Assay for PhosphoRab activation of LRRK2 Kinase

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ABSTRACT

MST kinase phosphorylates Rab proteins at the same site as LRRK2 and has been used to phosphorylate Rab8A and Rab10 quantitatively. This protocol includes a method to produce phosphoRab8A protein and remove as much contaminating MST3 as possible, to enable use of the phosphoRab to test subsequent activation of LRRK2 kinase. See these references for details on MST3 phosphorylation of Rab GTPases:

Axel Knebel, Kerryn Berndsen, Pawel Lis, Paul Davies, Dario R Alessi. Expression and purification of Rab8A (1-181) stoichiometrically phosphorylated at pThr72 (the LRRK2 site). http://dx.doi.org/10.17504/protocols.io.butinwke

Berndsen K, Lis P, Yeshaw WM, Wawro PS, Nirujogi RS, Wightman M, Macartney T, Dorward M, Knebel A, Tonelli F, Pfeffer SR, Alessi DR (2019). PPM1H phosphatase counteracts LRRK2 signaling by selectively dephosphorylating Rab proteins.. eLife. https://doi.org/pii:e50416.10.7554/eLife.50416

Dhekne HS, Yanatori I, Vides EG, Sobu Y, Diez F, Tonelli F, Pfeffer SR (2021). LRRK2-phosphorylated Rab10 sequesters Myosin Va with RILPL2 during ciliogenesis blockade.. Life science alliance. https://doi.org/pii:e202101050.10.26508/lsa.202101050

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MATERIALS TEXT

His-Rab8A Q67L full length

His-GFP-Rab10 Q68L 1-181

His-MST3 protein (pET15b 6HIS MST3 TV1; MRC-PPU DU62980)

GST-PreScission protease (plasmid MRC-PPU DU2328)

Reaction buffer: 50 mM HEPES pH 8, 150 mM NaCl, 10 mM MgCl2, 0.2 mM TCEP, 250 µM GTP, 2 mM ATP, 5% (v/v) glycerol, 5 µM BSA

LRRK2 G2019S (Thermo Fisher Scientific #A15200)

11 To cleave the HIS tag from MST3, bind GST-PreScission protease (**50 µg**) to **100 µL** glutathione agarose slurry (pre-washed and pelleted) **02:00:00** at **4 °C** in a total volume of **500 µL** . Wash the resin 3X with **1 mL** reaction buffer. Add His-MST3 kinase (**0.5 mg**) and incubate **Overnight** on a rotator at **4 °C** . 1d

11 Spin beads **3000 rpm, 4°C, 00:05:00** and collect supernatant, which contains free MST3 and uncleaved His-MST3. Bind supernatant to Nickel-NTA agarose (**100 µL** of a 50% slurry, prewashed and pelleted) for **02:00:00** at **4 °C** to trap uncleaved His-MST3. 2h 5m

11 Spin beads **3000 rpm, 4°C, 00:05:00** and collect supernatant, which contains free MST3 without the HIS tag. 5m

11 Phosphorylate His-Rab8A Q67L full length with free MST3 using a molar ratio of 1:6 (kinase:substrate) at **30 °C** **Overnight** in reaction buffer.

11 Phosphorylated His-Rab8A is then separated from MST3 by gel filtration using a **24 mL** Superdex 75 10/300 column (Cytiva Life Sciences, #17517401). Collect the relevant Rab-containing fractions determined by SDS-PAGE. 16h

11 Further purify His-Rab8A by binding to Nickel-NTA agarose (**100 µL** of a 50% slurry, prewashed and pelleted) and elute with **500 millimolar (mM)** imidazole (3 X **100 µL**)

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after washing with 60 column volumes of reaction buffer.

11 Incubate [M]**88 nanomolar (nM)** LRRK2 G2019S with [M]**3 micromolar (µM)** His-GFP-Rab10 Q68L 1-181 or His-SUMO-Rab10 wild type full length substrate ± [M]**6 micromolar (µM)** phosphoRab8A Q67L in reaction buffer in a total volume of **100 µL** .

11 Incubate reaction in a **30 °C** water bath and collect **20 µL** time points at 0, 10, and 20 minutes. Stop reactions with addition of **5 µL** SDS-PAGE sample buffer. Add [M]**200 nanomolar (nM)** MLi-2 for control conditions to ensure that pRab10 detected is due to LRRK2 activity.

11 Analyze samples by SDS-PAGE and immunoblot for phosphoRab10; image blot with Li-COR and quantify bands using ImageJ (see below for more details).

Francesca Tonelli, Dario Alessi. Quantitative Immunoblotting Analysis of LRRK2 Signalling Pathway.
http://dx.doi.org/10.17504/protocols.io.bsgrnbv6

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