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## OPEN ACCESS

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

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## Cell culture and Western blot

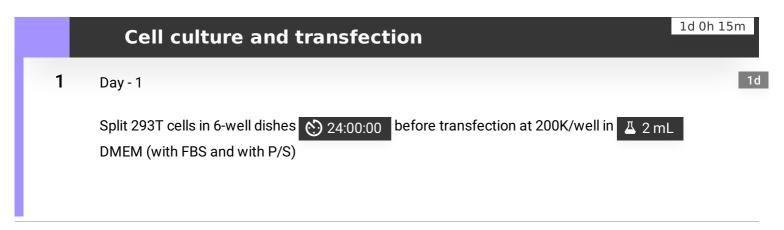
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**ABSTRACT** 

Protocol for detecting Rab7a phosphorylation at S72 by LRRK1 and LRRK1 mutants in HEK 293T cells.



**2** Day - 2

Transfection

2.1 Warm PEI and Opti-MEM at RT for 00:15:00 Label a tube for each well.

15m

- 2.2 Add DNA (  $\pm$  500 ng of GFP-Rab7 +  $\pm$  1  $\mu g$  of LRRK1 construct of interest) to  $\pm$  150  $\mu L$  of Opti-MEM and gently vortex for 5 sec

15m

- 2.4 In the meantime...remove media from 6-well dish containing cells and replace with fresh 1 mL DMEM (with FBS and <u>without</u>P/S)...put back in 37 deg incubator until ready to add transfection mixture
- 2.5 After 15 min incubation, add DNA/Optimem/PEI mixture dropwise Δ 150 μL onto each well Give a bit of a light swirl before putting back in incubator

## **Cell lysis**

1d 12h

3 after transfection, begin cell lysis

1d 12h

3.1 Wash plate on ice with cold PBS 1x

- 3.2 Add A 300 µL RIPA buffer (0.5% Triton, 50 mM Tris pH7.5, 150 mM NaCl, 0.1%SDS) with protease and phosphatase inhibitors (cOmplete mini EDTA free + PhosSTOP tablets) 3.3 Lift with cell lifters on ice 3.4 Pipet up lysate, put in eppendorf tube, and shake 15 mins in the cold room 3.5 Spin at MAX at 4 deg for 15 mins 3.6 10m Remove supernatant and make sample (boil at \$\\$ 95 °C for \( \) 00:10:00 ), store in  $\S$  -80  $^{\circ}\mathrm{C}$  . I like to store the lysate and take some out to make a sample -I use the 4xNuPage LDS sample buffer: Δ 65 μL lysate + Δ 10 μL 10x Reducing Agent + Δ 25 μL 4x LDS buffer 1h **Western blot** 4 SDS-PAGE with Bis-Tris gel and MOPS running buffer 4.1 Load a 4-12% Bis-Tris gel with 🗸 25 µL of prepared lysate in sample buffer and run at 180V 50m for  $\sim$  00:50:00 or until dye front has reached the bottom of the gel.
- 4.2 Assemble gel with Immobilon-FL PVDF membrane for transfer according to instructions from your western blot transfer apparatus. When using fluorescence detection for Western blot it is important to use low fluorescence background membrane (Immobilon-FL or equivalent).

We activate membrane with MeOH and rinse with water.

Transfer in Western transfer buffer with Tris/Glycine and 20% MeOH at 200 mA for 4 hr at **4** °C 4.3 After transfer is complete, rinse membrane with water and allow to dry between sheets of Whatman paper. 4.4 Block in 5% milk in TBS (no Tween 20) 4.5 Dilute primary antibody in 5% milk with TBST (with Tween 20) -rabbit anti Rab7 phospho-S72 (MJF-38) at 1:1000 -mouse anti-GFP at 1:2500 (Santa Cruz) (for total Rab quantification) -rabbit anti-LRRK1 (ab228666) at 1:500 -rabbit anti-GAPDH at 1:3000 (Cell signaling technologies) 4.6 Rock Overnight at \$4°C 4.7 5m Rinse 3x with TBST for 00:05:00 4.8 Rinse 1x with 5% milk in TBST 4.9 Add secondary antibodies in 5% milk with TBST and incubate at B Room temperature for 1 hr -LiCor mouse and rabbit secondary IRdye antibodies at 1:5000 4.10 5m Rinse 3x with TBST for 00:05:00 4.11 Image on LiCor Odyssey CLx