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Protocol status: In development
We are still developing and optimizing this protocol

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LENTIVIRAL TITRATION FOR EARLY POST- MITOTIC DOPAMINERGIC NEURONS

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ABSTRACT

iPSCs- derived neurons are particularly challenging cells for genetic screening. Hence we develop a protocol for lentiviral titration of mDA neurons where mDA neuronal cell suspension combined with concentrated lentiviral supernatant are added at different dilutions in 48 well plates. Subsequent centrifugation (spinfection) was performed to achieve high efficiency transduction. The transduction efficiency is determined as a percentage of BFP- positive cells through FACS (Fluorescence Activated Cell Sorting).

ATTACHMENTS

LENTIVIRAL TITRATION FOR EARLY POST-MITOTIC DOPAMINERGIC NEURONS.docx

PROTOCOL integer ID: 95925

Keywords: ASAPCRN, Lentiviral Production, CRISPRi, dopaminergic neuron differentiation, Perturb-Seq

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MATERIALS

	A	B	C
	MATERIAL	COMPANY	CATALOG
	48 well TC treated plate	Falcon	353078
	15ml polypropylene centrifuge tubes	Falcon	352096
	5ml serological pipettes	Corning	4487
	10ml serological pipettes	Corning	4488
	DNA Low-bind tubes 1.5ml	Eppendorf	022431021
	P1000 tip	Neptune	BT1250
	FBS	Bovogen	2008A
	DPBS	ThermoFisher Scientific	14040133
	Hank's Balanced Salt Solution (HBSS)	ThermoFisher Scientific	14175-095
	Neuronal Isolation Neuronal Enzyme (with Papain)	ThermoFisher Scientific	88285
	Neurobasal Media	ThermoFisher Scientific	21103049
	B27 w/o vit A	ThermoFisher Scientific	12587-010
	L-glutamine	ThermoFisher Scientific	L3000015
	Pen-Strep	ThermoFisher Scientific	12260
	BDNF (Brain Derived Neurotrophic Factor)	R&D	248-BDB
	GDNF (Glial Cell line Derived Neurotrophic Factor)	Peprotech	450-10
	Ascorbic Acid	Sigma	4034
	cAMP	Sigma	D0627

	A	B	C
	TGF-B (Transforming Growth Factor - b)	R&D	243-B3
	DAPT	Tocris	2634
	Polyornithine (PO)	Sigma	P3655
	Cultrex Mouse Laminin I	R&D	3400-010-1
	Fibronectin	Corning	FAL356008

REAGENT COMPOSITION

	A	B
	MEDIA 2	
	REAGENT	VOLUME IN ML
	Neurobasal Media	480
	B27 without Vit A (10x)	10
	Pen-Strep	5
	L-Glutamine	5

	A	B	C	D
	MATURATION MEDIA (MM)			
	REAGENT	STOCK SOLUTION	WORKING SOLUTION	VOLUME IN ul
	Media 2	-	-	24796
	BDNF	10 ug/ml	20ng/ml	50
	GDNF	10 ug/ml	20 ng/ml	50
	AA	100mM	200uM	50
	cAMP	100mM	200uM	50
	DAPT	100mM	10uM	2.5
	TGF-B	20 ug/ml	1ng/ml	1.25

	A	B
	FACS BUFFER (PBS +2% FBS)	
	REAGENT	VOLUME IN mL
	PBS	49
	FBS	1

BEFORE START INSTRUCTIONS

hESC CRISPRi dCAS9 are differentiated to D25 according to the following protocol:

CITATION

Tae Wan Kim, Jinghua Piao, So Yeon Koo, Sonja Kriks, Sun Young Chung, Doron Betel, Nicholas D. Socci, Se Joon Choi, Susan Zabierowski, Brittany N. Dubose, Ellen J. Hill, Eugene V. Mosharov, Stefan Irion, Mark J. Tomishima, Viviane Tabar, Lorenz Studer. Biphasic Activation of WNT Signaling Facilitates the Derivation of Midbrain Dopamine Neurons from hESCs for Translational Use. protocols.io.
LINK
https://protocols.io/view/biphasic-activation-of-wnt-signaling-facilitates-t-bu7znzp6

At D25, the cells were sorted by MACs sorting to obtain pure population dopaminergic mDA neurons.

CITATION

Tae Wan Kim. Dopamine neuron enrichment using MACS. protocols.io.
LINK
https://protocols.io/view/dopamine-neuron-enrichment-using-macs-cyrfxv3n

Day -1: Coating wells with Poly - L ornithine(PO)

1 Coat 500 ul per well in a 48-well plate with 15 ug/ml PO in DPBS.

2 Incubate the plate overnight at 37ºC with 5% CO2 and 20.9% O2.

Day 0: Coating wells with Laminin and Fibronectin

3 Thaw Fibronectin and Laminin on ice.

4 Aspirate 250ul of coated PO from each well of the 48 well plate and wash the wells with 1 ml of DPBS. Repeat two more times for a total of 3 x DPBS washes.

> **Note**
>
> Do not let the wells dry out.

5 Aspirate DPBS and add 500 ul of 2ug/ml Fibronectin and 1ug/ml Laminin in cold DPBS.

Day 1: Titration of D25 midbrain dopaminergic neurons(mDA neurons) wit…

6 Thaw the viral stock on ice.

7 Prepare 15 ml tubes with 200000 D25 pure population mDA neuronal suspension (CD49 neg) with concentrated lentiviral supernatants in serial dilutions in the 48 well plate in the following manner.

> **Note**
>
> Make sure to mix well by gentle pipetting. Change tips after making up each dilution. Titration was done in triplicates.

	A	B	C	D	E	F	G	H	I	J	K
	DILUTION										
		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
	Cell+MM media	600ul	600ul	600ul	600ul	600ul	600ul	600ul	600ul	600ul	600ul

	A	B	C	D	E	F	G	H	I	J	K
	Viral supernatant	600ul	600ul of 1/2	600ul of 1/4	600ul of 1/8	600ul of 1/16	600ul of 1/32	600ul of 1/64	600ul of 1/128	600ul of 1/256	600ul of 1/512

Table: 1 Serial dilution of concentrated lentiviral supernatant to determine lentiviral titer in TU/mL

8 Aspirate the fibronectin/laminin coating and proceed immediately to the next step.

9 Add 200 ul/well for each viral dilution with the cells.

10 To increase the transduction efficiency, centrifuge the plate at 300g for 20 minutes at 25 C.

11 Incubate the cells at 37°C with 5% CO2 and 20.9% O2 for 16-18 hours.

Day 2: Replace media

12 Aspirate the viral supernatant media gently and immediately add maturation media.

13 Return the plate back to the incubator.

Day 4: FACs Analysis

14 Aspirate the spent media.

15 Wash the cells 10 times with DPBS to remove the viral particles from the mDA neurons.

16 Note: The neurons are sturdy and do not lift off during the washes. However look under the microscope during the washes to avoid the neurons lifting off.

17 Add 100ul HBSS +papain and incubate the neurons for 45 mins in the incubator.

Note

Ideally the neurons should dissociate as single cells.

18 Neutralize the papain with maturation media and collect the cells into 1.5ml eppendorf tubes.

Note

If the neurons are still present as a sheet or have clumps, use a P1000 tip, pipette the cells up and down to break them into single cell suspension.

19 Centrifuge the cells at 300 g for 5 minutes.

20 Aspirate the spent media gently without disturbing the pellet.

21 Resuspend the cells in 300 ul of FACs buffer.

22 Transfer the cells with the FACs buffer into FACs tubes.

23 Analyze the cells through flow cytometry to determine BFP positive cells.

24 The MOI for CRISPRi screen was quantified as the 10%-30% of BFP-positive cells to ensure one gRNA enters one cell.

Calculating the Lentiviral Titer in TU/ml

25 **Method 1: Calculating using dilution Factor**
T= (NXFXD)/Vt

Where
T= Titer, (TU/mL)
N=Number of cells transduced
F= Fraction of cells with fluorescence
D=Dilution Factor
Vt=Transduction volume in mL

Method 2: Calculating using volume of virus
T= (NxF)/Vv

Where,
T= Titer, (TU/mL)
N=Number of cells transduced
F= Fraction of cells with fluorescence
Vv=Virus volume

Detailed protocol for lentiviral titration for the virus can be found in the following link:
https://www.addgene.org/protocols/fluorescence-titering-assay/

Calculating Virus volume for required MOI

26 For Perturb seq, to restrict the viral integration in such a way that one virus infects one cell, we keep the MOI between 0.1-0.3.

27 Calculating the virus volume, for MOI (0.1-0.3).
MOI=(T xVv)/N

Where,
T= Titer, (TU/mL)
N=Number of cells transduced
Vv=Virus volume

Detailed protocol for calculating MOI can be found in the following link:
https://info.abmgood.com/multiplicity-of-infection-moi