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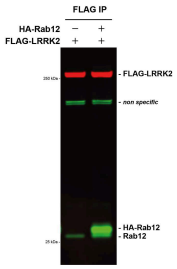
Co-immunoprecipitation protocol to study LRRK2 binding to Rab12 in a cell-based assay



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Francesca Tonelli[1,2]

[1]Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815

Francesca Tonelli
MRC-PPU at The University of Dundee

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Abstract

This protocol describes the immunoprecipitation (IP) of FLAG-tagged LRRK2 from whole cell lysates to assess its interaction with Rab12. This method can be used to screen the impact that LRRK2 mutations have on its binding to Rab12 in cells, as well as the effect of any compound or cell treatment on LRRK2 interaction with Rab12.

*Note: The IP products can be analysed by quantitative immunoblotting (as described in **dx.doi.org/10.17504/protocols.io.bsgrnbv6**) using an antibody targeting Rab12.*

Attachments

Figure 1.tif
24.6MB

Guidelines

This protocol describes the immunoprecipitation (IP) of FLAG-tagged LRRK2 from whole cell lysates to assess its interaction with Rab12. This method can be adapted to immunoprecipitate HA-tagged Rab12 from whole cell lysates to assess its interaction with LRRK2 by replacing ANTI-FLAG® M2 Affinity Gel with an anti-HA antibody-coupled matrix (Pierce™ Anti-HA Agarose, ThermoFisher Scientific, Cat #26181, or equivalent).

Materials

Materials and reagents:

- HEK293 cells (ATCC #CRL-1573) cultured in complete growth medium: Dulbecco's Modified Eagle's Medium (DMEM), High Glucose, no glutamine (Gibco™, catalog number: 11960044, or equivalent) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Sigma #F7524, or equivalent), 2 mM L-glutamine (Gibco™, catalog number: 25030024, or equivalent), Penicillin-Streptomycin 100U/mL (Gibco™, catalog number: 15140122, or equivalent).
- N-terminus FLAG-tagged LRRK2 wild-type or mutant cDNA and FLAG-empty vector cDNA (negative control) in a pCMV5 vector for mammalian expression; N-terminus HA-tagged Rab12 wild-type or mutant cDNA and HA-empty vector cDNA (negative control) in a pCMV5 vector for mammalian expression.

All plasmids used for our studies are available from the MRC PPU Reagents and Services (https://mrcppureagents.dundee.ac.uk). These include FLAG-tagged wild-type LRRK2 (DU62804) and HA-tagged wild-type Rab12 (DU48963).

- Invitrogen™Lipofectamine™2000 Transfection Reagent (ThermoFisher Scientific #11668019)
- Transfection medium: Opti-MEM Reduced Serum Medium (ThermoFisher Scientific #31985062)
- Lysis Buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 0.27 M sucrose, 0.1% (v/v) NP-40 Alternative (Merck #492016), 1X phosSTOP phosphatase inhibitor cocktail (PhosSTOP tablet: Roche, REF# 04906837001; to be added just before use) and 1X protease inhibitor cocktail (cOmplete EDTA-free protease inhibitor cocktail tablet: Roche, REF# 11873580001; to be added just before use)
- Bradford assay kit (Pierce™ Coomassie Plus (Bradford) Assay Kit, ThermoFisher Scientific 23236, or equivalent)
- Resin for LRRK2 immunoprecipitation: ANTI-FLAG® M2 Affinity Gel (Millipore, A2220)
- IP wash buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl
- NuPAGE™ LDS SampleBuffer (4X) (Invitrogen™ cat# NP0007)
- 2-mercaptoethanol
- Anti-FLAG Antibody: DYKDDDDK Tag (D6W5B) Rabbit mAb (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody) (Cell Signaling Technology, Cat #14793) (RRID:AB_2572291). Use at 1:1,000 dilution.
- Anti-total Rab12 Antibody: Sheep polyclonal antibody (MRC PPU Reagents and Services, University of Dundee; Cat #SA227) (RRID: AB_2921227). Use at 1 µg/ml dilution.
- Tissue culture Petri dishes (100 mm) (BD Biosciences, Falcon®, catalog number: 351029)
- Cell lifters (Sigma-Aldrich CLS3008, or equivalent)
- Corning™ Costar™ Spin-X™ Centrifuge Tube Filters 0.22-µm-pore-size (FisherScientific 10104101)

Equipment:

- CO_2 incubator for growing cells
- Laminar flow hood for cell culture
- Refrigerated bench-top centrifuge (Eppendorf microcentrifuge 5417R, or equivalent).
- Plate reader for Protein quantification (BioTek Epoch, or equivalent)
- Rotator disk with adjustable speed (FisherScientific 13407669, or equivalent).
- Dry bath/heat block (Thermo Scientific™ 88870005, or equivalent).

Troubleshooting

Transient transfection of HEK293 cells:

1 Seed HEK293 cells to be 70-90% confluent at transfection. Proceed to the next step the day after seeding cells.

2 Dilute FLAG-tagged LRRK2 (wild-type or mutant) DNA and HA-tagged Rab12 (or HA-empty control) DNA in Opti-MEM™Reduced Serum Medium in a sterile Eppendorf tube.
For a 10 cm dish: Dilute 8 µg of FLAG-tagged LRRK2 (wild-type or mutant) DNA and 2 µg of HA-tagged Rab12 (or HA-empty control) DNA in 0.5 mL Opti-MEM™Reduced Serum Medium.

Note: The method described here will also enable to assess the interaction of FLAG-LRRK2 with endogenous Rab12, independent of HA-Rab12 overexpression.

3 Dilute Lipofectamine®2000 Reagent in Opti-MEM™ Reduced Serum Medium in another sterile Eppendorf tube.
For a 10 cm dish: Dilute 30 µl of Lipofectamine®2000 Reagent in 0.5 mL of Opti-MEM™ Reduced Serum Medium.

4 Add the diluted DNA to the diluted Lipofectamine®2000 Reagent and mix gently.

5 Incubate at room temperature for 5 minutes.

6 Add the transfection mix to the culture medium in each dish drop by drop using a pipette.

7 Transfer the plates to a humidified incubator maintaining 37°C and 5% (v/v) CO_2.

8 Lyse cells 20-24 hours after transfection, as detailed in the next section.

Preparation and quantification of cell lysates:

9 Quickly rinse cells in the tissue culture dish by carefully pouring room temperature culture media without Foetal bovine serum (FBS) into the dish.

Note: As HEK293 cells are loosely attached to the dish surface, extra care should be taken during the washing step.

10 Pour off media from the culture dish and completely aspirate any residual media. Immediately add freshly prepared ice-cold lysis buffer, ensuring that the entire surface is covered by lysis buffer.
Note: As a guideline, use 1 ml of lysis buffer for a 10 cm dish for HEK293 cells.

11 Immediately transfer the cell dishes to ice.

12 Scrape the cells on the dish using a cell lifter to ensure all cells are detached from the dish.

13 Using a pipette, transfer cell lysate to an Eppendorf tube on ice.

14 Leave samples on ice for 20 minutes to allow for efficient lysis.
Note: Mix the samples by inverting the tubes. Do not vortex.

15 Clarify lysates by centrifugation at 15,000 g for 10 min at 4°C.

16 Transfer the supernatants into new Eppendorf tubes and discard the pellet. Keep the lysates on ice.

17 Determine the protein concentration of cell lysates by Bradford assay according to the manufacturer's instructions, performing measurements in triplicate.

Note: Ensure the concentration of the samples is in the linear range for the Bradford assay. If it isn't, prepare appropriate dilutions in water of each lysate. Generally, protein concentrations of near confluent cells lysed as described above should result in protein concentrations of at least 2 mg/ml.

18 Proceed to FLAG-LRRK2 immunoprecipitation, as detailed in the next section.

Note:
-We recommend confirming the expression of the transiently expressed proteins (FLAG-LRRK2 and HA-Rab12) by performing quantitative immunoblotting analysis as described in dx.doi.org/10.17504/protocols.io.bsgrnbv6.
-When comparing multiple FLAG-tagged variants of LRRK2, we recommend assessing the expression levels of LRRK2 in the whole lysates by immunoblotting prior to immunoprecipitation and adjusting how much cell lysate is to be used to

immunoprecipitate LRRK2 accordingly, to ensure that the amount of LRRK2 between samples is as close as possible.

Immunoprecipitation of FLAG-tagged LRRK2 from cell lysates:

19 Wash the required amount of ANTI-FLAG® M2 Affinity Gel beads (25 µl of packed gel beads are required for each immunoprecipitation reaction):

19.1 Mix the affinity gel beads by vortexing until completely resuspended, and immediately aliquot the required volume into a 1.5 mL microcentrifuge tube on ice.
Note: Cut 1 mm off the end of a pipette tip when dispensing/aliquoting the affinity gel beads.

19.2 Add IP wash buffer to the beads, vortex and centrifuge for 3 minutes at 1,500 x g. Remove supernatant, being careful not to disturb the beads. Repeat this step for a total of 3 washes.
Note: Each wash should be performed with a volume of IP wash buffer of at least 20 times the total packed gel volume.

19.3 Resuspend the washed beads in IP wash buffer to make a 1:1 slurry (e.g., add 100 µl IP wash buffer to 100 µl packed beads). Leave on ice until use.

20 Add 25 µl of packed gel beads from Step 19.3 (= 50 µl of a 1:1 slurry) to 1 mg of whole cell extract from Step 16.
Note: The immunoprecipitation conditions (amount of resin and amount of cell extract) might need optimisation.

21 Incubate at 4°C for one hour on a rotator, under mild agitation (20 rpm).
Note: Ensure the resin and lysate are mixing properly. If necessary, top up with lysis buffer.

22 Collect the resin by centrifugation at 1,500 g for 3 minutes at 4^{O}C. Discard supernatant (flow-through).
Note: The flow-through may be retained if desired to assess efficient depletion of FLAG-LRRK2 from cell lysates.

23 Resuspend the resin in IP wash buffer. Disperse the resin by gently inverting the tubes. Do not vortex.
Note: Each wash should be performed with a volume of IP wash buffer of at least 20 times the total packed gel volume (e.g. 500 µl IP wash buffer per 25 µl beads).

24 Repeat steps 22 and 23 for a total of 3 washes.

25 Collect the resin by centrifugation at 1,500 g for 3 minutes at 4^{O}C. Discard supernatant.

26 Elute FLAG-LRRK2 from the resin by resuspending the resin in 2X Loading buffer (prepared by diluting 4X Loading buffer 1:1 in milliQ water) and incubating at 70°C on a heat block for 10 min.
Note: To ensure optimal elution, use a minimum of 25 µl of 2X Loading buffer for 25 µl of resin.

27 Collect the eluent by centrifugation through a 0.22-µm-pore-size Spin-X column.

28 Supplement the samples with 2-Mercaptoethanol to 1% (v/v).

29 Incubate the samples for 5 min at 70°C on a heat block.

30 Proceed to quantitative immunoblotting analysis of the IP products, as detailed in the next section.

Analysis of immunoprecipitation products by quantitative immunoblotting analysis:

31 Perform quantitative immunoblotting analysis of the IP products following the method described in **dx.doi.org/10.17504/protocols.io.bsgrnbv6** (Quantitative Immunoblotting Analysis of LRRK2 Signalling Pathway).
Load 95% of each IP sample to detect the interacting protein (HA-Rab12 and endogenous Rab12). Loading 5% of each IP sample should be sufficient to detect the bait (FLAG-LRRK2). Use the following primary antibodies for immunoblotting analysis of the IP products:

- Anti-FLAG Antibody: DYKDDDDK Tag (D6W5B) Rabbit mAb (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody) (Cell Signaling Technology, Cat #14793) (RRID:AB_2572291). Use at 1:1,000 dilution.
- Anti-total Rab12 Antibody: Sheep polyclonal antibody (MRC PPU Reagents and Services, University of Dundee; Cat #SA227) (RRID: AB_2921227). Use at 1 µg/ml dilution.

Protocol references

Quantitative Immunoblotting Analysis of LRRK2 Signalling Pathway: **dx.doi.org/10.17504/protocols.io.bsgrnbv6**