Jun 02, 2022

# Quantitative immunoblot analysis of LRRK1 signalling pathway

DOI
dx.doi.org/10.17504/protocols.io.6qpvr68e3vmk/v1

Asad Malik[1], Athanasios Karapetsas[1], Francesca Tonelli[1], Dario Alessi[1]

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

asap

Dario R Alessi
Dundee





**DOI: https://dx.doi.org/10.17504/protocols.io.6qpvr68e3vmk/v1**

**External link: https://doi.org/10.1042/BCJ20220308**

**Protocol Citation:** Asad Malik, Athanasios Karapetsas, Francesca Tonelli, Dario Alessi 2022. Quantitative immunoblot analysis of LRRK1 signalling pathway. **protocols.io https://dx.doi.org/10.17504/protocols.io.6qpvr68e3vmk/v1**

**Manuscript citation:**
Malik AU, Karapetsas A, Nirujogi RS, Chatterjee D, Phung TK, Wightman M, Gourlay R, Morrice N, Mathea S, Knapp S, Alessi DR, PKC isoforms activate LRRK1 kinase by phosphorylating conserved residues (Ser1064, Ser1074 and Thr1075) within the COR GTPase domain. Biochemical Journal 479(18). doi: 10.1042/BCJ20220308



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

**Created:** March 11, 2022

**Last Modified:** May 31, 2024

**Protocol Integer ID:** 59358

**Keywords:** immunoblot analysis, LRRK1, Rab7A, LRRK1 signalling pathway, ASAPCRN, quantitative immunoblot analysis of lrrk1, characterized antibodies for lrrk1, analysis of the lrrk1, immunoblotting analysis, quantitative immunoblot analysis, pathway components in cell, signalling pathway component, quantitative analysis of protein expression, mouse tissue extract, protein expression, signalling pathway, characterized antibody, phosphorylation, antibody, lrrk1, such as phosphorylation, protein transfer, different cell component, antibody incubation, protein, gel electrophoresi

## 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 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.

## Guidelines

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

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

## Materials

**Reagents:**

*Lysis buffer*:

| | A | B |
|---|---|---|
| | 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 (Roche, 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

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

*4X SDS loading buffer*:

| | A | B |
|---|---|---|
| | Tris-HCl, pH6.8 | 250mM |
| | SDS | 8% (w/v) |

| | A | B |
|---|---|---|
| | 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*:

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

*Protein transfer buffer*:

| | A | B |
|---|---|---|
| | Tris-HCl | 48 mM |
| | glycine | 39 mM |
| | freshly supplemented with 20% Methanol (v/v) | |

*TBS-T*:

| | A | B |
|---|---|---|
| | 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.

cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail **Merck Millipore (EMD Millipore) Catalog #**11836170001

Microcystin-LR **Enzo Life Sciences Catalog #**ALX-350-012

Pierce™ Coomassie Plus (Bradford) Assay Kit **Thermo Fisher Catalog #**23236

Invitrogen™ NuPAGE™ LDS Sample Buffer (4X) **Thermo Fisher Scientific Catalog #**NP0007

Invitrogen™ NuPAGE™ 4 to 12% Bis-Tris 1.0 mm Midi Protein Gels **Thermo Fisher Scientific Catalog #**WG1402BOX

NuPAGE™ MOPS SDS Running Buffer (20X) **Thermo Fisher Catalog #**NP000102

IRDye® 680LT Goat anti-Mouse IgG Secondary Antibody **LI-COR Catalog #**926-68020

IRDye® 800CW Goat anti-Mouse Secondary Antibody **LI-COR Catalog #**925-32210 926-32210

IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody **LI-COR Catalog #**926-32211

IRDye® 680LT Donkey anti-Mouse IgG Secondary Antibody **LI-COR Catalog #**926-68022

IRDye® 800CW Donkey anti-Mouse IgG Secondary Antibody **LI-COR Catalog #**926-32212

IRDye® 800CW Donkey anti-Rabbit IgG Secondary Antibody **LI-COR Catalog #**926-32213

IRDye® 800CW Donkey anti-Goat IgG Secondary Antibody **LI-COR Catalog #**926-32214

Anti-Rab7 antibody Mouse monoclonal **Merck MilliporeSigma (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

**Equipment:**

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

Equipment

| Refrigerated Centrifuge | NAME |
|---|---|
| Centrifuge | TYPE |
| Eppendorf | BRAND |
| EP-5417R | SKU |
| https://www.marshallscientific.com/Eppendorf-5417R-Refrigerated-Centrifuge-p/ep-5417r.htm | LINK |

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

Equipment

| Digital Dry Baths/Block Heaters | NAME |
|---|---|
| Dry bath/heat block | TYPE |
| Thermo Scientific™ | BRAND |
| 88870005 | SKU |
| https://www.thermofisher.com/order/catalog/product/88870005 | LINK |

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.

## Troubleshooting

## Preparation of lysates from cultured cells

30m

1

Note

**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

**Note**: The amount of lysis buffer to use will depend on cell type and cell confluency. As a guideline, use 0.1 mL to 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 µL 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

**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 On ice .

Note

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

## Preparation of samples for immunoblot analysis

5m

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

Note

**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 above should result in protein concentrations of at least 2 mg/ml.

10 Prepare samples for immunoblotting to achieve the same protein concentration for all samples (ideally, 0.5 µL to 2 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.

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

## SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 2h

12 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.

Note

**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

14 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 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

**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.

18 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.

19 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

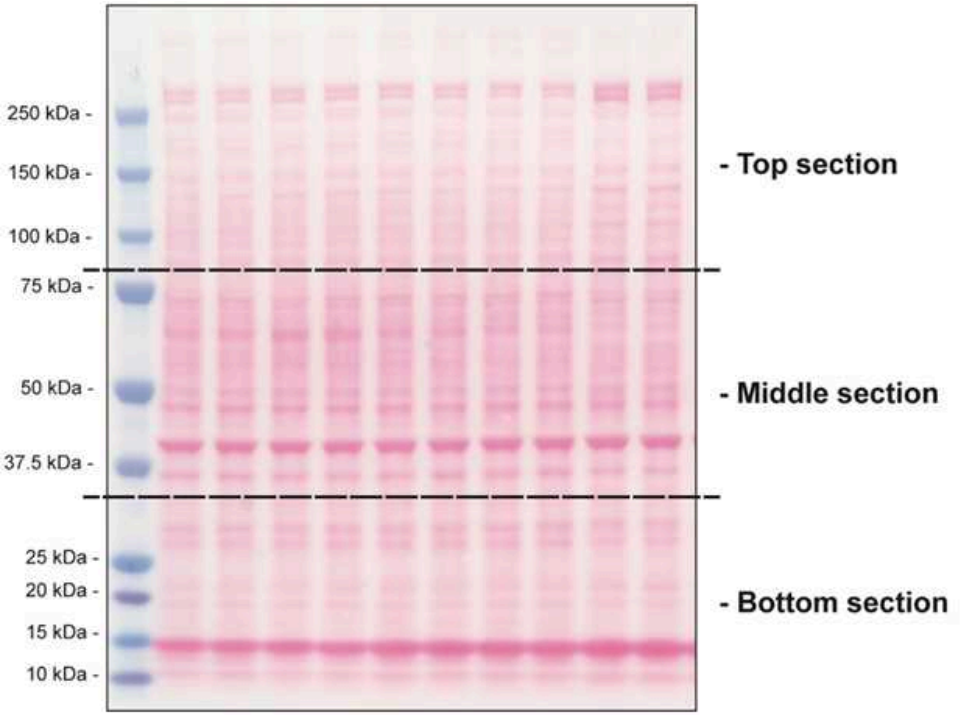


**Figure 1.** Ponceau-stained membrane.

Note

**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 | B | C | D | E |
|---|---|---|---|---|---|
| | **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 |

22 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 01:00:00 at Room temperature on a see-saw rocker.

Note

**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 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.

**Table 2:**

| | 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 |

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

## Image acquisition and Analysis

25 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

**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.

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

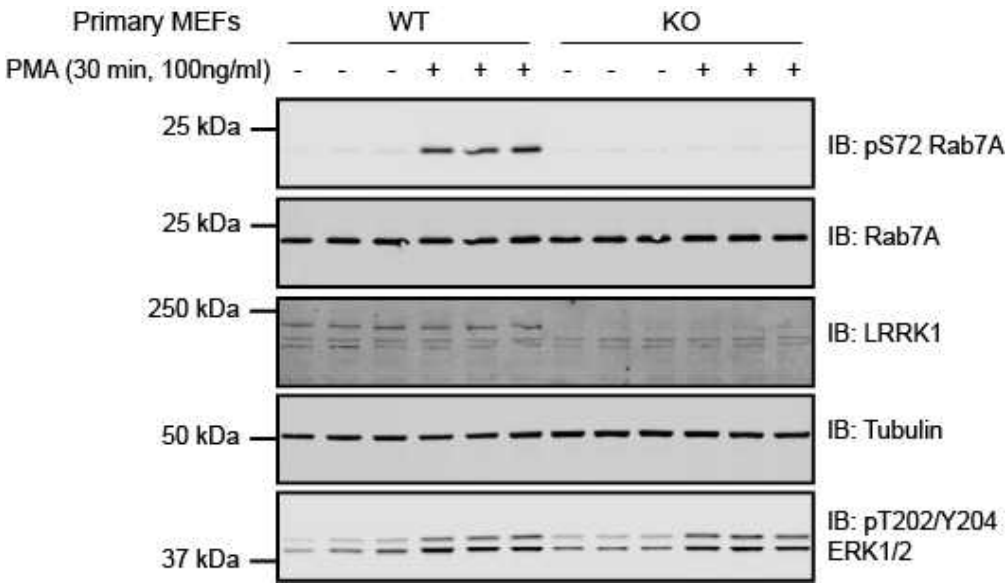


**Figure 2.** Representative results of quantitative immunoblotting analysis of pSer72 Rab7A/total Rab7A and total LRRK1 levels performed in extracts from wild-type (WT) and homozygous LRRK1 knock-out (KO) mouse embryonic fibroblasts treated +/- PKC activator PMA (100 ng/ml, 30 min) according to the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