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Immunoblotting protocol for dopamine and cortical iPSC-derived neurons with different Parkinsonian mutations

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol details the method for immunoblotting of differentiated VMDA and cortical iPSC's from different Parkinson's mutations for quantification of Parkin, α-syn, Rab-10 and glucocerebrosidase proteins.

Materials

Solutions

- *1 x MES buffer*

50 mL 20x MES Buffer
950 mL dH2O
*make 1 L at a time

- *10 x Transfer buffer (1L):*

105 g Glycine
22.5 g Tris
1L dH2O

- *1 x Transfer Buffer (15% methanol)*

100 mL 10x Transfer buffer
750 mL dH2O
150 mL Methanol
Note: Should be made up fresh (place in cold room or on ice to cool prior to transfer)

- *10x TBS buffer (1L):*

87 g Sodium Chloride
12 g Tris
* pH to 7.4

- *1x TBS + 0.1% Tween 20 (TBS-T)*

100 mL 10x TBS buffer
1 mL Tween-20
900 mL dH2O
*use magnetic stirrer to dissolve Tween into solution

- *5% Skim Milk in TBST*

5 g skim milk powder
100 mL TBS-T

- *Stripping buffer (1L)*

15 g glycine
1 g SDS
10 ml Tween20
- Adjust pH to 2.2
- Bring volume up to 1 L with ultrapure water.

Antibodies

	Antibody	Company	Cat. No.	Dil. Factor	Molecular weight
	alpha synuclein	BD Biosciences	610787	1:1000	14kDa
	P62/SQSTM 1	Abcam	ab56416	1:1000	62 kDa
	GBA	Abnova	H00002629 -M01	1:1000	62 kDa
	Rab-10	Cell Signalling Technologie s	8127S	1:1000	23 kDa
	B-actin	Abcam	ab6276	1:50000	37 kDa

Primary antibodies

	Antibody	Company	Cat. No.	Dil. Factor
	Anti-Mouse IgG (H+L) HRP Conjugate	Bio-Rad	1706516	1:5000
	Anti-Rabbit IgG (H+L) HRP Conjugate	Bio-Rad	1706515	1:5000

Secondary antibodies

Troubleshooting

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet) for each of the raw materials used.

Ethics statement

Experiments using human iPSCs were approved by the Human Research Ethics Committee at the University of Sydney (2017/094). Application of this protocol may need prior approval by the users' institutional review board (IRB) or equivalent ethics committee(s).

Before start

Ventral midbrain progenitors and Cortical neural progenitors were generated from iPSC according to the methods of Gantner and colleagues (1, 2).

BCA Assay and Sample preparation

1 Thaw samples and centrifuge at 12000 rpm, 4°C , 4°C, 00:20:00

2 Transfer supernatant to new microcentrifuge tubes and discard pellet.

3 Perform BCA assay as per manufacturer's protocol to determine protein concentration of lysates

4 Prepare 30 µL sample for loading by adding 20 ug of protein lysate to required volme of dH2O
and 7.5 µL of 4X NuPage LDS sample Buffer

5 Store samples at -20 °C until ready to run western blot

Running Gels

1h 10m

6 Thaw samples from freezer on ice and place on heating block for 00:10:00 at 70 °C

10m

7 While waiting for samples to heat up remove the white sticker and gel comb carefully from
gel(s) and rinse with dH2O.

8 Place 12-well gel(s) into casket and clip in place. Fill up the centre part with 1 X MES buffer and
check for any leaks. Once in place, add 1 X MES to the outer space until half way full.

9 After samples have heated, quickly vortex and spin down.

10 Load 4 µL of the ladder and 25 µL of the samples prepared at 1 mg/mL (or as needed)

11 Run gel at 180 V (constant voltage) for 01:00:00 at Room temperature

1h

Transfer and blocking

2h 30m

12 Obtain large container and fill with 1 X transfer buffer

13 For each gel obtain a sandwich apparatus, 2 X sponges, 2 X filter paper, 1 X nitrocellulose membrane (0.45 um)

14 Open sandwich apparatus and place components in the following order: sponge, filter paper, gel, nitrocellulose membrane, filter paper, sponge

15 Perform transfer at 90 V (constant voltage) for 01:30:00 in cold room 1h 30m

16 Place membrane in container and cover with 5% skim milk in TBS-T solution, place on rocking platform for 01:00:00 1h

Primary and secondary antibody incubations

1h 20m

17 Prepare primary antibody in 5% skim milk in TBS-T solution (dilution 1:1000)

18 Cut blots using scissors into smaller strips as desired

19 Incubation at 4 °C overnight with gentle rocking

20 Wash blots with TBS-T every 3 times for 00:10:00 10m

21 Prepare secondary antibody (anti-mouse or anti-rabbit HRP conjugated antibody) in 2.5% skim milk in TBS-T solution

22 Incubation at Room temperature for 01:00:00 with gentle rocking 1h

23 Wash blots with TBS-T every 3 times for 00:10:00 10m

Blot imaging

24 Visualise bands using BioRad ChemiDoc MP Imaging System chemiluminescence mode

25 Band analysis and quantication is performed in ImageLab, all proteins are normalised to B-
actin used as a loading control

Stripping and

1h 20m

26 If stripping membrane is required to reprobe withanother antibody, add Abcam mild stripping
buffer incubate for 00:10:00 at Room temperature with rocking. 10m

27 Wash membrane 3 times with TBS-T for 00:10:00 each, them block in 5% skim milk in
TBS-T solution for 01:00:00 1h 10m

28 Repeat step 17 onwards with new primary antibody as required.

Protocol references

1. Gantner, C.W., et al., An optimized protocol for the generation of midbrain dopamine neurons under defined conditions. Star Protocols, 2020. 1(2): p.100065.
2. Gantner, C.W., et al., FGF-MAPK signaling regulates human deep-layer corticogenesis. Stem cell reports, 2021. 16(5): p. 1262-1275