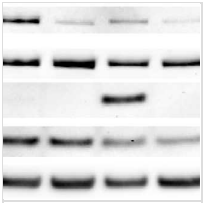

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Western blotting for LRRK2 signalling in macrophages

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ABSTRACT

This protocol describes the immunoblotting for components of the LRRK2 signalling pathway (LRRK2, LRRK2 pS935 and phospho-Rabs) using Invitrogen NuPage SDS-PAGE reagents and the BioRad Turbo Blot transfer system.

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KEYWORDS

Western Blot, LRRK2, Rab GTPase, phosporylation, NuPAGE, Turbo Blot

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MATERIALS TEXT

Reagents:

- Lysis buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% (v/v) Triton X-100

NOTE: add protease and phosphatase inhibitors fresh each time (eg Halt™ Protease and Phosphatase Inhibitor Cocktail (100X), #78440, ThermoFisher Scientific)

- Loading buffer: NuPAGE LDS sample buffer (#NP0007, ThermoFisher Scientific)
- Sample reducing agent: NuPAGE sample reducing agent (#NP0009, ThermoFisher Scientific)
- 4-12% Bis-Tris NuPAGE gels (eg #NP0321BOX, ThermoFisher Scientific)
- SDS-PAGE running buffer: MES running buffer (#NP0002, ThermoFisher Scientific)
- Trans-Blot® Turbo™ PVDF Transfer Packs: eg #1704157, BioRad
- TBS-T: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20.
- Blocking and antibody dilution buffer: 5 % (w/v) non-fat milk powder in TBS-T
- Primary and secondary antibodies (see table 1 for primary antibody suggestions)

Equipment:

- Gel tank (#A25977, ThermoFisher Scientific; or similar)
- Trans-Blot® Turbo™ Transfer System (1704150, BioRad)
- + standard lab equipment such as a shaker, bench top centrifuge and heat block

Preparation of protein lysates

1 Culture cells as usual in a 12-well or 6-well plate.

We recommend including a control using an LRRK2 kinase inhibitor (eg MLi-2, 100 nM for 1 hr) in your sample preparation to verify phospho-Rab immunoblots.

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2

Wash cells once in PBS

3 Add 100 µl per 12-well or 200 µl per 6 well of lysis buffer containing protease and phosphatase inhibitors. Keep samples **On ice** from now on.

4 Scrape cells using a cell lifter and transfer lysate to a 1.5 ml Eppendorf tube

5 Vortex and incubate on ice for **00:10:00** . 10m

6 Clarify lysate by spinning at **17000 x g** at **4 °C** for **00:15:00** 15m

7 Transfer supernatant into a fresh 1.5 ml Eppendorf tube and store at -20 °C.

1. Determine protein concentration using an assay of choice (eg Pierce™ Coomassie Plus Assay Kit)

SDS-PAGE

8 Prepare protein lysates by adding sample loading buffer and reducing agent (eg NuPAGE LDS sample buffer and reducing agent).

The final protein concentration should be in the range of **1-2 µg/µL** . We recommend loading **10-15 µg** of total protein per lane.

9 Denature samples at **80 °C** for **00:08:00** 8m

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10 Prepare the SDS gel (4-12% Bis-Tris Nu-PAGE gel) by assembling the apparatus, filling the tank with MES running buffer and flushing all the wells with MES running buffer.

11 Spin down the samples quickly in case of condensation and load wells carefully. Include a broad range molecular weight marker (eg Broad molecular weight ladder, ab116028, Abcam).

12 Run the gel at 180 V for **00:35:00** . 35m

Transfer

13 Transfer SDS gel into 20% EtOH while preparing the transfer stack. Make sure the gel is submerged.

14 Prepare the transfer stack according to the manufacturer's instructions.

15 Transfer proteins using BioRad pre-programmed protocols: Mixed MW setting (2.5A for **00:07:00**). 7m

Immunolabelling and detection

16 Transfer PVDF membrane into 5 % milk/TBS-T and block for **01:00:00** at RT with constant shaking. 1h

> We routinely cut the membrane into three parts to be able to blot for LRRK2, a loading control and Rabs at the same time.

17 Prepare primary antibodies in 5 % milk/TBS-T and incubate membranes in antibody solution at **4 °C** **Overnight** with constant shaking

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A	B	C
Target	Catalogue no (Supplier)	Dilution
pan-phospho-Rab	ab230260 (Abcam)	1:1000
Rab8A	6975S (Cell Signaling)	1:1000
Rab10 pT73	ab230261 (Abcam)	1:1000
Rab10	8127S (Cell Signaling)	1:1000
LRRK2 pS935	ab133450 (Abcam)	1:1000
LRRK2	ab133474 (Abcam)	1:1000
beta-Actin	A1978-200UL (Sigma-Aldrich)	1:5000

Table 1: Antibodies which are commonly used in our lab. Note: multiplexing for phopsho- and total protein signal is possible depending on the antibodies used. We strip and re-plot for total protein after detection of the phospho signal as all LRRK2 and Rab antibodies listed here are raised in rabbit.

18 Wash membranes by shaking in TBS-T for 5-10 min

19 

Repeat the wash step 2x

20 

Repeat washing steps as above.

21 Detect signal using appropriate detection equipment.

Optional: stripping and re-blot 10m

22 Depending on the primary antibodies and the detection method used, it might be necessary to strip the blot using a Western Blot Stripping buffer (eg #T7135A, Takara Bio) for **00:10:00** at RT and repeat the primary and secondary antibody incubation steps. 10m

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