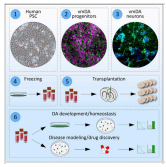

Jul 21, 2021

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

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Benjamin Trist

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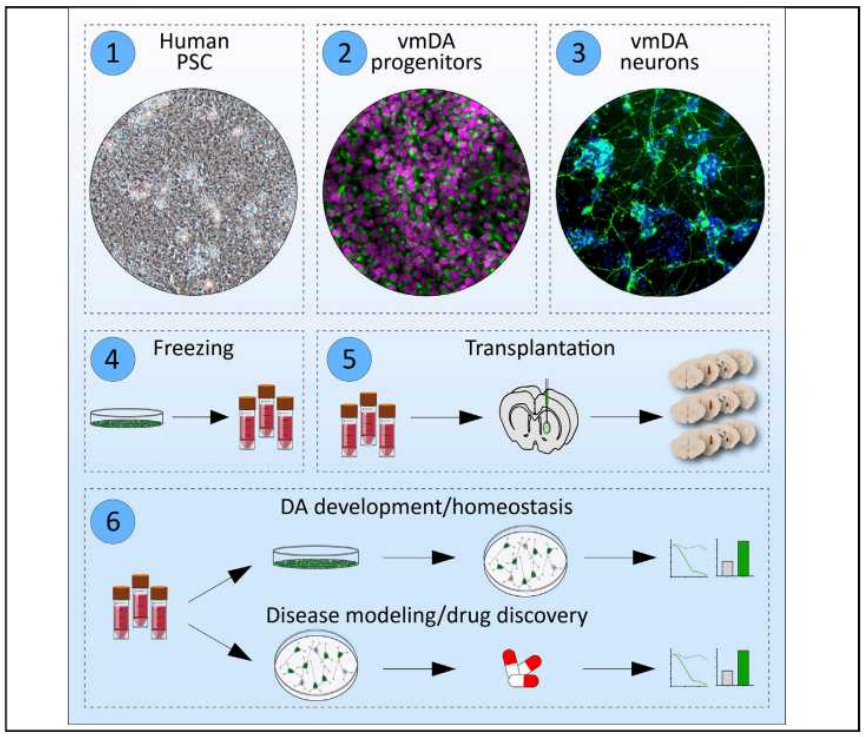
ABSTRACT



Here, we describe a xeno-free, feeder-free, and chemically defined protocol for the generation of ventral midbrain dopaminergic (vmDA) progenitors from human pluripotent stem cells (hPSCs). This simple-to-follow protocol results in high yields of cryopreservable dopamine neurons across multiple hPSC lines. Wnt signaling is the critical component of the differentiation and can be finely adjusted in a line-dependent manner to enhance production of dopamine neurons for the purposes of transplantation, studying development and homeostasis, disease modeling, drug discovery, and drug development.
For complete details on the use and execution of this protocol, please refer to Gantner et al. (2020) and Niclis et al. (2017a).

HIGHLIGHTS

- Reproducible differentiation of human dopamine neurons from multiple hPSC lines
- Dopamine progenitors can be cryopreserved for downstream applications
- Dopamine neurons mature *in vitro*, enabling screening or developmental studies
- Transplanted dopamine progenitors are capable of restoring motor function

ATTACHMENTS

An Optimized Protocol for the Generation of Midbrain Dopamine Neurons under Defined Conditions.pdf

DOI

dx.doi.org/10.17504/protocols.io.bu7ynzpw

PROTOCOL CITATION

Carlos W. Gantner, Agustín Cota-Coronado, Lachlan H. Thompson, Clare L Parish 2021. An Optimized Protocol for the Generation of Midbrain Dopamine Neurons from human iPSCs under Defined Conditions. **protocols.io**
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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Gantner et al., An Optimized Protocol for the Generation of Midbrain Dopamine Neurons under Defined Conditions, STAR Protocols (2020), https://doi.org/10.1016/j.xpro.2020.100065

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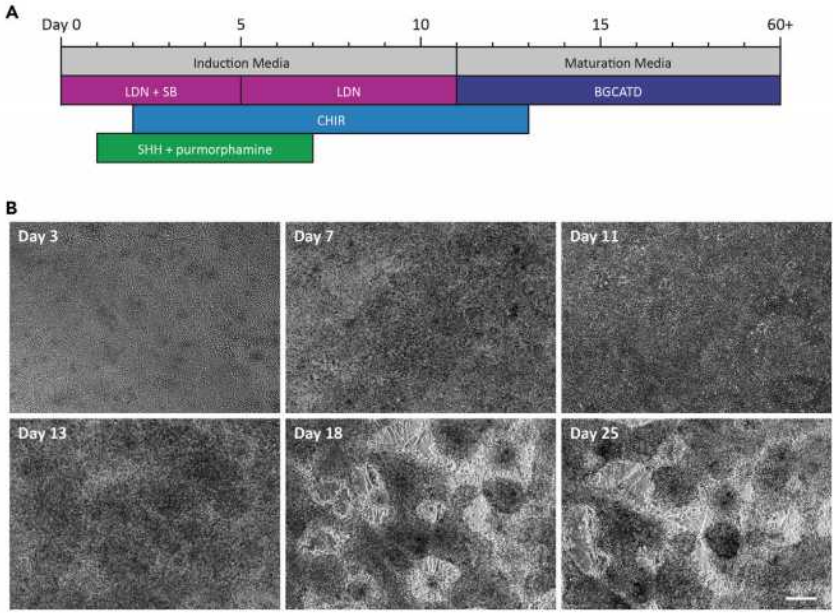

Figure 1. Differentiation Overview and Morphological Changes during vmDA Differentiation
(A) Schematic representation of the differentiation protocol indicating when each factor is added to the base media to appropriately pattern hPSC toward the vmDA lineage.
(B) Morphological changes during differentiation. At day 3, the cells are a flat, uniform monolayer. By day 11, the density has increased, and the wells appear darker under the microscope. By days 18–20, fibers begin to appear as neurons accumulate and aggregate together and by day 25 vmDA neurons display extended axonal processes. Scale bar, 100 µm.

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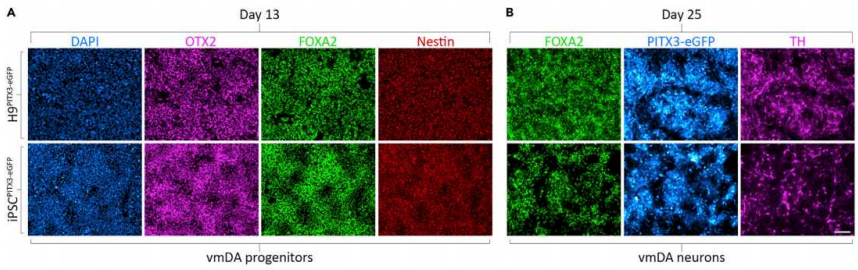

Figure 2. Characterization of Developing vmDA Progenitors and Neurons with 2.5 mM CHIR99021
(A) Immunofluorescence analysis of developing vmDA progenitors differentiated from either H9:PITX3-eGFP or RM3.5:PITX3-eGFP using 2.5 mM CHIR99021. At day 13, vmDA progenitors should homogenously express OTX2, FOXA2 and Nestin, indicating correct midbrain floorplate lineage acquisition.
(B) At day 25, vmDA neurons expressing FOXA2, PITX3, and TH should be readily observed if differentiation has been successful. Scale bar, 100 mm.

A	B	C	D	E	F	G	H	I	J	K
	Nestin	OTX2	FOXA2	LMX1A	EN1	NURR1	PITX2	BARHL1	(PITX3)eGFP	TH
	1:1000	1:1000	1:500	1:200	1:50	1:200	1:500	1:500	1:1000	1:1000
Day 13	+++	+++	+++	++	+/-	-/+	+/-	+/-	-	-
Day 16	+++	+++	+++	++	++	++	+/-	+/-	+/-	+/-
Day 25	++	+++	+++	++	+/-	+	+/-	+/-	++	++
Day 60	+/-	++	++	+	+/-	+/-	+/-	+/-	+++	+++

Table 5. Expression of vmDA Genes during Differentiation
Expression level: - none; +/- low; + moderate; ++ high; +++ very high.

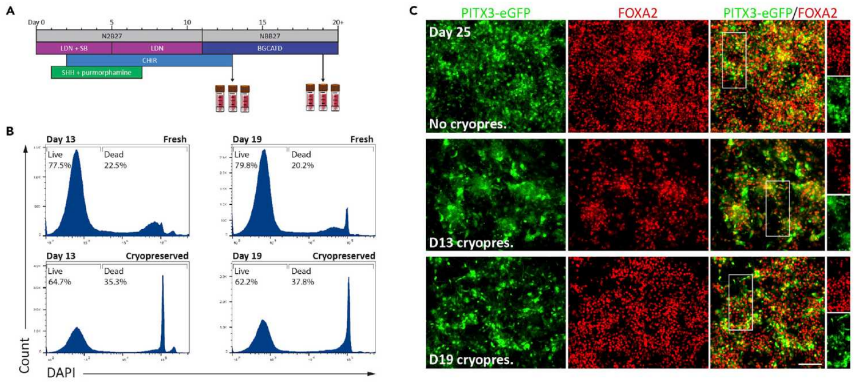

Figure 3. vmDA Progenitors Can Be Cryopreserved and Thawed with High Viability
(A) Differentiation schematic highlighting two time points where vmDA progenitors can be frozen and/or transplanted with high viability.
(B) Live/dead analysis of DNA content using FACS highlights that cells are viable after cryopreservation at days 13 or 19.
(C) By day 25, thawed and replated vmDA progenitors mature into FOXA2+/PITX3-eGFP+ vmDA neurons in vitro. Scale bar, 100 µm.

EXPECTED OUTCOMES

One day after seeding, undifferentiated hPSC should tessellate the culture surface such that there are no (or very small) gaps between cells. As differentiation proceeds cells will actively proliferate and appear denser, until individual cells can no longer be resolved under the microscope (typically day 5). At the early stages of differentiation there should be very little cell death. By day 13 the majority of the culture should appear homogenous in distribution and density across the culture well, co-express FOXA2 and OTX2 (≥80%) and will begin to appear darker under the microscope as cell density increases (Figures 1B and 2; Table 5). Note, some death may occur following the transition from Induction to Maturation Media. Addition of the g-secretase inhibitor DAPT, which drives cell cycle exit, often leads to clear neuronal processes by day 20, although this is cell line-dependent and does not necessarily reflect the final TH+ vmDA neuron yield.
By day 20 TH+ (or PITX3-eGFP+) neurons are readily observable and are increasingly observed until peaking at

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day 40 (D20: ~5%; D25: ~25%; D40: ~50%) (Figure 2; Table 5; See Niclis et al. (2017a) for more detailed quantification across numerous hPSC lines). vmDA neurons should also co-express FOXA2, OTX2, NURR1, EN1, LMX1A (Figure 2; Table 5). vmDA progenitors and neurons will increasingly clump together after day 30 as they continue to mature. At day 40 vmDA neurons in culture are capable of synthesizing and metabolizing dopamine, and by day 60 vmDA neurons display characteristic vmDA electrophysiological properties (Niclis et al., 2017a).

For the purpose of transplantation, we typically obtain total cell yields of 1–2 x 10^6/cm^2 at time points amenable to implantation (days 13–22). Demonstrative of the functionality and integration of these cells, we observe correction in the unilateral 6-hydroxydopamine rat model of DA depletion/Parkinson's disease in amphetamine-induced rotational asymmetry after 12–16 weeks.

LIMITATIONS

We have successfully utilized this protocol to generate vmDA neurons from multiple hESC and hiPSC cell lines (including H9, HES3, RUES2, 409B2 parental lines and several subclones). However, differentiation efficiency does vary between hPSC lines. We have found that the majority of this variation is Wnt-dependent. As such, we recommend optimization of the CHIR99021 concentration for each cell line. Typically, titration between 2–3 µM is sufficient to optimize vmDA yield (Figure 4). This is particularly important for iPSC-based disease modeling studies, that typically derive numerous cell lines. Even under optimal conditions, where OTX2+/FOXA2+, putative vmDA progenitors make up ≥90% of the culture, only ~50% of these will mature into TH+ vmDA neurons. This is likely due to the aberrant maintenance of an immature state in vitro or the generation of adjacent midbrain lineages. Typically, we do not observe significant 5-HT, GABA or Glutamate (≥2%) contamination within vmDA cultures. If specific analysis of the vmDA population is desired (e.g. for in vitro disease modeling of vmDA phenotypes, vmDA-centric drug development, or grafting in vivo), we suggest the use of an appropriate fluorescent reporter (such as PITX3-GFP or TH-GFP) to allow for isolation (e.g., for multi-omic analyses) and/or tracking in vitro or after transplantation. See Niclis et al. (2017b) for an example of how a fluorescent reporter can augment analysis.

Finally, the use of fully defined media, utilizing xenogeneic-free reagents (amenable to clinical translation), results in less variability and better efficiency between differentiations and cell lines (Niclis et al., 2017a). However, the use of animal product-free reagents can add unnecessary expense when such stringency may not be required. For other applications we recommend using the more traditional media formulations, which are typically less expensive and work equally well. We regularly interchange xenogeneic and xenogeneic-free reagents (e.g. mTeSR2/mTeSR1) without altering the protocol, although this should be determined on a case-by-case basis by the end user.

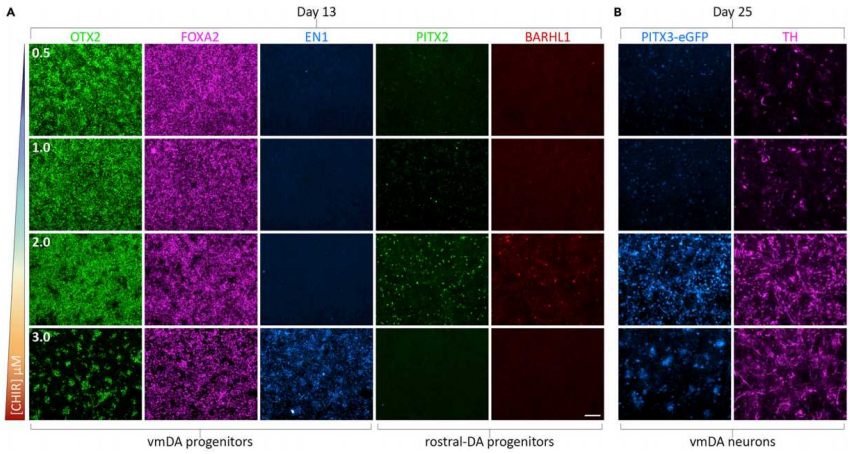

Figure 4. CHIR99021 Concentration Determines the Rostrocaudal Positioning of Developing Neural Progenitors
Titrating CHIR99021 is advisable for each individual cell line.
(A) Neural progenitors at day 13 exposed to increasing levels of [CHIR99021] (0.5–3.0 µM) demonstrating a loss of fore/midbrain marker OTX2 and increased EN1 at higher concentrations. PITX2 and BARHL1 are expressed in the rostral midbrain and are helpful to fine-tune CHIR99021 concentration. We have found that 2.5 mM consistently generated vmDA progenitors from multiple hPSC cell lines.
(B) PITX3-eGFP+/TH+ vmDA neurons are born only after appropriate CHIR99021 addition and are progressively lost when [CHIR99021] is too high and cultures are overcaudalized. Scale bar, 100 µm

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For **TROUBLESHOOTING**, **RESOURCE AVAILABILITY**, **ACKNOWLEDGMENTS**, **AUTHOR CONTRIBUTIONS**, **DECLARATION OF INTERESTS** and **REFERENCES**, please take a look at the attachment.

MATERIALS TEXT

KEY RESOURCES TABLE

A	B	C
REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
Antibodies		
Rabbit polyclonal anti-BARHLl1	Novus Biologicals	NBP1-86513, RRID:AB_11034569
Mouse monoclonal anti-EN1	DSHB	4G11, RRID:AB_528219
Goat polyclonal anti-FOXA2	Santa Cruz Biotechnology	sc-6554, RRID:AB_2262810
Chicken polyclonal anti-GFP	Abcam	ab13970, RRID:AB_300798
Rabbit polyclonal anti-LMX1A	Millipore	AB10533, RRID:AB_10805970
Rabbit polyclonal anti-NURR1/NUR77	Santa Cruz Biotechnology	Sc-990, RRID:AB_2298676
Goat polyclonal anti-OTX2	R&D Systems	AF1979, RRID:AB_2157172
Sheep polyclonal anti-PITX2	R&D Systems	AF7388, RRID:AB_11128639
Rabbit polyclonal anti-TH	Pel-Freez Biologicals	P40101-0, RRID:AB_461064
Sheep polyclonal anti-TH	Pel-Freez Biologicals	P60101-0, RRID:AB_461070
Chemicals, Peptides, and Recombinant Proteins		
Accutase®	Innovative Cell Technologies	AT104
Ascorbic acid	Sigma-Aldrich	A4403
B27™ Supplement or CTS™ B27™ Supplement	Gibco	17504044 A1486701
B27™ Supplement -vitA or CTS™ B27 Supplement -vitA	Gibco	12587010 A3353501
Brain-derived neurotrophic factor	R&D Systems	248-BDB
CHIR99021	Miltenyi Biotech	130-103-926
Dibutyryl cAMP	Tocris Bioscience	1141
DMEM/F-12 or CTS™ DMEM/F-12	Gibco	11320033 A1370801
Dimethyl sulfoxide	Sigma-Aldrich	D2650
Glial cell line-derived neurotrophic factor	R&D Systems	212-GD
GlutaMax™ Supplement	Gibco	35050061

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Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A)	Gibco	51300044
KnockOut™ serum replacement or CTS™ KnockOut™ serum replacement	Gibco	10828010 12618012
Laminin-521	BioLamina	LN521
Laminin - mouse	Sigma-Aldrich	L2020
LDN193189	Miltenyi Biotech	130-103-925
MEM Non-Essential Amino Acids	Gibco	11140035
mTeSR™1 or TeSR™2	STEMCELL Technologies	85880 05860
N2 Supplement or CTS™ N2 Supplement	Gibco	17502048 A1370701
N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT)	Sigma-Aldrich	D5942
Neurobasal™ or CTS™ Neurobasal™	Gibco	A3582901 A1371201
$PBS^{+/+}$ (with Calcium/Magnesium)	Gibco	13492609
$PBS^{-/-}$	Gibco	14190144
Pierce™ 16% formaldehyde	Thermo Fisher	28906
Purmorphamine	Miltenyi Biotech	130-104-465
ReLeSR™	STEMCELL Technologies	05873
SB431542	R&D Systems	1614
SHH C25II	R&D Systems	464-SH
TGFb3	Peprotech	100-36E
Trypan blue	Sigma-Aldrich	T8154
Y27632 (ROCK inhibitor) or Y27632-GMP	Tocris Bioscience	1254 TB1254-GMP
Critical Commercial Assays		
MycoAlert™ Mycoplasma Detection Kit	Lonza	LT07-218
Experimental Models: Cell Lines		
H9 PITX3-eGFP hESC	Colin Pouton Laboratory. (Watmuff et al., 2015)	N/A
RM3.5 PITX3-eGFP hiPSC	In house	N/A
Other		
48-well plate – Nunclon Delta surface	Thermo Fisher	150687
Bright-Line™ hemocytometer	Sigma-Aldrich	Z359629
Cryovials – 1.5 mL	Thermo Fisher	366656
Mr. Frosty™ freezing container	Thermo Fisher	5100-0001
T25 Flask	Corning	353108

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MATERIALS AND EQUIPMENT

Laminin-521 Dilution (Tables 1 and 2)

A	B	C	D
	Laminin-521 (µL)	**PBS+/+ (µL)**	**Final Volume (µL)**
T25	150	2850	3000
48-well plate	7.5	142.5	150
96-well plate	3.5	66.5	70

Table 1. Laminin-521 Dilution for 5 µg/mL

A	B	C	D
	Laminin-521 (µL)	**PBS+/+ (µL)**	**Final Volume (µL)**
T25	300	2700	3000
48-well plate	15	135	150
96-well plate	7	63	70

Table 1. Laminin-521 Dilution for 10 µg/mL

Base Media Composition (Tables 3 and 4)

A	B
Induction media (N2B27)	**Volume (mL)**
DMEM/F12	47.75
Neurobasal media (NBM)	47.75
B27 -Vitamin A (1x)	2
N2 (1x)	1
Glutamax (1x)	1
Penicillin/Streptomycin (0.5x)	0.5
Total	100 mL

Table 3. Induction Media Composition

A	B
Maturation Media (NBB27)	**Volume (mL)**
DMEM/F12	46.75
Neurobasal media (NBM)	46.75
B27 +Vitamin A (1x)	2
N2 (1x)	1
Non-essential amino acids (1x)	1
ITS-A (1x)	1
Glutamax (1x)	1
Penicillin/Streptomycin (0.5x)	0.5
Total	100 mL

Table 4. Maturation Media Composition

SAFETY WARNINGS

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

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BEFORE STARTING

Note: All steps should be carried out under sterile conditions and according to local regulatory requirements (e.g. Australian Std 2243 requiring all human tissues to be confined to Class II biosafety cabinets)

Note: Cells are cultured throughout at **37 °C** in **5 % CO2** .

BEFORE YOU BEGIN: **Pluripotent Stem Cell Thawing** 2h 18m

1 2h

Coat a T25 flask with **5 µg/mL** – **10 µg/mL Laminin-521** and leave in the incubator for ≥ **02:00:00** (see Tables 1 and 2 for laminin-521 dilution).

Note: **5 µg/mL of Laminin-521** is sufficient for cell lines that have been adapted to Laminin-521. For non-adapted lines (i.e. hPSC lines maintained on substrates other than laminin-521) **10 µg/mL** may be required to ensure attachment during initial passages. Laminin-521 can also be added for **12:00:00** – **18:00:00** at **4 °C** , although ≥ **02:00:00** at **37 °C** is preferable.

CRITICAL: Laminin-521 must be diluted in PBS+/+. Diluting in $PBS^{-/-}$ will result in poor attachment and survival. Ensure that enough laminin solution is made to coat the entire flask throughout the coating period. Do not use flasks which have dried as cells will not attach in these areas.

2 15m

Equilibrate **15 mL pre-made mTeSR1** in a tightly closed 15 mL tube in the incubator for ≥ **00:15:00** .

3

Rapidly thaw a cryovial of hPSC in a **37 °C** water bath and transfer the contents to **10 mL pre-warmed mTeSR1 solution** using a P1000 pipette.

4 3m

Centrifuge cells at **300 x g, 21°C, 00:03:00** , aspirate supernatant, and resuspend the cell pellet in **5 mL mTeSR1 media** , supplemented with **10 Micromolar (µM) Y27632** .

CRITICAL: Addition of the ROCK inhibitor Y27632 is essential for hPSC survival, particularly if the cells have been cryopreserved as single cells rather than aggregates.

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5 Aspirate laminin from the T25 flask and immediately plate the cell suspension, gently rocking the flask multiple times up-and-down and then side-to-side to evenly distribute the cells.

BEFORE YOU BEGIN: **Pluripotent Stem Cell Culture**

6

One day after thawing, wash once with $PBS^{-/-}$ to remove debris, and replace with fresh media.

7 Continue to culture hPSC until they reach 70%–80% confluence, changing media every day.

Note: Generally, cells will recover quickly after thawing and should reach confluency within 5 days. However, if cells are growing slowly it is best to passage at a lower density (50%– 60% confluence) or after 5–6 days.

8 15m

To passage hPSC, wash once in $PBS^{-/-}$ and add **2 mL ReLeSR** . Leave at **Room temperature** and monitor cell morphology under the microscope until colonies are loosely attached (**00:05:00** – **00:10:00**).

9 Remove ReLeSR carefully and rapidly tap the flask hard 3–4 times on each long edge to detach colonies.

10

Resuspend by dissociating colonies in **2 mL equilibrated mTeSR1** using a P1000 pipette. Dilute cells (1:10–1:20) and transfer using a 5 mL serological pipette to a Laminin-521 coated T25 flask.

Note: Morphology during passage can vary between cell lines and the optimal timing of ReLeSR should be determined accordingly. The majority of cells should remain as 10–50 cell aggregates, removing the need for Y27632. Add Y27632 if passaged as single cells. Likewise, the growth rate of each line will differ, and passage ratios should be adjusted to compensate. We recommend passaging every 3–5 days.

Optional: Cryopreserve stem cells at low passage number by adding [M]**10 % DMSO** , [M]**45 % KnockOut Serum Replacement** and [M]**45 % mTeSR** to isolated cell pellets (after ReLeSR dissociation) and slow-freeze at **-80 °C** . 3–4 cryovials can be generated from a single, confluent T25 flask. Transfer to liquid nitrogen for long-term storage.

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Pause Point: hPSC can be maintained by passaging every 3–5 days until the desired experimental start time. Thinking ahead and knowing the growth characteristics of the cell line are helpful in aligning cells at 70%–80% confluence with the desired start date.

CRITICAL: Always passage thawed cells ≥2 times before beginning a differentiation experiment, or until the cell line displays normal morphology and growth rate characteristics.

CRITICAL: Routine mycoplasma testing is essential to maintaining high-quality hPSC stocks that will consistently differentiate. The quality of stem cells will dictate differentiation efficiency and should be closely monitored by testing, morphology and immunostaining (e.g. ≥ 95% OCT4+). In addition, we do not recommend using high passage number hPSC.

BEFORE YOU BEGIN: **Preparing Differentiation Media** 20m

11 Prepare media as outlined in the Materials and Equipment section below (Tables 3 and 4).

Note: Always prepare media as close to use as possible and use within 2 weeks. It is best to make Induction Media the day of the experiment and Maturation Media at day 11. Where possible, add each factor (SHH, CHIR, etc.) to the base media on the day to minimize degradation.

Plating hPSC for Differentiation 1h

12 **Timing: 1 h**

This section describes the passaging of hPSC cultures into monolayers appropriate for differentiation.

13 2h

Coat a 48-well plate with **[M]5 µg/mL Laminin-521** and leave in the incubator for ≥ **02:00:00** (see Tables 1 and 2 for laminin-521 dilution).

Note: Differentiation can proceed in any size multi-well plate or flask. Adjust the Laminin-521 dilution, seeding density, and media volume accordingly.

14 10m

Passage hPSC at 70%–80% confluency by washing once in $PBS^{-/-}$, adding **2 mL Accutase**, and placing in the incubator for **00:04:00** – **00:06:00**. Monitor the morphology of the cells closely during this period. Once colonies show a characteristic bright halo and are close to detaching, gently pipette using a P1000 until ≥90% of the flask has detached.

15 3m

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Rapidly dilute Accutase solution in ≥ **3 mL mTeSR1** and centrifuge at **300 x g, 21°C, 00:03:00** .

16

Aspirate supernatant, resuspend the cell pellet in fresh **37 °C warmed** mTeSR1 supplemented with **10 Micromolar (µM) Y27632** and count live cells using a hemocytometer.

Note: Resuspend hPSC at 1–2 x 10^6 cells/mL to enable rapid, accurate counting. Typically, we resuspend hPSC in 1 mL/5–10 cm^2 . Each cell line grows at a different density and therefore the correct volume to resuspend cells in should be determined individually.

17

25m

Seed 0.3 x 10^6 cells/cm^2 (48 well surface area = ~1 cm^2) in ≥ **400 µl mTeSR1 + Y27632** . Rock the plate in a "figure eight" movement to evenly distribute cells and leave to settle for **00:10:00** – **00:15:00** before placing in the incubator.

Note: It is crucial to start the differentiation with an even monolayer of cells. Carefully dissociating to a single cell solution is important as it allows for an accurate count and an even distribution upon seeding. It is equally important not to prolong exposure of the hPSCs to enzymatic dissociation as this will impair survival. The duration of enzymatic treatment and the balance between optimal dissociation and survival may vary between cell lines.

Note: Initial seeding density should be optimized for each cell line to ensure confluency 24 h after seeding. Typically, we seed 0.2–0.4 x 10^6 cells/cm^2 . Trypan blue exclusion dye can be utilized during cell counting to ensure that only viable cells are included in the final cell seeding calculation.

CRITICAL: Seeding too many cells can lead to early detachment during differentiation, while seeding an insufficient number of cells can lead to poor survival and off-target differentiation.

vmDA Progenitor Differentiation

18 **Timing: 13 days**

This step describes the steps needed to differentiate hPSCs into vmDA progenitors.

See *Figure 1A* for a schematic of the differentiation.

19

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Day 0: 24 h after seeding check under the microscope that hPSC have survived and appear as a single, confluent (≥95%) monolayer with no obvious differentiation or uneven distribution.

Note: No (or very few small) gaps should be present the day after seeding the cells. If it is hard to see, wash twice with $PBS^{-/-}$ and check under the microscope. In addition, there should be very little death. If there is a large amount of death or cells are not confluent, it is best to discard the cells and start again.

20 Prepare Induction Media and supplement with [M]**10 Micromolar (µM) SB431542** and [M]**200 Nanomolar (nM) LDN193189** , SMAD inhibitors of the Transforming Growth Factor-b and Bone Morphogenetic Protein pathways, respectively.

21

Gently wash each well twice with $PBS^{-/-}$ to remove residual mTeSR and replace media with **500 µL supplemented Induction Media** .

22 Day 1: Change media to **500 µL Induction Media** supplemented with [M]**10 Micromolar (µM) SB431542** , [M]**200 Nanomolar (nM) LDN193189** , as well as ventralizing factors [M]**100 ng/mL C25II SHH** and [M]**2 Micromolar (µM) purmorphamin** .

23 Days 2, 3 and 4: Change media to **500 µl** supplemented with [M]**10 Micromolar (µM) SB431542** , [M]**200 Nanomolar (nM) LDN193189** , [M]**100 ng/mL C25II SHH** , [M]**2 Micromolar (µM) purmorphamine** and [M]**2.5 Micromolar (µM) Wnt-agonist CHIR99021** to caudalize developing progenitors.

Note: Media is changed every 24 h and 500 mL/48-well is added at days 0–4. At day 5 and beyond media is changed every other day and the volume is increased to 1 mL/48-well.

24 Day 5: Change media to **1 mL Induction Media** supplemented with [M]**200 Nanomolar (nM) LDN193189** , [M]**100 ng/mL C25II SHH** , [M]**2 Micromolar (µM) purmorphamine** and [M]**2.5 Micromolar (µM) CHIR99021** .

25 Days 7 and 9: Change media to **1 mL Induction Media** supplemented with [M]**200 Nanomolar (nM) LDN193189** and [M]**2.5 Micromolar (µM) CHIR99021** .

26

Day 11: Wash cultures twice in $PBS^{-/-}$ and switch base media to Maturation Media with [M]**20 ng/mL rhBDNF** , [M]**20 ng/mL rhGDNF** , [M]**0.1 Milimolar (mM) dibutyryl cAMP** , [M]**200 Nanomolar (nM) Ascorbic Acid** , [M]**1 ng/mL rhTGFb3** , and [M]**10 Micromolar (µM) DAPT (Maturation Media + BGCATD)** , supplemented with [M]**2.5 Micromolar (µM) CHIR99021** .

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Optional: Analyze cells at the end of progenitor patterning, day 13, for markers of vmDA progenitors by fixation and immunofluorescence or collection of cells for RT-qPCR. See below for the immunostaining protocol. By day 13 the majority (≥80%) of cells should co-express OTX2, FOXA2 and Nestin (Figure 2A; Table 5). Together, these markers indicate correct specification of ventral midbrain progenitors.

Note: If vmDA progenitors are too dense at day 13 for accurate quantification, it is possible to passage the cells with Accutase. If so, replate cells in Maturation Media + BGCATD, supplemented with **[M]10 Micromolar (µM) Y27632** at 0.3–0.45 x 10^6/cm^2 . Fix 24 h later, after cells have attached, for analysis or continued culture.

CRITICAL: Developing neural progenitors are extremely sensitive to CHIR99021 concentration. Take care when diluting new vials of CHIR99021 and precisely pipette the correct volume during the experiment. It is best to always add CHIR99021 on the day if possible.

Terminal Differentiation and Long-Term Culture of vmDA Neurons

27 **Timing: 12+ days**

This step describes the long-term culture of vmDA progenitors and the generation of vmDA neurons.

28

Days 13–30. Change media every other day to Maturation Media + BGCATD.

Optional: Transplantation of vmDA progenitors is optimal between days 13–22. Wash cells twice in $PBS^{-/-}$, add 400 mL/48-well of Accutase and incubate for **00:07:00** – **00:10:00** at **37 °C** . Monitor the wells closely under a microscope and test detachment every few minutes by gently pipetting the Accutase directly onto the cells. Once cells begin to detach, gently pipette using a P1000 to collect ≥90% of the well and further dissociate vmDA progenitors. Accutase can be left on for up to 20 min for slowly detaching, more dense cultures. Arrest enzymatic digestion when cells have dissociated into small (5–20 cell) aggregates by adding Maturation Media + BGCATD, supplemented with **[M]10 Micromolar (µM) Y27632** and centrifuge at **300 x g, 21°C, 00:03:00** . Aspirate the supernatant, resuspend the cell pellet in (1 mL/cm^2) Maturation Media + BGCATD + **[M]10 Micromolar (µM) Y27632** and count viable progenitors using a hemocytometer and Trypan blue exclusion. Centrifuge at **300 rpm, 21°C, 00:03:00** , aspirate supernatant, and resuspend at 0.1–0.2 x 10^6/µL in Maturation Media + BGCATD + **[M]10 Micromolar (µM) Y27632** and store **On ice** prior to transplantation.

Note: From day 18–20, vmDA neurons will become increasingly abundant and can be indirectly observed by the formation of neuronal bunches and axonal projections. By day 25, these neuronal bundles become dense and highly arborized (**Figure 1B**), making dissociation challenging and resulting in poor survival after plating and following transplantation. Viability when counting cells for further culture or transplantation should be ≥90%.

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Cells should not be stored on ice for >5 h.

Optional: We routinely characterize our cultures at days 13, 16, and 25 to confirm the degree of vmDA specification and neuronal identity within the cultures. If desired, fix cultures or recover cells from wells at these stages for immunofluorescence and/or RT-qPCR. vmDA progenitors co-expressing LMX1A, EN1, NURR1 should be apparent by D16, and vmDA neurons co-expressing FOXA2 along with the postmitotic lineage markers PITX3 and TH should be readily apparent by D25 (Figure 2B; Table 5).

A	B	C	D	E	F	G	H	I	J	K
	Nestin	OTX2	FOXA2	LMX1A	EN1	NURR1	PITX2	BARHL1	$(PITX3)_eGFP$	TH
	1:1000	1:1000	1:500	1:200	1:50	1:200	1:500	1:500	1:1000	1:1000
Day 13	+++	+++	+++	++	+/-	-/+	+/-	+/-	-	-
Day 16	+++	+++	+++	++	++	++	+/-	+/-	+/-	+/-
Day 25	++	+++	+++	++	+/-	+	+/-	+/-	++	++
Day 60	+/-	++	++	+	+/-	+/-	+/-	+/-	+++	+++

Table 5. Expression of vmDA Genes during Differentiation

29 From day 30 onwards, perform half-media changes, such that ~ **400 µL** is removed from each well and ~ **500 µL** of fresh, equilibrated media is added to maintain ~ **1 mL** /48-well. At these late stages of the differentiation, media changes can be done 2–3 times per week.

Note: For in vitro studies of DA development, homeostasis, disease modelling, or for drug screening approaches, vmDA neurons can be cultured until at least day 60, at which point electrophysiologically active vmDA neurons will be present.

Cryopreservation of vmDA Progenitors 23m

30 **Timing: 1 h**

This step describes the protocol for generating frozen stocks of vmDA progenitors for downstream applications.

Note: Cryopreserving vmDA progenitors between days 13 and 19 is ideal to balance lineage acquisition and survival.

31 Label cryovials and cool freezing container to **4 °C** .

32 Prepare freeze media containing [M]**20 % DMSO** and [M]**60 % KnockOut serum replacement** and [M]**20 % fresh Maturation Media** + BGCATD + [M]**10 Micromolar (µM) Y27632** .

Note: **0.5 mL freeze media** and **0.5 mL Maturation Media** are needed per 1 mL cryovial. Typically,

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we cryopreserve vmDA progenitors at 3 x 10^6/mL.

33 17m

Wash wells twice in $PBS^{-/-}$ and add **400 µL** /48-well of Accutase. Place in incubator for **00:07:00** – **00:10:00** . Monitor cell morphology closely under the microscope.

34

Dissociate cells by gentle pipetting using a P1000 and dilute Accutase in Maturation Media + BGCATD + Y27632.

35 3m

Centrifuge cells at **300 x g, 00:03:00** , aspirate supernatant and resuspend in Maturation Media + BGCATD + **10 Micromolar (µM) Y27632** (1 mL/cm^2) at approximately 1 mL x 10^6/mL for accurate counting using a hemocytometer and Trypan blue exclusion.

36 3m

Centrifuge at **300 x g, 00:03:00** , aspirate supernatant and resuspend in fresh Maturation Media + BGCATD + **10 Micromolar (µM) Y27632** at 6 x 10^6/mL.

37

Transfer **500 µL of the cell solution** to each cryovial and add 500 mL of freeze media, pipetting twice to gently mix.

38 Rapidly move vials to the freezing container and place at **-80 °C** .

39 2d

After ≥ **48:00:00** , transfer cryovials to LN_2 for long-term storage.

Pause Point: vmDA progenitors can be stored for periods ≥1 year.

CRITICAL: Ensure cells are exposed to Accutase for the minimum required time that still allows for accurate quantification. Maximizing the presence of small aggregates (2–10 cells) will improve cell survival. In contrast, over-dissociation in Accutase, leading to a high number of single cells, will significantly impair survival during thawing.

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Recovery of Cryopreserved vmDA Progenitors 2h 18m

40 **Timing: 30 min**

See Figure 3 for an overview of vmDA progenitor maturation following thawing.

This step describes how to thaw cryopreserved vmDA progenitors for use in downstream applications.

41 2h

Coat 48-well plates with **10 µg/mL Laminin-521** and place in incubator for ≥ **02:00:00** .

42 15m

Equilibrate **15 mL Maturation Media** + BGCATD + **10 Micromolar (µM) Y27632** at **37 °C** for ≥ **00:15:00** .

43

Rapidly thaw a cryovial of vmDA progenitors in a **37 °C** water bath and transfer the contents to **10 mL prewarmed media** using a P1000 pipette.

44 3m

Centrifuge at **300 x g, 00:03:00** , aspirate supernatant and resuspend in Maturation Media + BGCATD + **10 Micromolar (µM) Y2732** .

45

Plate progenitors at 0.3–0.45 x 10^6/cm^2.

Optional: Count vmDA progenitors using a hemocytometer to ascertain an accurate count of surviving cells. Live/dead analysis using FACS can also be used with a subfraction of the cells, in addition to Trypan blue exclusion. Viability immediately post-thaw is typically 60%–70%, although a further proportion may undergo apoptosis after re-plating.

Optional: Transplant vmDA progenitors directly after thawing. Wash once in $PBS^{-/-}$ after counting, centrifuge, aspirate supernatant and resuspend in Maturation Media + BGCATD + **10 Micromolar (µM) Y27632** at 0.1–0.2 x 10^6/µL for transplantation.

Optional: For long-term culture of vmDA neurons for studying development, drug screening, and/or disease modeling, wash cells once in $PBS^{-/-}$ the day after thawing and replace media with **1 mL fresh Maturation Media** + BGCATD. Proceed with steps 28–29 to continue the differentiation.

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Note: The concentration of rhGDNF and Y27632 can be doubled to [M]**40 ng/mL** and [M]**20 Micromolar (µM)** , respectively, if survival upon thawing is poor.

Immunostaining of vmDA Progenitors and Neurons 1h 42m

46 **Timing: 2 days**

This step outlines how to undertake immunocytochemical profiling of generated vmDA progenitors.

47 10m

Wash wells once with PBS$^{-/-}$ to remove debris and add **200 mL 4% paraformaldehyde** . Leave at **Room temperature** for **00:10:00** .

48 2m

Aspirate paraformaldehyde and wash 3 times with PBS$^{-/-}$, leaving wells in PBS$^{-/-}$ for at least **00:02:00** between washes.

Pause Point: Fixed plates can be stored for several months at **4 °C** . Addition of [M]**0.05 % Na- Azide** to PBS$^{-/-}$ can reduce possible bacterial or fungal contamination if storing long-term.

48.1 Aspirate paraformaldehyde.

48.2 Wash with PBS$^{-/-}$.

48.3 Leave wells in PBS$^{-/-}$ for at least **00:02:00** . 2m

48.4 Wash with PBS$^{-/-}$.

48.5 Leave wells in PBS$^{-/-}$ for at least **00:02:00** . 2m

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48.6 Wash with PBS$^{-/-}$.

49 Prepare blocking buffer by adding **1 mL species-specific serum** (same as secondary antibodies) and **0.6 mL 10% Triton X-100** to **18.4 mL PBS-Azide 0.05%** .

50 Add **200 µL** /48-well of blocking buffer to each well for **00:30:00** – **01:00:00** . 1h 30m

51 1d 6h

Dilute the primary antibodies in blocking buffer. Aspirate blocking buffer and add **200 µL** /48- well of primary antibody solution. Incubate at **4 °C** for **12:00:00** – **18:00:00** .

52 1h 30m

Wash wells 3 times in PBS$^{-/-}$ to remove primary antibodies and block cells again for **00:30:00** – **01:00:00** by adding **200 µL blocking buffer** .

52.1 Wash wells in PBS$^{-/-}$ to remove primary antibodies.

52.2 Wash wells in PBS$^{-/-}$ to remove primary antibodies.

52.3 Wash wells in PBS$^{-/-}$ to remove primary antibodies.

52.4 Block cells again for **00:30:00** – **01:00:00** by adding **200 µL blocking buffer** . 1h 30m

53 3h 30m

Dilute appropriate secondary antibodies in blocking buffer and add **200 µL secondary antibody solution** to each well. Incubate cultures in the dark at **Room temperature** for **01:30:00** – **02:00:00** .

54 10m

Wash wells in PBS$^{-/-}$ and add [M]**1 µg/mL DAPI (diluted in PBS-/-)** for **00:10:00** .

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55

Wash wells twice in PBS$^{-/-}$ and image.

Pause Point: Stained samples can be stored for a week at **4 °C** before imaging. It is best to store them in PBS$^{-/-}$ + **0.05 % Na-Azide** .

Note: It is optimal to perform immunofluorescence immediately after fixation of the cells. If long-term storage is necessary, ensure sufficient PBS-Azide is added to each well (**1 mL**), to account for evaporation during refrigeration and minimize exposure to light to prevent fluorochrome fading.

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