **500 µl PBS-/-** .

67.1 Wash with **500 µl PBS-/-** .

67.2 Wash with **500 µl PBS-/-** .

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68 DAPI stain for **00:08:00** - **00:10:00** . 8m

69

Wash DAPI with 1-2x PBS-/- wash.

69.1 Wash DAPI with PBS-/- wash.

69.2 Optional: Wash DAPI with PBS-/- wash.

70 Fill well with PBS/Azide for long-term storage.

71

Image.

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