Reagent table:

Item	Vendor	Catalog #
DMEM/F12	Gibco	11320033
mTESR-plus Medium	StemCell Tech	100-0276
Neurobasal medium	Gibco	21103049
N2 supplement	Gibco	17502048
B27 supplement without vitamin A	Gibco	12587-010
L-Glutamine	Sigma	G8450
Penicillin-Streptomycin	Gibco	15140122
Y-27632 Dihydrochloride Rock inhibitor	ToCris	CD0141
DPBS (No calcium, No magnesium)	Cytiva	SH30028
Accutase	Innovative Cell Tech	AT104
Matrigel	Corning	354230
Laminin	R&D Systems	3400-010-02
Poly-L-Ornithine	Sigma	P3655
LDN	Stemgent	04-0074
SHH C25II	R&D Systems	1845-SH-100
SB431542	SelleckChem	S1067
CHIR99021	ToCris	4423
GDNF	PeProtech	450-10
BDNF	PeProtech	450-02
Dibutyryl-cAMP (Bucladesine)	SelleckChem	S7858
Sodium L-Ascorbate	Sigma	A40-34
TGF□3	R&D Systems	8420-B3-005
DAPT	Tocris	2634

Protocol:

Note1: This protocol has been used to differentiate dopaminergic neurons from hPSC adapted to feeder free culture systems. Our version of the protocol has been adapted from Kim et al 2021; Cell Stem Cell 28, 343–355 e5, February 4, 2021 and Piao et al. 2021; Cell Stem Cell 28, 217-229 e7, February 4, 2021.

Note2: We have used two alternative protocol variants with minor modifications which will be outlined at the respective steps as **SP** (Soldner Protocol) and **HP** (Hockemeyer Protocol).

Note3: It is important to start the differentiation from pristine, undifferentiated feeder free cultures. For more details consult: https://doi.org/10.17504/protocols.io.b4mcqu2w

Plate preparation

- **Matrigel/Geltrex coating**: Prepare matrigel/geltrex (1:30) in cold DMEM/F12 or DPBS in a 15 ml tube as described by the manufacturer. Coat each well with 1-1.5 ml (6 well plate size) or 0.5 ml (12w plate size) of solution and incubate at 37 C incubator for at least 30-60 min.
- **Laminin coating**: Prepare laminin (2 µg/ml) in cold DPBS and coat each well of a 6-well plate with 1.5ml laminin solution (2 µg/ml) and incubate overnight at 37 C.
- **Poly-L-Ornithine (PLO) + Fibronectin (F) + Laminin (L) coating**: Prepare PLO (15 µg/ml) in sterile water and coat each well with 0.5-1 ml making sure it covers the entire surface. Incubate at 37 C for 6 hours to overnight. The day after, wash each well with sterile water 4 times and add a new solution with fibronectin (1 µg/ml) and laminin (2 µg/ml) (in water) and incubate overnight at 37 C. Do not let the wells dry.
- **Poly-L-Ornithine (PLO) + Laminin (L) coating**: Prepare PLO (15 µg/ml) in sterile water and coat each well of a 12-well plate with 0.5-1 ml making sure it covers the entire surface. Incubate at 37 C for 6 hours to overnight. The day after, wash each well with sterile water 2 times and add a new solution with laminin (2 µg/ml) (in cold DPBS) and incubate overnight at 37 C. Do not let the wells dry.

Media recipes

- **Media A**: Neurobasal media + N2 supplement (1% vol/vol) + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + SHH (200 ng/ml) + CHIR99021 (0.7 µM) + LDN (250 nM) + SB431542 (10 µM).
- **Media B**: Neurobasal media + N2 supplement (1% vol/vol) + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + SHH (200 ng/ml) + CHIR99021 (7.5 µM) + LDN (250 nM) + SB431542 (10 µM).
- **Media C**: Neurobasal media + N2 supplement (1% vol/vol) + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + CHIR99021 (7.5 µM).

- **Media D**: Neurobasal media + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + BDNF (20 ng/ml) + GDNF (20 ng/ml) + Ascorbic acid (200 µM) + Dibutyryl-cAMP (0.5 mM) + TGF□3 (1ng/ml) + CHIR99021 (3 µM).
- **Precursor splitting media**: Neurobasal media + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + BDNF (20 ng/ml) + GDNF (20 ng/ml) + Ascorbic acid (200 µM) + Dibutyryl-cAMP (0.5 mM) + TGF□3 (1ng/ml).
- **Maturation media**: Neurobasal media + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + BDNF (20 ng/ml) + GDNF (20 ng/ml) + Ascorbic acid (200 µM) + Dibutyryl-cAMP (0.5 mM) + TGF□3 (1ng/ml) + DAPT (10 µM).

Dopaminergic neuron differentiation:

1. Day 0: Dissociate hPSCs using accutase (10 min - 37C). Quench dissociation by diluting the accutase solution with mTeSR-plus media + 10 µM Y-27632 and collect the cells into a 15ml conical tube. Spin the cells down at 105 rcf/g - 5 min. Remove the supernatant, resuspend the cells in mTeSR-plus media + 10 µM Y-27632 and count them.

   SP: Plate the cells at 400-600k/cm2 in media A + 10 µM Y-27632 onto geltrex coated plates (adjust cell number to the plate size being used).

   HP: Plate the cells at 400-600k/well in a 6-well plate in mTeSR-plus + 10 µM Y-27632 onto matrigel coated plates.
2. Day 1-3: Change the media - media A (change daily or every other day as necessary).
3. Day 4 and day 6: Change the media- media B (change daily or every other day as necessary).
4. Day 7 and Day 9: Change the media - media C (change daily or every other day as necessary).
5. Day 10: Change the media - media D.
6. Day 11: Passage #1. Dissociate cells using accutase (approximately 10 min - 37 C but longer incubations may be necessary to properly detach the cells). Quench dissociation by diluting the accutase solution with 1 ml/well media D + 10 µM Y-27632. Collect cells in 15ml conical tubes and spin them down at 300g - 5 min. Remove the supernatant, resuspend the cell in media D + 10 µM Y-27632.

   SP: Count the cells and plate them at 800k/cm2 in media D + 10 µM Y-27632 onto PLO+F+L coated plates.

   HP: Split 1:2 in precursor splitting media + 10 µM Y-27632 onto laminin-coated plates.
7. Day 12-15: Change the media - maturation media (change daily or every other day as necessary).
8. Day 16: Passage #2. Dissociate cells using accutase (approximately 10 min - 37 C but longer incubations may be necessary to properly dissociate collect the cells). Quench dissociation by diluting accutase with 1 ml/well precursor splitting media + 10 µM Y-27632. Collect the cells in 15 ml conical tubes and spin them down at 300g - 5 min. Remove the supernatant, resuspend the cell in precursor splitting media + 10 µM Y-27632 and count them.

   SP: Plate the cells at 800k/cm2 in precursor splitting media + 10 µM Y-27632 onto PLO+F+L coated plates.

   HP: Plate the cells at >1 million cells per well of 12-well plate in maturation media + 10 µM Y-27632 onto PLO+L coated plates.

9. Day 17-24: Change the media - maturation media (change daily or every other day as necessary).
10. Day 25: Passage #3.

    SP: Dissociate cells using papain solution (30 min - 37C). Inactivate papain solution using ovomucoid trypsin inhibitor (10mg/ml). Collect your cells in 15 ml conical tubes and spin them down at 300g - 5 min. Remove the supernatant, resuspend the cells in maturation media + 10 μM Y-27632 and count them. Plate the cells at 200-300k/cm2 in maturation media + 10 μM Y-27632 onto PLO+L+F coated plates.

    HP: Dissociate cells using accutase (approximately 10 min - 37C is the usual standard but longer incubations may be necessary to properly detach the cells). Quench accutase by adding maturation media + 10 μM Y-27632. Collect your cells in 15 ml conical tubes and spin them down at 300g - 5 min. Remove the supernatant, resuspend the cells in maturation media + 10 μM Y-27632 and count them. Plate the cells at >1 million cells per well of 12-well plate in maturation media + 10 μM Y-27632 onto PLO+L coated plates.
11. Day >26: Change media to maturation media (perform media changes every 2-3 days). Cells can be maintained in this media for several months or until experiment.