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Quantitative immunoblot analysis of LRRK1 signalling pathway

Asad Malik¹, Athanasios Karapetsas¹, Francesca Tonelli ¹, Dario R Alessi¹

¹Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Do w Street, Dundee DD1 5EH, UK

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	Dario Alessi

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 LRRK1 signalling pathway components in cell and mouse tissue extracts. Specifically, we immunoblot using rigorously validated and characterized antibodies for LRRK1-total, Rab7A and pS72 Rab7A, pT202/Y204 ERK1/2, phosphor(Ser)-PKC Substrates and GAPDH or Tubulin (loading controls), 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.

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immunoblot analysis, LRRK1, Rab7A, LRRK1 signalling pathway, ASAPCRN

_____ protocol,

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Mar 25, 2022 Dario Alessi

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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.

Note: This protocol was adapted from a similar method describing immunoblot analysis of the LRRK2 pathway dx.doi.org/10.17504/protocols.io.bsgrnbv6



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Reagents:

Lysis buffer.

A	В	
Tris-HCl pH 7.5	50 mM	
Triton X-100	1%(v/v)	
EGTA	1 mM	
Na3VO4**	1 mM	
NaF	50 mM	
β-glycerophosphate	10 mM	
sodium pyrophosphate	5 mM	
sucrose	0.27 M	
cOmpleteTM, EDTA-free Protease Inhibitor Cocktail (Ro	che,	
11836170001)**		
Microcystin-LR (Enzo Life Sciences, ALX-350-012)**		

^{**:} To be added fresh before use.

Bradford assay kit (Pierce™ Coomassie Plus (Bradford) Assay Kit, ThermoFisher Scientific 23236, or equivalent).

4X Loading buffer. Invitrogen™ NuPAGE™ LDSSampleBuffer,catnoNP0007; 4X SDS loading buffer

Note: Supplement with 5% (v/v) beta-mercaptoethanol before use.

4X SDS loading buffer.

Α	В
Tris-HCl, pH6.8	250mM
SDS	8% (w/v)
glycerol	40% (v/v)
bromophenol blue	0.02% (w/v)

NuPAGE 4-12% Bis-Tris Midi Gels (Thermo Fisher Scientific, Cat# WG1402BOX or Cat# WG1403BOX) or self-cast 10% Bis-Tris gels.

SDS-PAGE buffer.

Α	В	С			
For NuPAGETM Bis-Tris	NuPAGE MOPS SDS running buffer (ThermoFisherScientific, Cat#NP000102)				
gels					
For self-cast Bis-Tris gels	MOPS	50 mM			
	Tris	50 mM			
	SDS	0.1% (w/v)			
	EDTA	1 mM			

Protein transfer buffer.

Α	В			
Tris-HCl	48 mM			
glycine	39 mM			
freshly supplemented with 20% Methanol (v/v)				

TBS-T:



Α	В
Tris-HCl, pH 7.5	50 mM
NaCl	150 mM
Tween 20	0.1% (v/v)

- Membrane blocking solution: 5% (w/v) non-fat milk powder in TBS-T.
- Antibody dilution buffer: 5% (w/v) bovine serum albumin (BSA) in TBS-T.
- Primary antibodies and near-infrared fluorescent IRDye secondary antibodies (See Table 1 and Table 2).
- For cell treatment: PKC activator PMA (1000X concentration stock in DMSO; e.g.: 100 mg/ml stock of PMA in DMSO for treatment at 100 nM final concentration) and DMSO as control vehicle

treatment at 100		* .				130, e.g.	. 100 mg	/IIII SLOCK (DI FINIA III DINISO IC	וכ
⊗ c0mplete™	Mini	EDTA-	free	Protea	se	Inhibit	tor	Cocktail	Merck	
Millipore Catalo	g #118361700	001								
⊗ Micro	c y s t i n	- L R En	ZO		Life	2				
Sciences Catalo	g #ALX-350-0	12								
⊠ Pierce™	Coomassie	Plus	(Brad	dford)	Assay	Kit	Thermo	<u> </u>		
Fisher Catalog	#23236									
⊠ Invitrogen™	NuPAGE™	LDS	Sa	mple	Buffer	(4X)	Therm	o Fis	sher	
Scientific Catalo	og #NP0007									
⊠ Invitrogen™	NuPAGE™	4 to	12%	Bis-Tris	1.0	mm	Midi	Protein	Gels Thermo	Fisher
Scientific Catalo	og #WG1402B	ОХ								
⊗ NuPAGE™	MOPS	SDS	Runi	ning	Buffer	(20	X) Ther	mo		
Fisher Catalog	#NP000102									
⊠ IRDye®	680LT	Goat	а	nti-Mouse		gG	Seco	ndary		
Antibody Licor C	atalog #926-6	8020								
	Goat anti-Mous	e Secondar	y Antibo	ody LI-						
COR Catalog #92	5-32210 926-3	32210								
	Goat anti-Rabbi	t IgG Secon	idary							
Antibody Licor Cat	talog #926-32	211								
	Donkey anti-Mou	use IgG Sec	ondary							
Antibody Licor Cat	talog #926-68	022								
	Donkey anti-Mo	use IgG Se	condary							
Antibody Licor Cat	talog #926-32	212								
	Donkey anti-Rat	obit IgG Sec	condary							
Antibody Licor Cat	talog #926-32	213								
⊠ IRDye® 800CW	Donkey anti-Goa	at IgG Seco	ndary							
Antibody Licor Catalog #926-32214										
⊗ Anti-Rab7 antibo	⊠ Anti-Rab7 antibody Mouse monoclonal Sigma									
Aldrich Catalog #	R8779									
⊗ Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody Cell Signaling										
Technology Catalog ##9101										
⊠ Phospho-(Ser) PKC Substrate Antibody Cell Signaling										
Technology Catalog ##2261										
★ Anti-GAPDH Antibody										
(6C5) NeuroMab Catalog #sc-32233										
⊗α-Tubulin (DM1A) Mouse mAb Cell Signaling										

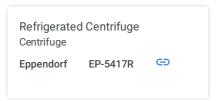


Technology Catalog #3873

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Equipment:

1. Refrigerated bench-top centrifuge (Eppendorf microcentrifuge or equivalent).



1. Plate reader for Protein quantification (BioTek Epoch, or equivalent)

Digital Dry Baths/Block Heaters
Dry bath/heat block
Thermo Scientific™ 88870005 ←

- 1. XCell4 SureLock Midi-Cell Electrophoresis System (if using Invitrogen NuPAGE precast midi gels), or equivalent gel electrophoresis apparatus.
- 2. Protein transfer apparatus: Trans-Blot® Cell (Bio-Rad), or equivalent wet transfer system.
- 3. See-saw rocker (VWR SSL4, or equivalent).
- 4. Odyssey CLx Imaging System paired with Image StudioTM Software.

Preparation of lysates from cultured cells

30m

1

Note: To ensure LRRK1 activation in cells, we recommend treating cells for at least **⊙ 00:30:00** ± PMA (a PKC activator) at a final concentration of **□100** ng/mL (or equivalent volume of DMSO) before lysis.

Quickly rinse cells in the tissue culture dish by carefully pouring **§ Room temperature** culture media without Foetal bovine serum (FBS) into the dish.

2



Pour off media from the culture dish and completely aspirate any 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 $\blacksquare 0.1 \text{ mL}$ to $\blacksquare 0.2 \text{ mL}$ of lysis buffer for each well of a 6-well plate, $\blacksquare 0.5 \text{ mL}$ for a 10- cm dish and



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■1 mL for a 15-cm dish. It is preferable to aim for protein concentrations of a least $\blacksquare 0.75 \ \mu g/\mu L$ as this will enable the appropriate amount of protein to be loaded onto a gel as detailed below.

- 3 Immediately transfer the cell dishes to ice.
- 4 Scrape the cells on the dish using a cell lifter (Sigma-Aldrich CLS3008, or equivalent) to ensure all cells are detached from the dish.
- 5 🕲 🎢 🔗

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.

- 6 Leave samples & On ice for © 00:20:00 to allow for efficient lysis.
- 7

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

8 Transfer the supernatants into new Eppendorf tubes and discard the pellet. Keep the tubes $\, \mathfrak{d} \,$ On ice .

Note: Cell lysates can be snap frozen in liquid nitrogen and stored at 8-80 °C for future use.

Preparation of samples for immunoblot analysis

Determine the protein concentration of cell or tissue 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 mg/ml (depending on cell type), while tissues lysed as described

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5

5m

10



Prepare samples for immunoblotting to achieve the same protein concentration for all samples (ideally, $\blacksquare 0.5 \ \mu g/\mu L$ to $\blacksquare 2 \ \mu g/\mu L$, 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 $\blacksquare 7.5 \ \mu L$ of lysate/lysis buffer mix, add $\blacksquare 2.5 \ \mu L$ of loading buffer). Mix by vortexing.

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Incubate samples for © 00:05:00 at & 70 °C heating block before immunoblot analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

2h

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

Notes:

- The amount of protein loaded for each sample ranges from □10 μg to □40 μg, depending on the cell/tissue 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 μg to □15 μg of protein for each cell extract for optimal signal; for mouse tissues like brain, spleen, kidney, lung and intestine we recommend loading □30 μg to □40 μg of protein for each tissue 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.

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

Protein transfer (Wet electroblotting)

1h 30m

- 4 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.
- 15 Assemble the gel and membrane transfer stack in a tray filled with transfer buffer to ensure that all components are

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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.

- 16 Carefully close the cassette holder and insert it in the transfer tank. Fill the tank with transfer buffer.
- 17 Electrophoretically transfer proteins from gel onto a nitrocellulose membrane at 100 V (constant voltage) for © 01:30:00 & On ice using a wet transfer system.
- 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.

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Each membrane can be divided into three sections by two horizontal cuts (one cut just above the 75 kDa ladder band and another cut between the 37.5 kDa and 25 kDa ladder bands) (Figure 1):

- 1. 'top section' (from the top of the membrane to the 75 kDa marker) to be probed for LRRK1
- 2. 'middle section' (between the 75 kDa and the 37.5 kDa marker) to be probed for GAPDH/tubulin (loading control)
- 3. 'bottom section' (from the 37.5 kDa marker to the bottom of the membrane) to be probed for pS72-Rab7A/Rab7A

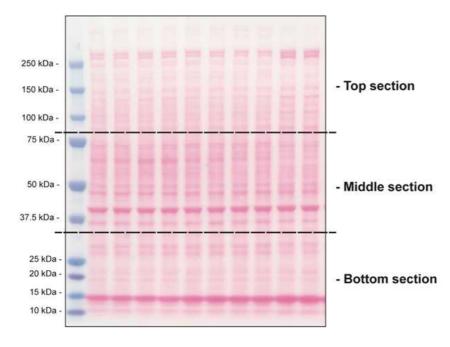


Figure 1. Ponceau-stained membrane.

Notes:

- When immunoblotting using anti-phospho(Ser)-PKC Substrates, it is preferable to blot the entire membrane to visualise all induced PKC substrates.
- The same blot can be simultaneously be blotted with a loading control like alpha tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pT202/Y204 ERK1/2 (to confirm that stimulation of cells with PMA has worked).

Membrane blocking and antibody incubation

1h 30m

20



Destain membranes from Step 19 by washing with TBS-T and incubate in blocking solution for at least © **00:15:00** at **§ Room temperature** on a see-saw rocker.

21



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:

Table 1:

A	В	С	D	Е
Antibody Target	Company	Cat. number	Host species	Dilution
pS72 Rab7A	Abcam Inc.	MJF-38, Clone 1	Rabbit	1 ug/ml
Rab7A (Total)	Sigma	R8779	Mouse	1 ug/ml
pT202/Y204 ERK1/2	Cell Signalling Technology	#9101	Rabbit	1 ug/ml
Phospho-(Ser) PKC Substrate Antibody	Cell Signalling Technology	#2261	Rabbit	1 ug/ml
LRRK1 (total) (C-terminus)	MRC-PPU Reagents and Services, University of Dundee	S405C	Sheep	1 ug/ml
GAPDH	Antibodies Inc./NeuroMab	sc-32233	Mouse	1:5,000
alpha-tubulin	Cell Signaling Technology	3873	Mouse	1:5,000

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

23



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

Note:

- If multiplexing primary antibodies, use secondary antibodies labelled with spectrally distinct near-infrared fluorescent dyes. Generally, we use IRDye 800CW (800 nm channel) secondary antibodies for the phosphoantibodies 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.

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Table 2:

Α	В	С	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

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Extensively wash membranes in TBS-T on a see-saw rocker (4 washes, 10-15 minutes per wash).

Image acquisition and Analysis

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.

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

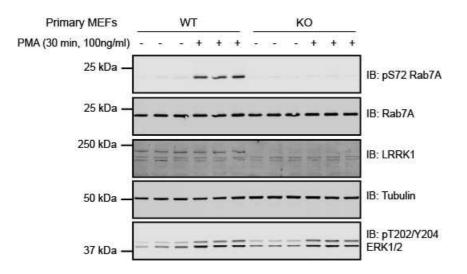


Figure 2. Representative results of quantitative immunoblotting analysis of pSer72 Rab7A/total Rab7A and total LRRK1 levels performed in extracts from wild-type (WT) and homozygous LRRK1 knock-out (KO) mouse embryonic fibroblasts treated +/- PKC activator PMA (100 ng/ml, 30 min) according to the protocol described here.