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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.

Cell culture and transfection

1d 0h 15m

1 Day - 1

1d

Split 293T cells in 6-well dishes 24:00:00 before transfection at 200K/well in 2 mL DMEM (with FBS and with P/S)

2 Day - 2

Transfection

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

2.2 Add DNA (500 ng **of GFP-Rab7** + 1 µg **of LRRK1 construct of interest**) to 150 µL of Opti-MEM and gently vortex for 5 sec

2.3 Add 3 µL room-temperature PEI to each tube containing Opti-MEM/DNA mix. Mix gently by pipetting and incubate DNA/DMEM/PEI mixture at room temperature in the hood for 00:15:00 . 15m

2.4 In the meantime…remove media from 6-well dish containing cells and replace with fresh 1 mL DMEM (with FBS and without 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 150 µL onto each well
Give a bit of a light swirl before putting back in incubator

Cell lysis 1d 12h

3 36:00:00 after transfection, begin cell lysis 1d 12h

3.1 Wash plate on ice with cold PBS 1x

3.2 Add 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 Remove supernatant and make sample (boil at 95 °C for 00:10:00), store in -80 °C . *I like to store the lysate and take some out to make a sample –I use the 4x NuPage LDS sample buffer:* 65 µL *lysate +* 10 µL *10x Reducing Agent +* 25 µL *4x LDS buffer* 10m

Western blot 1h

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 for ~ 00:50:00 or until dye front has reached the bottom of the gel. 50m

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 Rinse 3x with TBST for 00:05:00 5m

4.8 Rinse 1x with 5% milk in TBST

4.9 Add secondary antibodies in 5% milk with TBST and incubate at Room temperature for 1 hr
-LiCor mouse and rabbit secondary IRdye antibodies at 1:5000

4.10 Rinse 3x with TBST for 00:05:00 5m

4.11 Image on LiCor Odyssey CLx