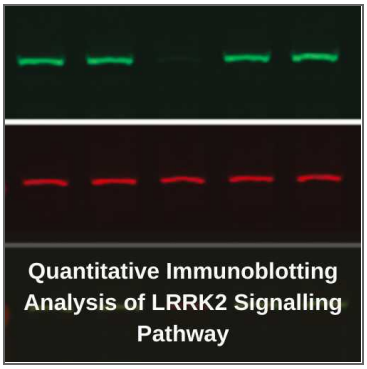

Quantitative Immunoblotting Analysis of LRRK2 Signalling Pathway V.2

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ABSTRACT

Accurate, quantitative analysis of protein expression and modifications (such as phosphorylation) is critical when studying cell signalling. Here we describe our method for efficient immunoblotting analysis of the LRRK2 signalling pathway components in cell and mouse tissue extracts. Specifically, we immunoblot using rigorously validated and characterized antibodies for LRRK2-total and LRRK2-pSer935 (multiplexed), Rab10-total and Rab10-pThr73 (multiplexed), Rab12-total and Rab12-pSer105 (multiplexed) and GAPDH or tubulin (loading control), although the protocol described here can also be applied to different cell components. Included are procedures for sample preparation from cultured cells and mouse tissue, gel electrophoresis, protein transfer, and antibody incubation.

Note: If analysing cells isolated from human blood (neutrophils, monocytes or PBMCs), please refer to the specific protocols deposited in Protocols.io on how to isolate these cells. These lysates can then be analysed by immunoblotting as described here (See Section "Preparation of samples for immunoblot analysis" to Section "Image acquisition and Analysis").

In this new version, the last step contains a supplemental video with extra context and tips, as part of the ASAP Protocol Particulars, featuring conversations with protocol authors

GUIDELINES

Figures

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

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Keywords: LRRK2, Rab GTPases, quantitative immunoblotting, lysate preparation, cells and tissues, ASAPCRN

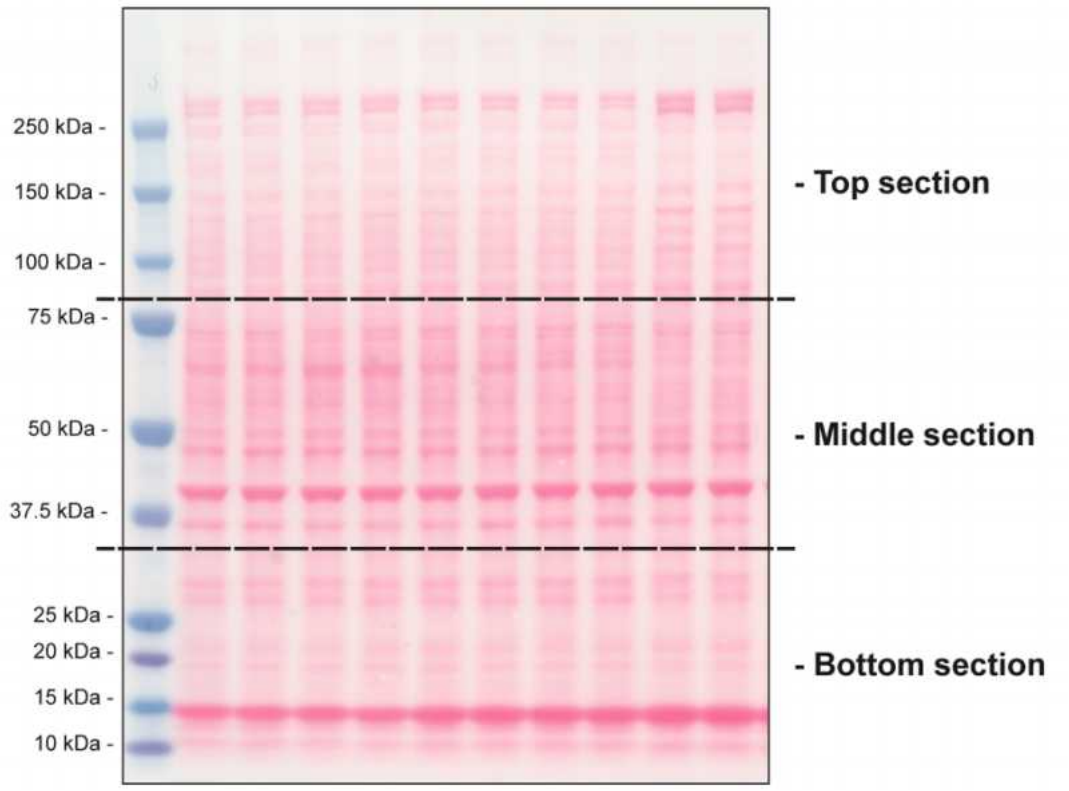

Figure 1: After protein transfer, each membrane can be divided into three sections as shown here:

1. 'top section' (to be blotted for LRRK2-total and LRRK2-pSer935),
2. 'middle section' (to be blotted for a loading control like alpha tubulin or GAPDH), and
3. 'bottom section' (to be blotted for Rab10-total and Rab10-pThr73, or Rab12-total and Rab12-pSer105).

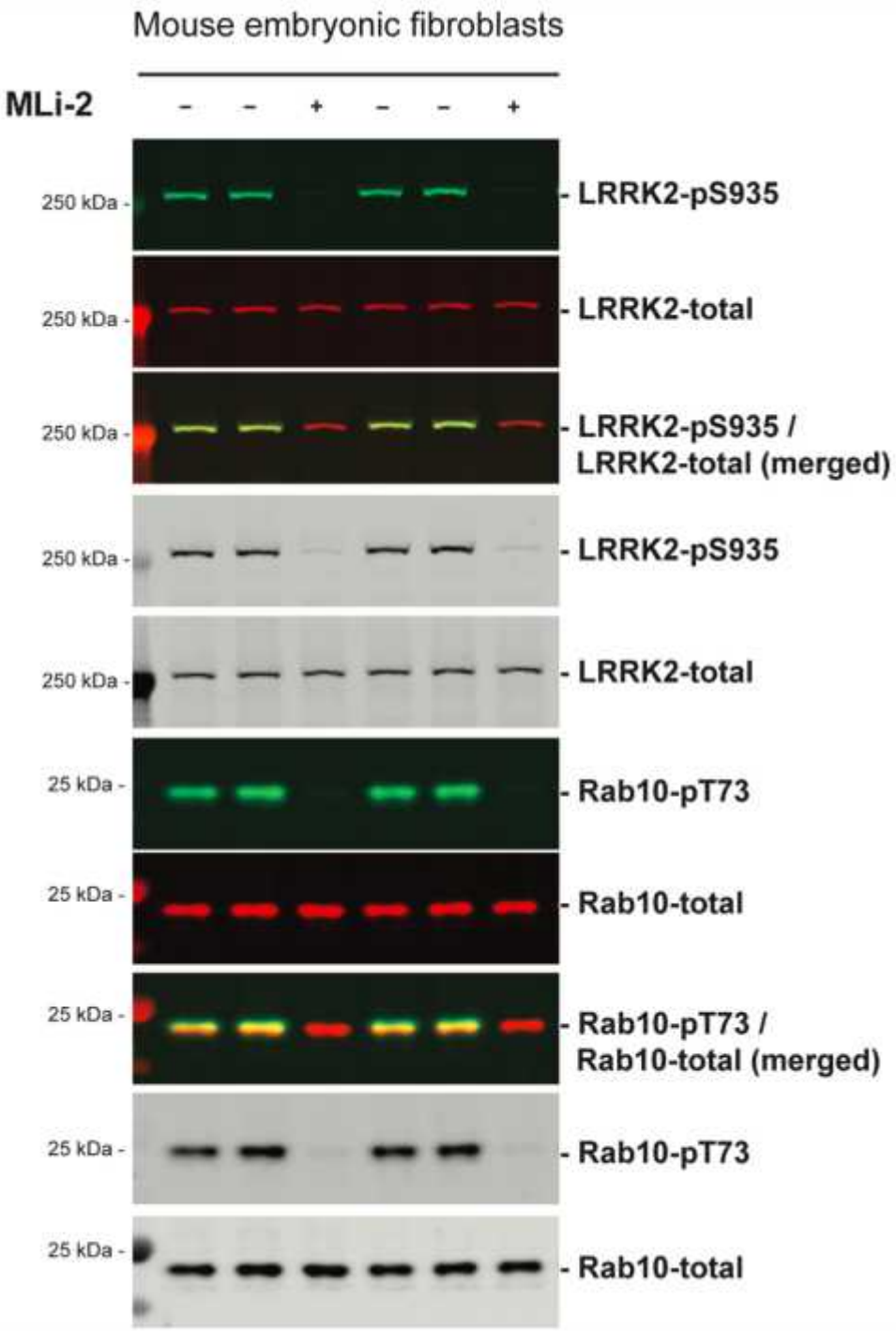

Figure 2: Representative results of quantitative immunoblotting analysis of pThr73 Rab10/total Rab10 and pS935 LRRK2/total LRRK2 signal performed in extracts from wild-type mouse embryonic fibroblasts treated +/- LRRK2 inhibitor MLi-2 (100 nM, 90 min) according to the protocol described here. Anti-LRRK2-total and LRRK2-pSer935 antibodies are multiplexed; anti-Rab10-total and Rab10-pThr73 antibodies are also multiplexed.

MATERIALS

Reagents:

1. Lysis buffer: 50 mM Tris-HCl pH 7.5, 1%(v/v) Triton X-100, 1 mM EGTA, 1 mM Na3VO4**, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.27 M sucrose, cOmpleteTM, EDTAfree Protease Inhibitor Cocktail (Roche, 11836170001)**, 1 µg/ml Microcystin-LR (Enzo Life Sciences, ALX-350-012)**. **: To be added fresh before use.
2. Bradford assay kit (Pierce™ Coomassie Plus (Bradford) Assay Kit, ThermoFisher Scientific 23236, or equivalent).
3. 4X Loading buffer: Invitrogen™ NuPAGE™ LDS Sample Buffer, cat no NP0007; 4X SDS loading buffer: 250mM Tris-HCl, pH6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue. Note: Supplement with 5% (v/v) beta-mercaptoethanol before use.
4. NuPAGE 4-12% Bis-Tris Midi Gels (Thermo Fisher Scientific, Cat# WG1402BOX or Cat# WG1403BOX) or self-cast 10% Bis-Tris gels.
5. SDS-PAGE buffer: For NuPAGETM Bis-Tris gels: NuPAGE MOPS SDS running buffer (ThermoFisherScientific, Cat#NP000102); for self-cast Bis-Tris gels: 50 mM MOPS, 50 mM Tris, 0.1% (w/v) SDS, 1 mM EDTA.
6. Protein transfer buffer: 48 mM Tris-HCl, 39 mM glycine; freshly supplemented with 20% Methanol (v/v).
7. TBS-T: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20.
8. Membrane blocking solution: 5% (w/v) non-fat milk powder in TBS-T.
9. Antibody dilution buffer: 5% (w/v) bovine serum albumin (BSA) in TBS-T.
10. Primary antibodies and near-infrared fluorescent IRDye secondary antibodies (See Table 1 and Table 2).
11. For cell treatment: LRRK2 inhibitor (1000X concentration stock in DMSO; e.g.: 100 µM stock of MLi-2 in DMSO for treatment at 100 nM final concentration) and DMSO as control vehicle.
12. For subcutaneous administration of MLi-2 to mice: MLi-2 sodium salt dissolved in 40% (w/v) (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich #332607) (6 mg/ml stock to achieve a 30 mg/kg final dose of MLi-2)

Equipment:

1. Cryogenic tissue pulveriser (Cellcrusher tissue pulveriser, or equivalent)
2. Refrigerated bench-top centrifuge (Eppendorf microcentrifuge 5417R, or equivalent).
3. Plate reader for Protein quantification (BioTek Epoch, or equivalent)
4. Dry bath/heat block (Thermo Scientific™ 88870005, or equivalent).
5. XCell4 SureLock Midi-Cell Electrophoresis System (if using Invitrogen NuPAGE precast midi gels), or equivalent gel electrophoresis apparatus.
6. Protein transfer apparatus: Trans-Blot® Cell (Bio-Rad), or equivalent wet transfer system.
7. See-saw rocker (VWR SSL4, or equivalent).
8. Odyssey CLx Imaging System paired with Image StudioTM Software

	A	B	C	D	E
	Antibody Target	Company	Cat. number	Host species	Dilution
	Rab10 pThr73	Abcam Inc.	ab230261	Rabbit	1 µg/ml
	Rab10 (total)	Nanotools	0680-100/Rab10-605B11	Mouse	1 µg/ml
	Rab10 (total)	Abcam Inc.	ab237703	Rabbit	1 µg/ml
	Rab12 pSer105 (mouse) / pSer106 (human)	Abcam Inc.	ab256487	Rabbit	1 µg/ml
	Rab12 (total)	MRC-PPU Reagents and Services, University of Dundee	SA227	Sheep	1 µg/ml
	Rab12 (total)	Proteintech	18843-1-AP	Rabbit	1 µg/ml
	LRRK2 pSer935	MRC-PPU Reagents and Services, University of Dundee	UDD2	Rabbit	1 µg/ml
	LRRK2 (total) (C-terminus)	Antibodies Inc./NeuroMab	75-253	Mouse	1 µg/ml
	LRRK2 (total) (N-terminus)	MRC-PPU Reagents and Services, University of Dundee	UDD3	Rabbit	0.1 µg/ml
	GAPDH	Antibodies Inc./NeuroMab	sc-32233	Mouse	1:5,000
	alpha-tubulin	Cell Signaling Technology	3873	Mouse	1:5,000

Table 1.

	A	B	C	D
	Secondary Antibodies	Company	Cat. number	Notes
	goat anti-mouse IRDye 680LT	LI-COR	926-68020	
	goat anti-mouse IRDye 800CW	LI-COR	926-32210	
	goat anti-rabbit IRDye 800CW	LI-COR	926-32211	
	donkey anti-mouse IRDye 680LT	LI-COR	926-68022	

	A	B	C	D
	donkey anti-mouse IRDye 800CW	LI-COR	926-32212	
	donkey anti-rabbit IRDye 800CW	LI-COR	926-32213	
	donkey anti-goat IRDye 800CW	LI-COR	926-32214	Reacts with Sheep primary Abs

Table 2.

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

Preparation of lysates

1 Please choose the relevant method for preparation of lysate:

1. Preparation of lysates from **cultured cells**
2. Preparation of lysates from **mouse tissues**

Step 1 includes a Step case.

1. Preparation of lysates from cultured cells

2. Preparation of lysates from mouse tissues

step case

1. Preparation of lysates from cultured cells

Choose this method if you will be preparing lysates from ***cultured cells.***

2

Note

To ensure the specificity of the phosphorylation signals detected by immunoblotting in cell lysates, we recommend treating cells for at least 30 min ± a LRRK2 kinase inhibitor such as MLi-2 at 100nM final concentration (or equivalent volume of DMSO vehicle) before lysis.

3 Quickly wash cells in the tissue culture dish by carefully pouring

Room temperature culture media without Foetal bovine serum (FBS) that the cells are currently growing in into the dish. We do not recommend using Phosphate Buffered Saline to wash cells prior to cell lysis as this may cause nutrient stress.

4 Pour off media without FBS from the culture dish, tilt dish and completely aspirate all residual media. Immediately add freshly prepared ice-cold lysis buffer, ensuring that the entire surface is covered by lysis buffer.

Note

The amount of lysis buffer to use will depend on cell type and cell confluency. As a guideline, use 0.1-0.2 ml of lysis buffer for each well of a 6 well plate, 0.5 ml for a 10 cm dish and 1 ml for a 15 cm dish. It is preferable to aim for protein concentrations of a least 0.75 µg/µl as this will enable the appropriate amount of protein to be loaded onto a gel as detailed below.

5 Immediately place the cell dishes On ice.

6 Scrape the cells on the dish using a cell lifter (Sigma-Aldrich CLS3008, or equivalent) to ensure all cells are detached from the dish.

7 Using a pipette, transfer cell lysate to an Eppendorf tube On ice.

Note

For non-adherent cells, transfer cells to a Falcon tube and pellet by centrifugation at 180 x g, 00:03:00; wash cells once with Room temperature culture media without FBS and pellet again as above. Discard supernatant and add freshly prepared ice-cold lysis buffer. Immediately transfer the Falcon containing the cell pellet to ice.

8 Leave samples On ice for 00:20:00 to allow for efficient lysis. 20m

9 Clarify lysates by centrifugation at 20800 x g, 4°C, 00:10:00. 10m

10 Transfer the supernatants into new Eppendorf tubes and discard the pellet. Keep the tubes On ice.

Note

Cell lysates can be snap frozen in liquid nitrogen and stored at -80 °C for future use.

Preparation of samples for immunoblot analysis

11 Determine the protein concentration of cell lysates by Bradford assay according to the manufacturer's instructions, performing measurements in triplicate.

Note

Ensure the concentration of the samples is in the linear range for the Bradford assay. If it isn't, prepare appropriate dilutions in water of each lysate. Generally, protein concentrations of near confluent cells lysed as described above should range from 0.5 to 5 µg/µl (depending on cell type).

12 Prepare samples for immunoblotting to achieve the same protein concentration for all samples (ideally, 0.5-2 µg/µl, depending on the sample at the lowest concentration) by combining the cell lysate with lysis buffer. Add a quarter of a volume of 4X SDS/LDS loading buffer freshly supplemented with beta-mercaptoethanol (i.e. for 7.5 µl of lysate/lysis buffer mix, add 2.5 µl of loading buffer). Mix by vortexing.

13 Incubate samples for 00:05:00 at 70 °C heating block before immunoblot analysis. 5m

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 2h 30m

Load samples onto a NuPAGE 4–12% Bis–Tris Midi Gel (ThermoFisherScientific,

14 Cat#WG1402BOX or Cat#WG1403BOX), or a self-cast 10% Bis-Tris gel, alongside pre-stained molecular weight markers (ranging from 10 kDa to 250 kDa). Rinse wells carefully with running buffer before loading samples.

Note

- The amount of protein loaded for each sample ranges from 10 to 40 µg, depending on the cell type and the protein(s) of interest. For cell lines like mouse embryonic fibroblasts, A549 cells and cells isolated from human peripheral blood (monocytes, neutrophils), we recommend loading 10-15 µg of protein for each cell extract for optimal signal.
- Be aware of maximum loading capacity of each well as per manufacturer's instructions and take care not to overload wells.
- If multiple gels are used for each set of experimental samples, an internal loading control should also be included for subsequent data normalization.

15 Electrophorese samples at 130V with MOPS SDS running buffer for 02:00:00 or until the blue dye runs off the gel. 2h

Protein transfer (Wet electroblotting) 2h 30m

16 Equilibrate the gel, one piece of nitrocellulose membrane (GE Healthcare, Amersham Protran Supported 0.45 µm NC) and two pieces of filter paper (WhatmanTM 3MM Chr Chromatography Paper, or equivalent) (all of the same size as the gel) by pre-soaking them in transfer buffer.

17 Assemble the gel and membrane transfer stack in a tray filled with transfer buffer to ensure that all components are submerged during the assembling. Place one sponge pad inside the cassette holder (on the side that will be facing the cathode). Place one piece of filter paper on top of the sponge pad, followed by the gel, nitrocellulose membrane, another piece of filter paper and another sponge pad.

Note

Carefully remove any air bubbles between layers using a roller after adding each layer.

18 Carefully close the cassette holder and insert it in the transfer tank. Fill the tank with transfer buffer.

19 Electrophoretically transfer proteins from gel onto a nitrocellulose membrane at 100 V (constant voltage) for 01:40:00 On ice using a wet transfer system. 1h 40m

20 After transfer, stain membranes with Ponceau solution to assess transfer efficiency and general quality of the samples. If an image is required for record, the Ponceau-stained membraned can be scanned.

21 Each membrane can be divided into three sections by two horizontal cuts (one cut just above the 75 kDa ladder band and another cut just below the 37.5 kDa ladder band):

1. 'top section' (from the top of the membrane to the 75 kDa marker),
2. 'middle section' (between the 75 kDa and the 37.5 kDa marker), and
3. 'bottom section' (from the 37.5 kDa marker to the bottom of the membrane) (Figure 1), to be incubated with primary antibodies as described in Step 23.

Note

Samples loaded onto one gel can be simultaneously blotted for LRRK2-total and LRRK2-pSer935, Rab10-total and Rab10-pThr73 and a loading control like alpha tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). If in addition Rab12-total and Rab12-pSer105 levels are analysed, duplicate gels are required.

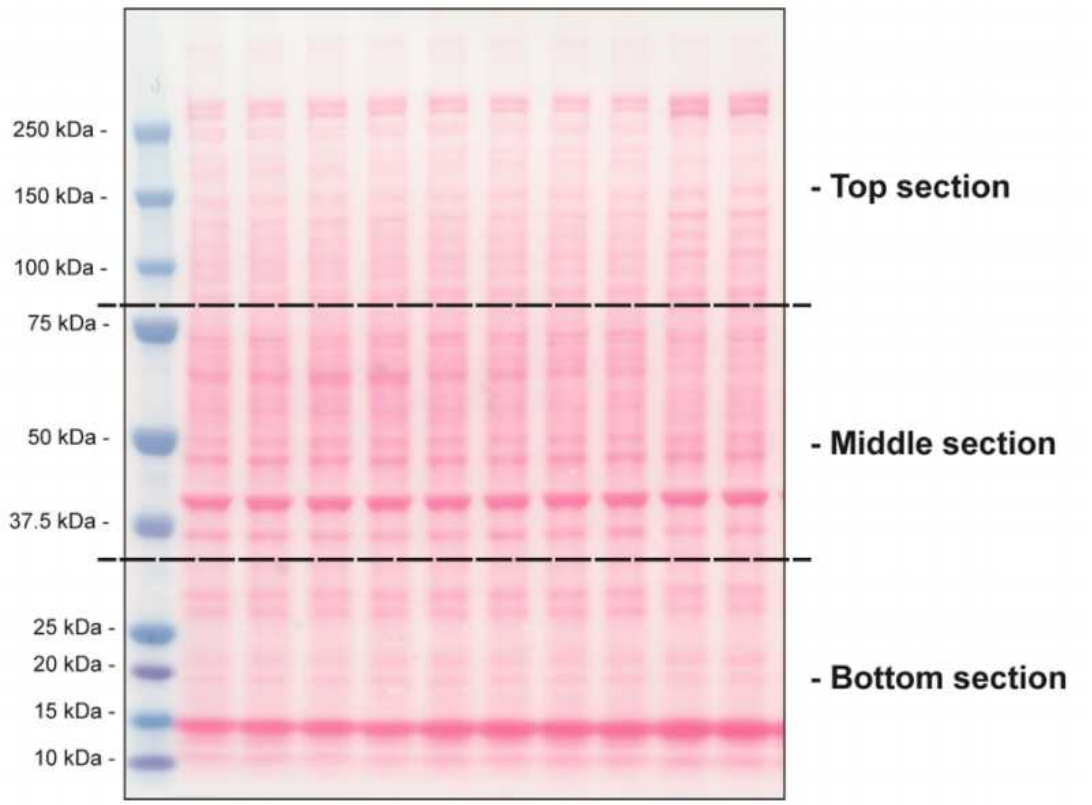

Figure 1: After protein transfer, each membrane can be divided into three sections as shown here:

1. 'top section' (to be blotted for LRRK2-total and LRRK2-pSer935),
2. 'middle section' (to be blotted for a loading control like alpha tubulin or GAPDH), and
3. 'bottom section' (to be blotted for Rab10-total and Rab10-pThr73, or Rab12-total and Rab12-pSer105).

Membrane blocking and antibody incubation

15m

22 Destain membranes from Step 21 by washing with TBS-T and incubate in blocking solution for at least 00:15:00 at room temperature on a see-saw rocker. 15m

23 Rinse the membrane in TBS-T and incubate Overnight at 4 °C with primary antibodies (diluted in 5% (w/v) BSA in TBS-T to their working concentration – Table 1), as follows: 15m

1. 'top section': incubate with anti-total and phospho-LRRK2 antibodies;
2. 'middle section': incubate with anti-alpha tubulin or GAPDH antibody;
3. 'bottom section': incubate with anti-total and phospho-Rab antibodies.

Note

- If assessing protein phosphorylation, we multiplex the phospho-specific antibody with the total antibody detecting the protein of interest.
- Table 1 lists the primary antibodies most commonly used in our lab to study LRRK2 signalling and their suggested working dilution. The selectivity and specificity of the antibodies suggested in Table 1 have been extensively validated using appropriate controls, including overexpression models and knock-out cell lines. All antibodies listed in Table 1 react with both human and mouse cells/tissues.

Note

	A	B	C	D	E
	Antibody Target	Company	Cat. number	Host species	Dilution
	Rab10 pThr73	Abcam Inc.	ab230261	Rabbit	1 µg/ml
	Rab10 (total)	Nanotools	0680-100/Rab10-605B11	Mouse	1 µg/ml
	Rab10 (total)	Abcam Inc.	ab237703	Rabbit	1 µg/ml
	Rab12 pSer105 (mouse) / pSer106 (human)	Abcam Inc.	ab256487	Rabbit	1 µg/ml
	Rab12 (total)	MRC-PPU Reagents and Services, University of Dundee	SA227	Sheep	1 µg/ml
	Rab12 (total)	Proteintech	18843-1-AP	Rabbit	1 µg/ml
	LRRK2 pSer935	MRC-PPU Reagents and Services, University of Dundee	UDD2	Rabbit	1 µg/ml
	LRRK2 (total) (C-terminus)	Antibodies Inc./NeuroMab	75-253	Mouse	1 µg/ml
	LRRK2 (total) (N-terminus)	MRC-PPU Reagents and Services, University of Dundee	UDD3	Rabbit	0.1 µg/ml
	GAPDH	Antibodies Inc./NeuroMab	sc-32233	Mouse	1:5,000
	alpha-tubulin	Cell Signaling Technology	3873	Mouse	1:5,000

Table 1.

24

After incubation with primary antibodies, wash membranes in TBS-T (3 washes, 5-10 minutes each, on a see-saw rocker):

24.1 (Wash 1/3): Wash membranes in TBS-T 00:05:00 - 00:10:00, on a see-saw rocker. 10m

24.2 (Wash 2/3): Wash membranes in TBS-T 00:05:00 - 00:10:00, on a see-saw rocker. 10m

24.3 (Wash 3/3): Wash membranes in TBS-T 00:05:00 - 00:10:00, on a see-saw rocker. 10m

25 

Incubate membranes with near-infrared fluorescent dye-labelled secondary antibodies (diluted to the working concentration: 1:20,000) for 01:00:00 at Room temperature on a see-saw rocker. 1h

Note

- If multiplexing primary antibodies, use secondary antibodies labelled with spectrally distinct nearinfrared fluorescent dyes. Generally, we use IRDye 800CW (800 nm channel) secondary antibodies for the phospho-antibodies multiplexed with IRDye 680LT (680 nm channel) secondary antibodies for the corresponding total antibody.
- Table 2 lists the near-infrared fluorescent dye-labelled secondary antibodies used in our lab.

Note

	A	B	C	D
	Secondary Antibodies	Company	Cat. number	Notes
	goat anti-mouse IRDye 680LT	LI-COR	926-68020	
	goat anti-mouse IRDye 800CW	LI-COR	926-32210	
	goat anti-rabbit IRDye 800CW	LI-COR	926-32211	
	donkey anti-mouse IRDye 680LT	LI-COR	926-68022	
	donkey anti-mouse IRDye 800CW	LI-COR	926-32212	
	donkey anti-rabbit IRDye 800CW	LI-COR	926-32213	
	donkey anti-goat IRDye 800CW	LI-COR	926-32214	Reacts with Sheep primary Abs

Table 2.

26 Extensively wash membranes in TBS-T (4 washes, 10-15 minutes each, with agitation):

26.1 (Wash 1/4): Extensively wash membranes in TBS-T 00:10:00 – 00:15:00, with agitation. 15m

26.2 (Wash 2/4): Extensively wash membranes in TBS-T 00:10:00 – 00:15:00, with agitation. 15m

26.3 (Wash 3/4): Extensively wash membranes in TBS-T 00:10:00 – 00:15:00, with agitation. 15m

26.4 (Wash 4/4): Extensively wash membranes in TBS-T 00:10:00 – 00:15:00, with agitation. 15m

Image acquisition and Analysis

27 Protein bands are acquired via near infrared fluorescent detection using the Odyssey CLx Imaging System and the signal intensity quantified using the Image Studio Software.

Note

To control for inter-gel variability, the signal intensity of each band can be normalised against the control sample loaded in each gel of a set of experiments.

28 Analyse immunoblotting data using a software for statistical analysis (Graphpad Prism, or equivalent).

ASAP Protocol Particulars: context and tips

29

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