VERSION 1

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

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PERTURB SEQ PROTOCOL FOR EARLY POST-MITOTIC DOPAMINERGIC NEURONS V.1

Renuka Ravi Gupta[1,2,3,4], n.farbehi[1,2,3,5], hendersa[3,6], Vikram Khurana[3,7], Gist Croft[3,8], Lorenz Studer[3,6], Joseph Powell[1,2,3,4]

[1]Garvan Weizmann Center for Cellular Genomics, Garvan Institute of Medical Research, Sydney, NSW 2010, Australia;
[2]Garvan Institute of Medical Research, Sydney, NSW 2010, Australia;
[3]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20815, USA;
[4]School of Medical Science, University of New South Wales, Sydney, NSW, 2052, Australia;
[5]Graduate School of Biomedical Engineering, University of New South Wales, Sydney, NSW, 2052, Australia;
[6]The Centre for Stem Cell Biology, Developmental Biology Program, Sloan Kettering Institute for Cancer Research, New York, NY, USA;
[7]Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA;
[8]The New York Stem Cell Foundation Research Institute, New York, NY, USA

Renuka Ravi Gupta

ABSTRACT

We have developed a protocol where genetic perturbations via CRISPRi machinery are introduced into early post mitotic dopaminergic neurons through lentiviral transduction . The selected cells were used to generate sequencing ready single cell libraries to assess gene expression profiles and CRISPR mediated perturbations.

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Keywords: ASAPCRN, Perturb-Seq, CRISPRi, Lentiviral Transduction, Stem Cells , Dopaminergic Neurons, Parkinson Disease

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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
	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
	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	

	A	B
	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) (25ml)			
	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

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 Aspirate the spent media.

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

4 Note: Ensure the robust adherence of neurons throughout the washing procedures. To mitigate the risk of neuronal detachment, it is imperative to conduct microscopic inspections during each wash step.

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

6 Note: Ideally the neurons should dissociate as single cells.

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

8 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.

9 Centrifuge the cells at 300g for 5 minutes.

10 Aspirate the spent media gently without disturbing the pellet.

11 Resuspend the cells in 300 ul of FACs buffer.

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

13 Sort the neurons to obtain 10% - 30% BFP positive cells and collect a minimum of 20000 cells for capture.

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

Day 0: Coating wells with Laminin and Fibronectin

15 Thaw Fibronectin and Laminin on ice.

16 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.

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

Day 1: Transduction of hESC CRISPRi dCAS9 D25 midbrain dopaminergic …

18 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

19 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

20 Prepare 15 ml tubes with 200k D25 pure population mDA neuronal suspension (CD49e neg) with concentrated lentiviral supernatants at an Multiplicity of Infection (MOI) of 10 - 30%.

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

22 Add 200 ul /well of the virus media cocktail with the cells from step iii.

23 To increase the transduction efficiency, centrifuge the plate at 300 g for 20 minutes at 25 C (Spinoculation method).

24 Incubate the cells at 37ºC with 5% CO2 and 20.9% O2 for 16-18 hours.

Day 2: Replace media

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

26 Return the plate back to the incubator.

Day 4: FACs Sort

27 Aspirate the spent media.

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

Note

Ensure the robust adherence of neurons throughout the washing procedures. To mitigate the risk of neuronal detachment, it is imperative to conduct microscopic inspections during each wash step.

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

Note

Ideally the neurons should dissociate as single cells.

30 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.

31 Centrifuge the cells at 300g for 5 minutes.

32 Aspirate the spent media gently without disturbing the pellet.

33 Resuspend the cells in 300 ul of FACs buffer.

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

35 Sort the neurons to obtain 10% - 30% BFP positive cells and collect a minimum of 20000 cells for capture.

Day 4: 10x Capture (Contd)

36 Sequencing specifications are as follows:
For the Gene Expression library. The standard read depth is 30000 reads per cell.

For the CRISPR library, the stand read depth is 7500 reads per cell.

To calculate the number of cells needed for the capture, we use the following formula

Total number of cells = Number of genes x Number of guides / genes x Number of cells/ guide, where

Number of genes =30

Number of guides/ genes = 2 (since we are using the dual guide strategy)

Number of cells per guide = 200

Total number of cells =30 genes x 2 dual guides x 200 cells = 12000 cells

37 Load a minimum of 12000 cells for the capture.

38 The 10x capture was performed using the Chromium Next GEM Single Cell 5’ Reagent Kit v2 (Dual Index with Feature Barcode technology for CRISPR screening.

39 Detailed protocol for 10X capture can be found in this link: https://cdn.10xgenomics.com/image/upload/v1684188462/support-documents/CG000510_ChromiumNextGEMSingleCell5_v2_CRISPR_UserGuide_Rev_C.pdf