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SDS-PAGE and Western Blotting

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We use this protocol and it's working.

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Abstract

This protocol describes the sample preparation and procedure to analyse protein abundance and phosphorylation of LRRK1, LRRK2 and Rab GTPases by SDS-PAGE and Western Blotting.

It is meant to accompany the manuscript and methods section of "Probing the proteome-wide interactomes of Leucine-rich Repeat Kinases 1 and 2 and alterations in their phosphorylation activity".

Materials

- Recombinant Anti-RAB7 (phospho S72) antibody [MJF-R38-1] (ab302494) **Abcam Catalog #**ab302494
- Anti-RAB10 (phospho T73) antibody [MJF-R21] **Abcam Catalog #**ab230261
- Anti-LRRK2 (phospho S935) antibody [UDD2 10(12)] **Abcam Catalog #**ab133450
- HRP-rabbit IgG **Jackson ImmunoResearch Laboratories, Inc. Catalog #**111-035-003
- PageRuler™ Prestained Protein Ladder 10 to 180 kDa **Thermo Fisher Scientific Catalog #**26616
- Spectra™ Multicolor High Range Protein Ladder **Thermo Fisher Catalog #**26625
- HRP-mouse IgG **Jackson ImmunoResearch Laboratories, Inc. Catalog #**115-035-062
- LRRK2 Antibody **Cell Signaling Technology Catalog #**5559
- Recombinant Anti-RAB10 antibody [MJF-R23] **Abcam Catalog #**ab237703
- Anti-RAB7 antibody [EPR7589] - Late Endosome Marker **Abcam Catalog #**ab137029
- Anti-RAB12 antibody [EPR28814-81] **Abcam Catalog #**ab316770
- Recombinant Anti-RAB12 (phospho S106) antibody [MJF-R25-9] **Abcam Catalog #**ab256487
- Monoclonal M2 antibody (anti-FLAG) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F1804-200UG
- Anti-alpha Tubulin antibody [DM1A] - Loading Control **Abcam Catalog #**ab7291
- anti-HA monoclonal antibody **Labcorp (formerly Covance) Catalog #**MMS-101R

Protocol materials

cOmplete™, EDTA-free protease inhibitor cocktail **Roche** **Catalog #**11873580001

Recombinant Anti-RAB7 (phospho S72) antibody [MJF-R38-1] (ab302494) **Abcam** **Catalog #**ab302494

Anti-RAB12 antibody [EPR28814-81] **Abcam** **Catalog #**ab316770

Monoclonal M2 antibody (anti-FLAG) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**F1804-200UG

anti-HA monoclonal antibody **Labcorp (formerly Covance)** **Catalog #**MMS-101R

Anti-LRRK2 (phospho S935) antibody [UDD2 10(12)] **Abcam** **Catalog #**ab133450

HRP-mouse IgG **Jackson ImmunoResearch Laboratories, Inc.** **Catalog #**115-035-062

Recombinant Anti-RAB12 (phospho S106) antibody [MJF-R25-9] **Abcam** **Catalog #**ab256487

Anti-alpha Tubulin antibody [DM1A] - Loading Control **Abcam** **Catalog #**ab7291

Recombinant Anti-RAB10 antibody [MJF-R23] **Abcam** **Catalog #**ab237703

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Anti-RAB10 (phospho T73) antibody [MJF-R21] **Abcam** **Catalog #**ab230261

PageRuler™ Prestained Protein Ladder 10 to 180 kDa **Thermo Fisher Scientific** **Catalog #**26616

Spectra™ Multicolor High Range Protein Ladder **Thermo Fisher** **Catalog #**26625

Sample preparation

35m

1 Lyse frozen cell pellets (from 6-well dishes and 35 mm diameter) in 100 µL RIPA buffer ([M] 50 millimolar (mM) Tris-HCl pH 8, [M] 150 millimolar (mM) NaCl, [M] 0.5 Mass / % volume sodium deoxycholate, [M] 0.1 Mass / % volume SDS, [M] 1 % (v/v) NP-40, [M] 1 millimolar (mM) DTT, 1x cOmplete™, EDTA-free protease inhibitor cocktail **Roche Catalog #**11873580001) with phosphatase inhibitors ([M] 5 millimolar (mM) NaF, [M] 5 millimolar (mM) beta-glycerophosphate, [M] 1 millimolar (mM) Na_3VO_4) on ice.

2 Incubate for 00:20:00 at 4 °C and gentle agitation. 20m

3 Pellet cell debris by centrifugation at 16000 x g for 00:10:00 and 4 °C . 10m

4 Take supernatant and determine protein concentration via BCA (bicinchoninic acid) assay (Pierce, Cat#23225) or similar.

4.1 Normalize samples to lowest protein concentration by adding lysis buffer.

5 Mix samples with 2x Laemmli sample buffer ([M] 62.5 millimolar (mM) Tris-HCl pH6.8, [M] 100 millimolar (mM) DTT, bromophenole blue).

5.1 Heat samples for 00:05:00 at 95 °C 5m

Samples are now ready for SDS-PAGE

SDS-PAGE (Sodium dodecyl sulfate - polyacrylamide gel electrophoresis)

3h 30m

6 Use clean glass plates for casting gels in SDS-PAGE and assemble everything according to your set-up.

7 Cast resolving gel with percentage with appropriate percentage, i.e. 7.5 % for LRRK1 and LRRK2 analysis and 12.5 % for Rab GTPases.

7.1 Incubate for 00:40:00 to 01:00:00 at Room temperature to allow polymerization. 1h 40m

8 Cast stacking gel (5 % acrylamide) and insert comb.

8.1 Incubate for at least 00:30:00 at Room temperature to allow polymerization. 30m

8.2 You can also incubate the gel longer to ascertain the best possible polymerization.

9 Remove comb and clean pockets with Laemmli running buffer to remove remaining gel residue.

10 Fill device with Laemmli running buffer.

11 Load 8 µL protein marker (

PageRuler™ Prestained Protein Ladder 10 to 180 kDa **Thermo Fisher Scientific Catalog #**26616

or

Spectra™ Multicolor High Range Protein Ladder **Thermo Fisher Catalog #**26625

), then load 25 µg per sample. Fill empty pockets with same volume of 2x Laemmli sample buffer as sample volume

12 Connect electrodes and run gel at 80V constant voltage for approx. 00:20:00 until samples have reached resolving gel. 20m

13 Run samples through resolving gel at 110 V constant voltage for approx. 01:00:00 until bromophenole blue has almost reached lower end of gel. 1h

Western Blot 5h 25m

14 Equilibrate gel from SDS-PAGE to Western Blot transfer buffer ([M] 12.5 millimolar (mM) Tris-HCl pH 8.3, [M] 100 millimolar (mM) glycine) shortly

15 Build Western Blot sandwich by placing a 0.22 or 0.45 µm PVDF membrane (0.22 µm for proteins <50 kDa) on gel and surrounding by wet Whatman paper.

15.1 Avoid and remove air bubbles between gel and membrane to allow an error-free transfer.

16 Place sandwich in Western Blot chamber, fill with transfer buffer and start transfer at 60 V for 01:30:00 . 1h 30m

16.1 Check if all marker bands have been transferred onto membrane. If not, continue electrophoretic transfer for another 00:10:00 to 00:15:00 . 25m

17 Block the membrane to inhibit unspecific binding with [M] 5 Mass / % volume milk in TNE-T buffer.

17.1 Incubate for 01:00:00 at Room temperature . 1h

17.2 Wash membrane multiple times with TNE-T buffer.

18 Add primary antibody in TNE-T (or [M] 5 Mass / % volume BSA in TNE-T for phosphosite antibodies). Dilute antibody according to manufacturer instructions, commonly 1:1000 or 1:5000 in the respective buffer.

18.1 Incubate at 4 °C Overnight while shaking. 1h

18.2 Wash multiple times

19 Dilute secondary antibody, e.g. HRP-coupled anti-mouse or anti-rabbit antibody, in TNE-T 1:5000 and add onto membrane.

19.1 Incubate for 01:30:00 at Room temperature while shaking. 1h 30m

19.2 Wash membrane multiple times

20 Place membrane on tray and add detection reagent for HRP-based chemiluminescence detection so membrane is covered.

21 Place in LAS-3000 (Fujifilm) and detect chemiluminscence signal.

22 Take picture of marker bands on membrane.

23 Overlay picture to determine molecular weight of bands and if necessary, select and show bands-of-interest and adjust contrast and brightness to make signals better visible using ImageJ.

24 If necessary, strip membrane to detect using another primary antibody. Otherwise store membrane at 4 °C .

Strip membrane 10m

25 After detecting signals of phosphosites, it is necessary to strip membrane of antibodies to detect signal of respective proteins using another primary antibody.

26 Wash membrane in TNE-T once to remove detection reagent.

27 Add 10 mL stripping buffer ([M] 20 millimolar (mM) Tris-HCl pH 7.4, [M] 6 Molarity (M) guanidine hydrochloride, [M] 0.02 % (v/v) NP-40, [M] 0.1 Molarity (M) β-mercapto ethanol).

27.1 Incubate membrane for 00:10:00 at Room temperature while shaking. 10m

28 Wash membrane in TNE-T and transfer to fresh box, and wash multiple times

29 Repeat steps 17-22 for new primary antibody detection.

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