

May 06, 2020

Midbrain dopaminergic differentiation of human pluripotent stem cells

 Neurobiology of Disease

DOI

dx.doi.org/10.17504/protocols.io.bddpi25n

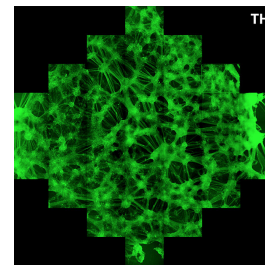
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DOI: <https://dx.doi.org/10.17504/protocols.io.bddpi25n>

External link: <https://onlinelibrary.wiley.com/doi/full/10.1111/ejn.14286>

Protocol Citation: Tilo Kunath, Szuping Chiu, Sophie Glendinning, Yixi Chen, Nicola Drummond, Maurice Canham 2020. Midbrain dopaminergic differentiation of human pluripotent stem cells. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.bddpi25n>

**Manuscript citation:**

Chen Y, Dolt KS, Kriek M, Baker T, Downey P, Drummond NJ, Canham MA, Natalwala A, Rosser S, Kunath T. (2019) Engineering synucleinopathy-resistant human dopaminergic neurons by CRISPR-mediated deletion of the SNCA gene. *Eur J Neurosci.* 49:510-524. doi: 10.1111/ejn.14286.

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Protocol status: Working

We use this protocol in our group and it is working.

Created: March 07, 2020

Last Modified: May 06, 2020

Protocol Integer ID: 33935

Keywords: hESC, iPSC, induced pluripotent stem cells, human embryonic stem cells, neuronal, midbrain, dopaminergic, mesencephalic, human pluripotent stem cells differentiation, midbrain dopaminergic, dopaminergic differentiation, mda neuron, mda neurons with mixed substantia nigra pars compacta, human pluripotent stem cell, human embryonic stem cell, induced pluripotent stem cell, pluripotent stem cell, embryonic stem cell, midbrain floor plate radial glia, ventral tegmental area identity, neuron, midbrain floor plate, cell line, radial glia, hipsc line, cell

Abstract

Differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) into midbrain dopaminergic (mDA) neurons is described in this protocol. This protocol has been adapted from published protocols from three laboratories, Lorenz Studer (New York), Malin Parmar/Agnete Kirkeby (Lund), and Su-Chun Zhang (Madison). The relevant papers from these labs are PMIDs: 20362538, 22056989, 22813745, 28094017, 28858290, 22696177. This protocol instructs hESCs and hiPSCs to adopt a midbrain floor plate radial glia-like identity before maturation into mDA neurons with mixed substantia nigra pars compacta and ventral tegmental area identity. It has been applied to more than 10 hESC and hiPSC lines in our laboratory, with some cell lines producing exceptional results, and others less so. Our version the protocol is published here PMID: 30472757.

Attachments



[ejn.14286.pdf](#)

2MB



[mDA protocol](#)

[Schemat...](#)

36KB





Materials


MATERIALS

- ✕ Biolaminin 111 LN **Biolamina Catalog #LN111**
- ✕ B27 supplement without retinoic acid (50x) **Gibco - Thermo Fisher Scientific Catalog #17504044**
- ✕ 24-well cell culture plate **Costar Catalog #3524**
- ✕ Neurobasal Medium (1x) **Gibco - Thermo Fisher Scientific Catalog #21103049**
- ✕ StemMACS™ iPS-Brew XF human: Basal medium and supplement **Miltenyi Biotec Catalog #130-104-368**
- ✕ StemMACS™ CHIR99021 **Miltenyi Biotec Catalog #130-103-926**
- ✕ Poly-L-Ornithine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P4957**
- ✕ Dulbecco's PBS (with calcium magnesium) **Gibco - Thermo Fisher Scientific Catalog #14040083**
- ✕ DMEM/F12 **Gibco - Thermo Fisher Scientific Catalog #21331020**
- ✕ UltraPure 0.5 M EDTA pH8 **Invitrogen - Thermo Fisher Catalog #15575020**
- ✕ Dulbecco's PBS (without calcium magnesium) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8537**
- ✕ N2 supplement (100x supplement) **Gibco - Thermo Fisher Scientific Catalog #17502048**
- ✕ L-Glutamine (200mM) **Thermo Fisher Scientific Catalog #25030024**
- ✕ Recombinant human BDNF **peprotech Catalog #450-02**
- ✕ Recombinant human GDNF **peprotech Catalog #450-10**
- ✕ Recombinant human FGF-8b **peprotech Catalog #100-25**
- ✕ Recombinant Human Sonic Hedgehog/Shh (C24II) N-Terminus **R&D Systems Catalog #1845-SH-500**
- ✕ L-Ascorbic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4403**
- ✕ DAPT **Tocris Catalog #2634**
- ✕ Dibutyl cAMP **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0627**
- ✕ StemMACS™ LDN-193189 **Miltenyi Biotec Catalog #130-103-925**
- ✕ Heparin sodium salt from porcine intestinal mucosa **Merck MilliporeSigma (Sigma-Aldrich) Catalog #H3149**
- ✕ Accutase® solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A6964**
- ✕ Trypan Blue **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154**
- ✕ STEM-CELLBANKER - GMP Grade **amsbio Catalog #11890**
- ✕ SB431542 **Merck Millipore (EMD Millipore) Catalog #616461-5MG**
- ✕ 7.5% Bovine Serum Albumin solution in DPBS **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A8412**
- ✕ DMSO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D2650**



 Biolaminin 521 LN **Biolamina Catalog #LN521**


 StemMACS™ LDN-193189 in solution **Miltenyi Biotec Catalog #130-106-540**

 Y-27632 dihydrochloride **Tocris Catalog #1254**

 200 mM L-glutamine **Invitrogen - Thermo Fisher Catalog #25030-024**

Troubleshooting

Before start

Before starting it is helpful to create a relatively large cryopreserved bank of hESCs or hiPSCs at one particular passage (20 or more vials). Upon thawing each vial perform the mDA differentiation protocol within one or two passages of recovering the cells. It is not advisable to keep the cells 'ticking' over, and initiating mDA differentiations at different passages of the cell line, as this reduces reproducibility. This method of working is well-described by Mark Tomishima's CryoPause publication.  [CryoPause_Tomishima.pdf](#)










Preparing reagents for mDA differentiation

1 Prepare 10 mM (1000x) stock of SB431542.

$C_{22}H_{16}N_4O_3 \cdot 2H_2O$. MW: 420.42 g/mol.

1.1 To prepare 1190 µl of 10 mM SB431542:





Add  1190 µL (2 x  595 µL) of **DMSO** (Sigma Aldrich, cat no. D2650) directly to vial containing  5 mg of **SB431542** (Merck Millipore, cat no. 616461-5MG) and mix by pipetting until powder is completely dissolved.

Prepare  20 µL -  50 µL aliquots of **10 mM SB431542** in sterile 1.5-ml Eppendorfs tubes and store at  -80 °C for up to one year. Once thawed, keep **SB431542** aliquot at  Room temperature in the dark for up to two weeks.

2 Prepare 10 mM (100,000x) and 0.1 mM (1000x) stocks of LDN193189.





$C_{25}H_{22}N_6$. MW: 406.48 g/mol.

2.1 To prepare 492 µl of 10 mM LDN193189:

Add  492 µL of **DMSO** (Sigma Aldrich, cat no. D2650) directly to vial containing  2 mg of **LDN193189** (Miltenyi Biotec, cat no. 130-103-925) to obtain a  10 millimolar (mM) solution. Warm briefly (3-5min) at  37 °C to dissolve powder.

Alternatively, **LDN193189** can be obtained in solution at 10 mM in DMSO (Miltenyi Biotec, cat no. 130-106-540).



Dilute **10 mM LDN193189** at 1 in 100 with **DMSO** (Sigma Aldrich, cat no. D2650) to create  0.1 millimolar (mM) (1000x) stocks.

Prepare  20 µL -  50 µL aliquots of **0.1 mM LDN193189** in sterile 1.5-ml Eppendorfs tubes and store at  -80 °C for up to one year. Once thawed, keep **LDN193189** aliquot at  Room temperature in the dark for up to two weeks.








3 Prepare 600 µg/ml (1000x) stock of Sonic Hedgehog (SHH-C24II).

3.1 To prepare 833 µl of 600 µg/ml SHH-C24II:

Add volume of  822 µL of **DPBS without Mg²⁺ and Ca²⁺** (Thermo Fisher, Cat no. 14190086) directly to vial containing  500 µg of **SHH-C24II** (R+D Systems, cat no. 1845-SH-500).





Add  11 µL of **7.5% BSA** (Sigma Aldrich, cat no. A8412-100ML) directly to the vial.

Mix by pipetting up and down several times until the powder is dissolved.




Prepare  20 µL -  50 µL aliquots of  600 µg/ml **SHH** in sterile 1.5-ml Eppendorfs tubes and store at  -80 °C for up to one year. Once thawed, keep **SHH** aliquot at  4 °C for up to two weeks.

4 Prepare 10 mM stock of CHIR99021.

4.1 To prepare 2414 µl of 10mM CHIR99021:



Add  2149 µL of **DMSO** (Sigma Aldrich, cat no. D2650) directly to vial containing  10 mg of **CHIR99021** (Miltenyi Biotech, Cat no 130-103-926) to obtain a  10 millimolar (mM) solution. Mix by pipetting up and down several times to dissolve the powder. If precipitate is observed, warm briefly (2-5min) at  37 °C .

Alternatively, **CHIR99021** can be obtained in solution at 10 mM in DMSO (Miltenyi Biotech, Cat no. 130-106-539)

Prepare  10 µL aliquots of **10 mM CHIR99021** in sterile 1.5-ml Eppendorfs tubes and store at  -80 °C for up to one year. Once thawed, keep **CHIR99021** aliquot at  Room temperature in the dark for up to two weeks.





5 Prepare 100 µg/ml (1000x) stock of recombinant FGF8b

5.1 To prepare 250 µl of 100 µg/ml FGF8b:

Add  246 µL of **DPBS without Mg²⁺ and Ca²⁺ (Thermo Fisher, Cat no 14190086)** directly to vial containing  25 µg of **FGF8b (R+D Systems, Cat no 423-F8-025)**

Add  3.4 µL of **7.5% BSA (Sigma Aldrich, cat no. A8412-100ML)** directly to the vial.

Mix by pipetting up and down several times to dissolve the powder.

Prepare  20 µL -  50 µL aliquots of **100 µg/ml FGF8b** in sterile 1.5-ml Eppendorfs tubes and store at  -80 °C for up to one year. Once thawed, keep **FGF8b** aliquots at  4 °C in the dark for up to two weeks.



6 Prepare 1 mg/ml (1000x) stock of Heparin Sodium Salt from porcine intestinal mucosa

6.1 To prepare 55 ml of 1 mg/ml Heparin:

Add  55 mL of **DPBS without Mg²⁺ and Ca²⁺ (Thermo Fisher, Cat no 14190086)** directly to vial containing  55 mg of **Heparin (Sigma Aldrich, Cat no H3149)**.



Mix by pipetting up and down several times to dissolve the powder.


Filter the reconstituted **Heparin solution** using an appropriately sized syringe for the volume and 0.33µM filter

Prepare  500 µL aliquots of **[1M] 1 mg/ml Heparin** in sterile 1.5-ml Eppendorfs tubes and store at  4 °C for up to two years.





7 Prepare 20 µg/ml (1000x) stock of recombinant Bone-Derived Growth Factor (BDNF).

7.1 To prepare 5 ml of 20 µg/ml BDNF:

Add  4930 μL of **DPBS without Mg^{2+} and Ca^{2+} (Thermo Fisher, Cat no 14190086)** directly to vial containing  100 μg of **BDNF (PeproTech, Cat no 450-02)**.



Add  70 μL of **7.5% BSA (Sigma Aldrich, cat no. A8412-100ML)** directly to the vial.


Mix by pipetting up and down several times to dissolve the powder.

Prepare  20 μL -  50 μL aliquots of [M] 20 $\mu\text{g}/\text{ml}$ **BDNF** in sterile 1.5-ml Eppendorfs tubes and store at  $-80\text{ }^{\circ}\text{C}$ for up to one year. Once thawed, keep **BDNF** aliquots at  $4\text{ }^{\circ}\text{C}$ for up to two weeks.

8 **Prepare 20 $\mu\text{g}/\text{ml}$ (2000x) stock of recombinant Glial Derived Growth Factor (GDNF).**

8.1 **To prepare 5 ml of 20 $\mu\text{g}/\text{ml}$ GDNF:**

Add  4930 μL of **DPBS without Mg^{2+} and Ca^{2+} (Thermo Fisher, Cat no 14190086)** directly to vial containing  100 μg of **GDNF (PeproTech, Cat no 450-10)**.



Add  70 μL of **7.5% BSA (Sigma Aldrich, cat no. A8412-100ML)** directly to the vial.

Mix by pipetting up and down several times to dissolve the powder.

Prepare  20 μL -  50 μL aliquots of [M] 20 $\mu\text{g}/\text{ml}$ **GDNF** in sterile 1.5-ml Eppendorfs tubes and store at  $-80\text{ }^{\circ}\text{C}$ for up to one year. Once thawed, keep **GDNF** aliquots at  $4\text{ }^{\circ}\text{C}$ for up to two weeks.





9 **Prepare 0.2 M (1000x) stock of Ascorbic Acid by Sigma Aldrich. $\text{C}_6\text{H}_8\text{O}_6$, MW: 176.12 g/mol.**

9.1 **To prepare 2841 μL 0.2 M Ascorbic Acid:**

Add  2841 μL of **sterile water** directly to vial containing  100 mg of **Ascorbic Acid (Sigma Aldrich, Cat no A4403)**.



Mix by pipetting up and down several times to dissolve the powder.

Filter the reconstituted **Ascorbic Acid** solution using an appropriately sized syringe for the volume and a 0.33µM filter.

Prepare  20 µL -  50 µL aliquots of [M] 0.2 Molarity (M) **Ascorbic Acid** in sterile 1.5-ml Eppendorfs tubes and store at  -80 °C for up to one year. Once thawed, keep **Ascorbic Acid** aliquots at  4 °C in the dark for up to two weeks.





10 **Prepare 0.5 M (1000x) stock of dcAMP** (N⁶,2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate sodium salt) **by Sigma Aldrich**. C₁₈H₂₃N₅O₈PNa, MW: 491.3 g/mol.

10.1 **To prepare 4070 µl 0.5 M dcAMP:**

Add  4070 µL of **sterile water** directly to vial containing  1000 mg of **dcAMP** (Sigma Aldrich, Cat no D0627).



Mix by pipetting up and down several times to dissolve the powder.


Filter the reconstituted **dcAMP** solution using an appropriately sized syringe for the volume and a 0.33µM filter.

Prepare  20 µL -  50 µL aliquots of [M] 0.5 Molarity (M) **dcAMP** in sterile 1.5-ml Eppendorfs tubes and store at  -80 °C for up to one year. Once thawed, keep **dcAMP** aliquots at  4 °C in the dark for up to two weeks.





11 **Prepare 10 mM (10,000x) stock of DAPT**
C₂₃H₂₆F₂N₂O₄, MW: 432.46 g/mol.

11.1 **To prepare 2312 µl 10 mM DAPT:**

Add  2312 µL of **DMSO** (Sigma Aldrich, cat no. D2650) directly to vial containing  10 mg of **DAPT** (Tocris, Cat no 2634).

Mix by pipetting up and down several times to dissolve the powder. If precipitate is observed, warm the solution to  37 °C for 2-5 mins.





Prepare  20 μL aliquots of  10 millimolar (mM) **DAPT** in sterile 1.5-ml Eppendorfs tubes and store at  $-80\text{ }^{\circ}\text{C}$ for up to one year. Once thawed, keep **DAPT** aliquots at  $4\text{ }^{\circ}\text{C}$ in the dark for up to 1 month.





12 **Prepare 10 mM (1000x) stock of Y-27632 dihydrochloride**

$\text{C}_{14}\text{H}_{21}\text{N}_3\text{O}_2\cdot 2\text{HCl}$. MW: 320.26 g/mol.

12.1 **To prepare 15.61 ml 10 mM Y-27632:**


Add  15.61 mL of **DPBS without Mg^{2+} and Ca^{2+}** (Thermo Fisher, Cat no 14190086) directly to vial containing  50 mg of **Y-27632 dihydrochloride** (Tocris, Cat no 1254).

Mix by pipetting up and down several times to dissolve the powder.

Prepare aliquots of  10 millimolar (mM) **Y-27632 dihydrochloride** in sterile 1.5-ml Eppendorfs tubes and store at  $-80\text{ }^{\circ}\text{C}$ for up to one year. Prepare smaller  50 μL aliquots for working use and larger  500 μL aliquots which can be aliquoted out at a later date.


Once thawed, keep **Y-27632** aliquots at  $4\text{ }^{\circ}\text{C}$ in the dark for up to 1 month.

13 **Prepare aliquots of N2**



N2 stocks should be stored at  $-20\text{ }^{\circ}\text{C}$. To avoid freeze thawing it is helpful to create working aliquots.

13.1 Prepare  0.5 mL aliquots of **100x N2** (Thermo Fisher, Cat 17502048) in sterile 1.5-ml Eppendorfs tubes and store at  $-20\text{ }^{\circ}\text{C}$ for up to one year.

14 **Prepare aliquots of B27 (without RA)**

B27 stocks should be stored at  $-20\text{ }^{\circ}\text{C}$. To avoid freeze thawing it is helpful to create working aliquots.




- 14.1 Prepare  1 mL aliquots of **50x B27 (without RA)** (Thermo Fisher, Cat no 17504044) in sterile 1.5-ml Eppendorfs tubes and store at  -20 °C for up to one year.

Preparation for mDA D0

- 15 On mDA day 0 cells hPSCs are lifted as clumps using EDTA and seeded onto plates that have been pre-coated with Laminin-111.

Plates need to be coated with Laminin 111 at least 12 hours prior to the beginning the differentiation.

15.1 Pre-Coating plates with Laminin 111:


Laminin-111 stocks (0.1mg/ml) (Biolamina, Cat no LN111) should be thawed at RT and then diluted 1:20 in **DPBS (with Ca²⁺ and Mg²⁺)** (ThermoFischer, cat no. 14040083) to create a final working stock solution of  5 µg/ml **Laminin-111**.

The diluted laminin should be mixed thoroughly and added to the wells at a volume recommended for the well size (see below table).

Plate type	Approx Area of Well (cm ²)	Volume of 5 µg/ml L521 (ul) required for optimal coating
24 well plate	2	300
48 well plate	1	150

Move the plate gently around to ensure the well is evenly covered

Wrap the plate in parafilm to reduce evaporation

Incubate the plate at  4 °C overnight for at least 12 hours (overnight incubation is recommended).


Coated plates can be stored at  4 °C for 3 weeks.


16 Preparation of 0.5mM EDTA from ThermoFisher.



To lift hPSCs as clumps for seeding, a stock of **0.5mM EDTA** is prepared from **50mM Ultra Pure EDTA** (ThermoFisher, Cat no 15575020).

16.1 To prepare 50ml of 0.5mM EDTA:

Take an  50 μ L aliquot of [M] 50 millimolar (mM) **Ultra Pure EDTA** (ThermoFisher, Cat no 15575020), and add to a 50ml falcon tube.

Add  50 mL of **DPBS (without Ca^{2+} and Mg^{2+})** (ThermoFischer, cat no. 14040083) directly to the 50ml falcon tube and mix to create a [M] 0.5 millimolar (mM) **EDTA** stock

Filter the diluted **EDTA** through a 0.33 μ M filter into a fresh 50ml falcon tube.


Label and store **0.5 mM EDTA** stock at  Room temperature .

0.5mM EDTA can be kept at  Room temperature for 6 months.






17 Preparation of Neural Induction Medium (NIM):

50% DMEM/F12 + 50% Neurobasal + 1x N2 + 1x B27 + 2mM L-Glutamine.

Between mDA D0 and mDA D4 cells are cultured within NIM.

The components which form the base media NIM (N2 supplement, B27 supplement, DMEM/F12 and Neurobasal media) are in themselves only stable for 2 weeks due to the presence of light sensitive components. Thus, fresh base media should be made up at the start of each differentiation and kept in the dark at  -4 °C for up to 2 weeks.

17.1 To prepare 50ml of NIM:

Add  24 mL of **DMEM/F12** (Thermo Fisher, Cat no 21331020) +  24 mL of **NeurobasalTM Medium** (Thermo Fisher, Cat no 21103049) +  500 μ L of **100x N2** (Thermo Fisher, Cat no 17502048) +  1 mL of **50x B27** (Thermo Fisher, Cat no 17504044) +  500 μ L of **200mM L-Glutamine** (Thermo Fisher, Cat no 25030032).

mDA Day 0

18 mDA Day 0 plating

On mDA Day 0 cells are lifted as clumps using 0.5mM EDTA and then seeded onto Laminin-111 coated plates.

Before beginning; self-renewing cultures of hPSCs must be free of spontaneous differentiation and confluency should be between 70% and 90% before use.




Prior to mDA D0 seeding, human ESC/iPSC cultures are grown on Laminin-521 (5µg/ml) matrix coated 6-well plates with daily feeding using 2 ml StemMACS iPS-BrewXF (Miltenyi Biotec). Each well of a 6-well plate should be pre-coated with 1200 µl of Laminin-521 overnight at 4°C before use to ensure even hPSC adherence and efficient growth. One well of a 6-well plate should provide enough cells to seed between 6 and 10 wells of a 24-well plate for mDA differentiation.

18.1 Preparation of wash media for lifted hPSCs:

Per each well of a 6 well plate of hPSCs prepare 4ml of wash media.

Wash media: 50% iPS-Brew XF medium (self renewal media for hPSCs)
+ 50% DMEM/F12 + 10 µM Y27632.

For example when lifting one well of hPSCs from a 6 well plate prepare:

 2 mL iPS-Brew XF medium (Miltenyibiotec, Cat no 130-104-368) +  2 mL DMEM/F12 (Thermo Fisher, Cat no 21331020) +  2 µL 10mM Y27632.

18.2

Prepare an adequate volume of plating medium:


Neural Induction Media (NIM) + 10 µM Y27632 + 10 µM SB431542, 100nM LDN, 600 ng/ml Shh and between 0.7 and 1.2 µM CHIR99021*.



To start differentiation in 24-well plates, you will need 400 µl per well.


**The concentration of CHIR99021 recommended can vary between cell lines. A change in 0.1 µM can have a suprisingly large effect on the puritiy of cells obtained. Therefore it is recommended when first starting to try out and compare a range of CHIR99021 concentrations.*

18.3 Lifting hPSCs with EDTA

Aspirate the **iPS-Brew XF medium** from the hPSCs (in 6-well plate).

Wash cells once with  1 mL of [M] 0.5 millimolar (mM) **EDTA** (prepared step 16.1).

Add  1 mL of [M] 0.5 millimolar (mM) **EDTA** to the cells and incubate at  37 °C for approximately 5-7 minutes or when most of the cells appear 'refractile' by light microscopy. **The incubation time should depend on the confluency of the cells.*


After the incubation period; remove the EDTA and use  1 mL of the Wash medium (*prepared in step 18.2*) to lift the cells gently from the well. Aim to lift cells in a few pipettes as possible to ensure optimal survival.

Transfer the lifted cells to the tube with remaining Wash medium.

Gently invert the tube a few times to ensure the cells are thoroughly mixed for cell counting.

18.4 Counting the cells:

By using EDTA the cells are lifted as clumps. For more accurate counting of the cells take an aliquot to be broken into single cells.

To count the cells, take $2 \times 10 \mu\text{L}$ aliquots of cells into new 0.5ml Eppendorfs. Mix each with  10 μL of Trypan Blue (**Sigma Aldrich, Cat no T8154**).

Count the cells using a haemocytometer*. Calculate the amount of cells within the wash media.

18.5 Plating cells:

On mDA Day 0 we recommend using seeding into a 24 well plate.

From the cell count, estimate the total volume of cells you will need to initiate differentiation at a density of $40,000 \text{ cells/cm}^2$.*.

For example, for 4 wells of a 24-well plate, you will need $4 \text{ (number of wells)} \times 2 \text{ (area of each well)} \times 40,000 \text{ (required density)} = 320,000 \text{ cells}$.



**Note the optimal plating density can vary depending on the cell line used. For example RC17 a plating density of 40,000 cells/cm² is recommended but for AST18 a plating density of 60,000 cells/cm² is recommended.*

Transfer 110% of the required volume (an extra 10% is transferred to account for any cell death) from the remaining cell suspension within the wash media (that has not been triturated) to a new tube for centrifugation 300 g for 3 minutes.

Aspirate the wash media and resuspend the colonies gently in the plating medium (*prepared in step 18.2*). It is recommended to plate cells into the wells of a 24 well plate in a volume of 400 µl per well. E.g if 4 wells of a 24-well plate are being seeding for example then cells should be resuspended in 4 × 400 µl.

Aspirate the Laminin-111 from the coated plates and seed the cell suspension into the well. Shake the plate with to ensure an even distribution of cells.

mDA Day 2

19 mDA Day 2 media change.

Fresh medium should be prepared from base media NIM and growth factors.

19.1 Prepare an adequate volume of fresh day 2 medium:

Neural Induction Media (NIM) + 10 µM SB431542, 100nM LDN, 600 ng/ml SHH and between 0.6 and 1.0 µM CHIR99021.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

For each well of a 24-well plate  400 µL of fresh media is required.


mDA Day 4

20 mDA Day 4 media change

Between mDA Day 4 and mDA Day 11 cells are no longer cultured within NIM as a base media but instead are cultured within neural proliferation media NPM.

**You should observe the cells becoming more confluent day by day. We expect cells to be close to 100% confluent between mDA D3 and mDA D7. How quickly cells become*






confluent varies between cell lines.

The components which form the base media NPM (N2 supplement, B27 supplement, DMEM/F12 and Neurobasal media) are in themselves only stable for 2 weeks due to the presence of light sensitive components. Thus, fresh base media should be made up at the start of each differentiation and kept in the dark at  4 °C for up to 2 weeks.

20.1 Preparation of NPM







50% NIM + 25% Neurobasal + 25% DMEM/F12 + 2mM L-Glutamine.

To prepare NPM from NIM:

For example, to make  50 mL **NPM** =  25 mL **NIM** +  12.25 mL **DMEM/F12** (Thermo Fisher, Cat no 21331020) +  12.25 mL **Neurobasal™ Medium** (Thermo Fisher, Cat no 21103049) +  250 µL **200mM L-Glutamine** (Thermo Fisher, Cat no 25030032).

Alternatively to make NPM de novo:

50% Neurobasal, 50% DMEM/F12 + 0.5x N2 + 0.5x B27 + 2mM L-Glutamine.

For example, to make  50 mL **NPM** =  24.4 mL of **DMEM/F12** (Thermo Fisher, Cat no 21331020) +  24.4 mL of **Neurobasal™ Medium** (Thermo Fisher, Cat no 21103049) +  250 µL of **100x N2** (Thermo Fisher, Cat no 17502048) +  500 µL of **50x B27** (Thermo Fisher, Cat no 17504044) +  500 µL of **200mM L-Glutamine** (Thermo Fisher, Cat no 25030032).

20.2 Prepare an adequate volume of fresh day 4 medium:

NPM + 10 µM SB431542, 100nM LDN, 600 ng/ml Shh and between 0.6 and 1.0 µM CHIR99021.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

For each well of a 24-well plate  600 µL of fresh media is required.

mDA Day 7



21 mDA Day 7 media change.

Cells should be confluent by mDA Day 7 at the latest.

21.1 Prepare an adequate volume of fresh day 7 medium:

NPM + 10 μ M SB431542, 100 nM LDN, 600 ng/ml Shh and between 0.6 and 1.0 μ M CHIR99021.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

For each well of a 24-well plate  600 μ L of fresh media is required.

mDA Day 9

22 mDA Day 9 media change

22.1 Prepare an adequate volume of fresh medium:

NPM + 100 ng/ml FGF8b + 1 μ M Heparin solution.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

For each well of a 24-well plate,  600 μ L of fresh media is required.

mDA Day 11: preparation


23 mDA Day 11

On mDA day 11 cells can either be replated or frozen for use later on. From mDA D11 onwards cells are cultured within new base media; Neural differentiation medium (NDM). mDA cells should be replated onto new plates freshly coated Laminin-111.

23.1 Pre-coating plates with Laminin-111





Plates should be coated with L111 following the same protocol as step ?

24 Preparation of Neural Differentiation Medium (NDM):

The components which form the base media NDM (B27 supplement, and Neurobasal media) are in themselves only stable for 2 weeks due to the presence of light sensitive components. Thus, fresh base media should be made up at the start of each differentiation and kept in the dark at  4 °C for up to 2 weeks.

24.1 Preparation of NDM:

Neurobasal + 1x B27 + 2mM L-Glutamine.

For example, to make  50 mL NDM =  48.5 mL of Neurobasal™ Medium (Thermo Fisher, Cat no 21103049) +  1 mL of 50x B27 (Thermo Fisher, Cat no 17504044) +  500 µL of 200mM L-glutamine (Thermo Fisher, Cat no 25030032).

mDA D11 replating

25 mDA D11 replating:

On day 11 cells can be (i) lifted and replated for continued differentiation, or (ii) cryopreserved

To replate; mDA D11 cells are lifted as single cells using Acctuase and replated into a new L111-coated plate at a density of 800,000cells/cm².

25.1 Preparation of wash medium:

NPM + 10 µM Y27632

For each well of a 24 well plate prepare  1.2 mL wash media. **This volume is 5x the volume of Acctuase added per well.*

25.2 Preparation of plating medium:

NDM + 10 µM Y-27632 + 20 ng/ml BDNF + 10 ng/ml GDNF 100 ng/ml FGF8b + 0.2 mM Ascorbic Acid + 1 µg/ml heparin solution.

Prepare plating medium according to the number of the wells to be re-plated, remember to account for extra dead volume.

It is recommended to re-plate cells into either a 24 or 48 well formatt.

Volume for day 11 plating:

	µl/well	area (cm ²)	Volume (µl)
	24 well plate	2	600
	48 well plate	1	300

25.3 Lifting cells as single cells with Accutase

Wash the cells once briefly Accutase (300 µl/well for 24-well plate) (**Sigma Aldrich, Cat no A6964**)

Add 300µl/well of Accutase (**Sigma Aldrich, Cat no A6964**) and incubate  37 °C for 10min.


Following the incubation period, to dissociate into single cells pipette up and down. *

**Place a P1000 tip in a P200 tip, set the P1000 to 600. Tilt the plate towards you, place the pipette tip in the bottom corner of the well . Pipetting the cells up and down against this corner will aid in breaking up the cells. Pipetting ~15 times is usually sufficient to break into single cells. Try to avoid the production of bubbles where possible.*

Transfer the lifted cells to the wash media.

Repeat the process for each well.

25.4 Counting cells

To count the cells, take 2 × 10 µl aliquots of cells into new 0.5ml Eppendorfs. Mix each with  10 µL of Trypan Blue (**Sigma Aldrich, Cat no T8154**).

Count the cells using a haemocytometer*. Calculate the amount of cells within the wash media.

Alternatively, can use automatic cell counter to count the cells. E.g TC20TM Automated Cell Counter Biorad (BioRad, Cat no 1450102**).*

25.5 Plating cells:



From the cell count, estimate the total volume of cells needed for re-plating. For mDA day 11 replate at a density of 800,000 cells/cm².

For example: cells needed = (number of wells) x 2cm² (area of a well in a 24-well plate) x 800,000 cells/cm² (required density). The volume to spin down = cells needed / cell count.

Transfer the required volume from the remaining cell suspension within the wash media (that has not been triturated) to a new tube for centrifugation 300 g for 3 minutes.

Aspirate the wash media and resuspend the colonies gently in the mDA Day 11 plating medium (*prepared in step 24.2*). E.g if 4 wells of a 24-well plate are being seeding for example then cells should be resuspended in 4 × 600 µl.

Aspirate the Laminin-111 from the coated plates and seed the cell suspension into the wells. Shake the plate to ensure even distribution of cells.

25.6 **Alternative to replating: freezing mDA Day 11 cells**

Instead of replating cells can be cryopreserved at this stage.

See cryopreservation protocol described in:

<https://www.biorxiv.org/content/10.1101/2020.02.11.944272v1>.

mDA Day 14

26 **mDA Day 14 media change.**

26.1 **Prepare an adequate volume of fresh medium:**

NDM + 20 ng/ml BDNF + 10 ng/ml GDNF + 100 ng/ml FGF8b + 0.2 mM Ascorbic Acid + 1 µg/ml heparin solution.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

Volume needed per well:

Plate Type	area (cm ²)	Volume
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			(μ l/ well)
	24 well plate	2	600
	48 well plate	1	300

Preparation for mDA Day 16

27 mDA Day 16 replating preparation

On mDA day 16 cells can either be replated or cryopreserved for use later on. mDA cells should be replated onto new plates freshly coated Laminin-111.

27.1 Pre-coating plates with Laminin-111

Plates should be coated with L111 following the same protocol as step 15.

mDA Day 16


28 mDA Day 16 replating


On day 16 cells can be (i) lifted and replated for continued differentiation, (ii) cryopreserved, or (iii) transplanted into an animal model.

As with mDA Day 11 replating, mDA Day 16 cells are lifted as single cells using Acctuse and replated into a new L111-coated plate at a density of 800,000 cells/cm².

28.1 Preparation of wash media:

Wash media is prepared from NDM + 10 μ M Y-27632.

For each well of a 24-well plate prepare  1.2 mL wash media.

For each well of a 48-well plate prepare  0.6 mL wash media.

**This volume is 5x the volume of Acctuse added per well.*

28.2

Preparation of plating media:

NDM + 10 μ M Y27632 + 20 ng/ml BDNF + 10 ng/ml GDNF + 0.2 mM Ascorbic Acid + 0.5 mM dcAMP + 1 μ M DAPT.

Prepare plating medium according to the number of the wells to be re-plated, remember to account for extra dead volume.

It is recommended to re-plate cells into either a 24 or 48-well format.


Volume for day 16 plating:

	Plate Type	area (cm ²)	Volume (μ l/well)
	24 well plate	2	600
	48 well plate	1	300

28.3 Lifting cells as single cells with Accutase

Aspirate off the NDM from each well.

Wash the cells once briefly Accutase (300 μ l/well for 24-well plate, 150 μ l/well for 48-well plate) (**Sigma Aldrich, Cat no A6964**) to remove excess medium.

Add Accutase (300 μ l/well for 24-well plate, 150 μ l/well for 48-well plate) (**Sigma Aldrich, Cat no A6964**) and incubate  37 °C for 10 mins.


Following the incubation period, to dissociate into single cells pipette up and down. ***

****Place a P1000 tip in a P200 tip, set the P1000 to 600. Tilt the plate towards you, place the pipette tip in the bottom corner of the well pipetting the cells up and down against this corner will aid in breaking up the cells. Pipetting ~15 times is usually sufficient. Try to avoid the production of bubbles.*

Transfer the lifted cells to the wash media.

Repeat the process for each well.

28.4 Counting cells

To count the cells, take $2 \times 10 \mu\text{L}$ aliquots of cells into new 0.5ml Eppendorfs. Mix each with  10 μL of Trypan Blue (**Sigma Aldrich, Cat no T8154**).

Count the cells using a haemocytometer*. Calculate the amount of cells within the wash media.

Alternatively, can use automatic cell counter to count the cells. E.g TC20TM Automated Cell Counter Biorad (BioRad, Cat no 1450102**).*

28.5 **Plating cells:**

From the cell count, estimate the total volume of cells needed for re-plating. For mDA day 16 replate at a density of 800,000 cells/cm².

For example: cells needed = (number of wells) \times 2 cm² (area of a well in a 24-well plate) \times 800,000 cells/cm² (required density). The volume to spin down = cells needed / cell count.

Transfer the required volume from the remaining cell suspension within the wash media (that has not been triturated) to a new tube for centrifugation 300 g for 3 minutes.

Aspirate the wash media and resuspend the colonies gently in the mDA Day 16 plating medium (*prepared in step 27.2*).

E.g if 4 wells of a 24-well plate are being seeding for example then cells should be resuspended in $4 \times 600 \mu\text{L}$.

Aspirate the Laminin-111 from the coated plates and seed the cell suspension into the wells with generous shaking to ensure even distribution.

28.6 **Alternative to replating: freezing mDA Day 16 cells**

Instead of replating, cells can be frozen at this stage.

See freezing protocol described in:

<https://www.biorxiv.org/content/10.1101/2020.02.11.944272v1>.

mDA Day 18

29 **mDA Day 18 media change**

29.1 **Prepare an adequate volume of fresh day 18 medium:**

NDM + 20 ng/ml BDNF + 10 ng/ml GDNF + 0.2 mM Ascorbic Acid + 0.5 mM dcAMP + 1 μM DAPT.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

Volume for day 18 media change:

	Plate Type	area (cm ²)	Volume (μl/well)
	24 well plate	2	600
	48 well plate	1	300

mDA Day 18 onwards

30 Media changes mDA day 18-day 45:

From mDA Day 18 onwards cells should be fed every 2-3 days until day 45. At day 45 the mDA cells are expressing high levels of mature mDA markers e.g TH.

30.1 Prepare an adequate volume of fresh medium:

NDM + 20 ng/ml BDNF + 10 ng/ml GDNF + 0.2 mM ascorbic acid + 0.5 mM dcAMP + 1 μM DAPT.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

Volume for media change:

	Plate Type	area (cm ²)	Volume (μl/well)
	24 well plate	2	600
	48 well plate	1	300



mDA neurons can be kept in culture for over 1 year feeding once or twice a week. DAPT should be removed from the feeding medium after day 45.