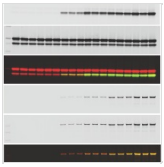

Aug 19, 2021

Assessment of in vitro kinase activity of over-expressed and endogenous LRRK2 immunoprecipitated from cells

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ABSTRACT

We describe a non-radioactive assay that we deploy for analysing LRRK2 protein kinase activity *in vitro* using Rab proteins as a substrate. This assay can be used to measure the intrinsic activity of LRRK2 immunoprecipitated from cells as well as to assess the impact of mutations on LRRK2 activity.

Protocol Overview:
1) Transient transfection of HEK293 cells for analysis of over-expressed LRRK2 activity.
2) Preparation and quantification of cell lysates (from HEK293 cells over-expressing LRRK2 or MEFs).
3) Immunoprecipitation of over-expressed FLAG-tagged LRRK2 from cell lysates for analysis of LRRK2 activity *in vitro*.
4) Immunoprecipitation of endogenous LRRK2 from MEFs for analysis of LRRK2 activity *in vitro*.
5) *In vitro* kinase assay using LRRK2 immunoprecipitated from cells.
6) Analysis of kinase reaction products by quantitative immunoblotting analysis.

ATTACHMENTS

LRRK2 kinase assay trial-2.tif

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PROTOCOL CITATION

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KEYWORDS

In vitro kinase activity, endogenous LRRK2, Immunoprecipitation, LRRK2, kinase assay, over-expressed LRRK2, LRRK2 activity

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Protocol Overview:
1) Transient transfection of HEK293 cells for analysis of over-expressed LRRK2 activity
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4) Immunoprecipitation of endogenous LRRK2 from MEFs for analysis of LRRK2 activity in vitro
5) In vitro kinase assay using LRRK2 immunoprecipitated from cells
6) Analysis of kinase reaction products by quantitative immunoblotting analysis

MATERIALS TEXT

Reagents:

- For transfection of FLAG-tagged LRRK2 in HEK293 cells: cDNA for expression of human FLAG-tagged LRRK2 in mammalian cells; Polyethylenimine “Max” (MW 4,000) (Polysciences, Inc., cat no 24885): 1 mg/ml stock in de-ionised H2O, filtered.

- **Lysis buffer**

  Protease Inhibitor Tablets cOmplete Mini EDTA free

- **Roche Catalog #11836170001**

  Microcystin-LR **Enzo Life**

- **Sciences Catalog #ALX-350-012**

A	B
Tris-HCl pH 7.5	50 mM
Triton X-100	1% (v/v)
EGTA	1 mM
Na3VO4**	1 mM
NaF	50 mM
β-glycerophosphate	10 mM
sodium pyrophosphate	5 mM
sucrose	0.27 M
Protease Inhibitor Tablets cOmplete EDTA free **	
Mycrocystin-LR **	1 µg/ml

**: To be added fresh before use.

  Pierce™ Coomassie Plus (Bradford) Assay Kit **Thermo**

- **Fisher Catalog #23236**

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ANTI-FLAG® M2 Affinity Gel **Millipore**

- Resin for LRRK2 immunoprecipitation: **Sigma Catalog #A2220** for FLAG-tagged LRRK2; protein A/G sepharose (Expedeon - AGA1000) for endogenous LRRK2;
- Anti-LRRK2 antibody (UDD3; available from MRC Reagents and Services: https://mrcppureagents.dundee.ac.uk/) for immunoprecipitation of endogenous LRRK2;
- IP wash buffers: Lysis buffer supplemented with 300 mM NaCl; 50 mM Tris-HCl pH 7.5;

- **Kinase assay buffer:**

A	B
Tris-HCl pH 7.5	50 mM
MgCl2	10 mM
ATP	1 mM

- Recombinant Rab proteins (available from MRC Reagents and Services: https://mrcppureagents.dundee.ac.uk/); For expression and purification of recombinant Rab8A, Rab10 and Rab12, see our protocols on protocols.io (dx.doi.org/10.17504/protocols.io.butinwke; dx.doi.org/10.17504/protocols.io.bvjxn4pn; dx.doi.org/10.17504/protocols.io.buy3nxyn)

NUPAGE LDS sample buffer (4x) **Thermo Fisher**

- 4X Loading buffer: **Scientific Catalog #NP0007** , or
- **4X SDS loading buffer:**

A	B
Tris-HCl pH6.8	250mM
SDS	8% (w/v)
glycerol	40% (v/v)
bromophenol blue	0.02% (w/v)

- LRRK2 inhibitor (1000X concentration stock in DMSO; e.g.: [M]**1 Milimolar (mM)** stock of MLi-2 in DMSO for treatment at [M]**1 Micromolar (µM)** final concentration) and DMSO as control vehicle.

Equipment:

- Refrigerated bench-top centrifuge (Eppendorf microcentrifuge 5417R, or equivalent).
- Plate reader for Protein quantification (BioTek Epoch, or equivalent)
- Thermo mixer (Eppendorf ThermoMixer, or equivalent)
- Digital Dry Bath/Block Heater, 2 block configuration, CN, EU, UK plugs, 200-240V **Thermo Fisher Catalog #88870005**

Transient transfection of HEK293 cells for analysis of over-expressed LRRK2 activity in vitro:

1

Transfect HEK293 cells at around 60-70% confluency. For a **10 cm** dish, add **10 µg** DNA (FLAG-tagged LRRK2 or FLAG empty vector) and **30 µl** of [M]**1 mg/ml** PEI solution to **1 mL** of Opti-MEM™ Reduced Serum Medium and vortex for 20/30 seconds.

Note: We recommend including a FLAG empty vector transfection as well as a FLAG-tagged LRRK2 D2017A

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(kinase dead) transfection to control for specificity of LRRK2 immunoprecipitation and activity.

2 

Incubate at **Room temperature** for **00:20:00** to allow the DNA/PEI complexes to form.

3 

Add the transfection mix to the culture medium in each dish drop by drop using a pipette and incubate cells at **37 °C** after transfection.

4 Lyse cells **20:00:00** - **24:00:00** after transfection.

Preparation and quantification of cell lysates (from HEK293 cells over-expressing LRRK2 or MEFs)

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

6 

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: The amount of lysis buffer to use will depend on cell type. As a guideline, use 1 ml of lysis buffer for a 10 cm dish for HEK293 cells and 750 µl of lysis buffer for a 15 cm dish for MEFs.

7 Immediately transfer the cell dishes to ice.

8 Scrape the cells on the dish using a cell lifter (Sigma-Aldrich CLS3008, or equivalent) to ensure all cells are detached from the dish.

9 Using a pipette, transfer cell lysate to an Eppendorf tube **On ice** .

10 Leave samples **On ice** for **00:20:00** to allow for efficient lysis.

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11

Clarify lysates by centrifugation at **20800 x g** for **00:10:00** at **4 °C** .

12 Transfer the supernatants into new Eppendorf tubes and discard the pellet. Keep the tubes **On ice** .

Note: Cell lysates can be snap frozen in liquid nitrogen and stored at -80^{O}C for future use. When assessing kinase activity of LRRK2 immunoprecipitated from cells, we do not recommend more than one freeze/thaw cycle.

13 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 µg/µl.

Immunoprecipitation of over-expressed FLAG-tagged LRRK2 from cell lysates for analysis of LRRK2 activity in vitro

14

Note: When comparing multiple FLAG-tagged variants of LRRK2, we recommend assessing the levels of LRRK2 in the lysates prior to immunoprecipitation by subjecting ~10 µg cell extract to immunoblotting, normalizing total LRRK2/Tubulin levels and adjusting how much cell lysate is to be used to immunoprecipitate LRRK2 based on this quantification, to ensure that the amount of enzyme between reactions is as close as possible.

Add **20 µl** of ANTI-FLAG® M2 Affinity Gel (washed 3 times in PBS and resuspended in PBS to make a 1:1 slurry so that 20 µl of 1:1 slurry correspond to 10 µl resin) to **1 mg** of cell extract.

Note: The immunoprecipitation conditions (amount of resin and amount of cell extract to be used) might need optimisation.

15

Incubate at **4 °C** for **01:00:00** , under mild agitation.

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16 

Collect the resin by centrifugation at **2500 x g** for **00:02:00** at **4 °C** . Discard supernatant.

17 Resuspend resin in **500 µl** of lysis buffer supplemented with **[M]300 Milimolar (mM)** NaCl.

18 Repeat step 16 and 17 twice.

19 

Collect the resin by centrifugation at **2500 x g** for **00:02:00** at **4 °C** . Discard supernatant.

20 Resuspend resin in **500 µl** of **[M]50 Milimolar (mM)** Tris-HCl **pH7.5** .

21 Repeat step 16 and 20.

22 

Collect the resin by centrifugation at **2500 x g** for **00:02:00** at **4 °C** . Discard supernatant.

23 Resuspend the resin in **[M]50 Milimolar (mM)** Tris-HCl **pH7.5** (1:1 ratio, i.e. resuspend 10 µl resin in 10 µl Tris-HCl).

24 Aliquot the resin into Eppendorf tubes kept **On ice** (one Eppendorf tube per reaction, **10 µl** resin each).

Immunoprecipitation of endogenous LRRK2 from MEFs for analysis of LRRK2 activity in vitro

25 - For each reaction, couple **10 µg** of anti-LRRK2 antibody (UDD3, available from MRC Reagents and Services: https://mrcppureagents.dundee.ac.uk/) to **20 µl** Protein A/G Sepharose resin (washed 3 times with PBS and resuspended in PBS to make a 1:1 slurry so that 20 µl of 1:1 slurry correspond to 10 µl resin) by incubating for **01:00:00** at **4 °C** under mild agitation. Include a control where UDD3 is replaced by pre-immune IgG.

26 

To get rid of any unbound antibody, wash the antibody/resin complexes: collect the resin by centrifugation at

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2500 x g for 00:02:00 at 4 °C , discard supernatant and resuspend in 250 µl of PBS.

27 Repeat step 26 twice more.

28 

Add the antibody/resin mix to 5 mg of cell extract per reaction.

Note: The immunoprecipitation conditions (amount of resin and amount of cell extract to be used) might need optimisation.

29 

Incubate at 4 °C for 01:00:00 , under mild agitation.

30 

Collect the resin by centrifugation at 2500 x g for 00:02:00 at 4 °C . Discard supernatant.

31 Resuspend resin in 250 µl of lysis buffer supplemented with [M]300 Milimolar (mM) NaCl.

32 Repeat step 30 and 31 twice more.

2m

33 

Collect the resin by centrifugation at 2500 x g for 00:02:00 at 4 °C . Discard supernatant.

34 Resuspend resin in 250 µl of [M]50 Milimolar (mM) Tris-HCl pH7.5 .

35 Repeat step 33 and 34.

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36 Collect the resin by centrifugation at **2500 x g** for **00:02:00** at **4 °C** . Discard supernatant.

37 Resuspend the resin in **50 Milimolar (mM)** Tris-HCl **pH 7.5** (1:1 ratio).

38 Aliquot the resin into Eppendorf tubes kept **On ice** (one Eppendorf tube per reaction, **20 µl** resin each).

In vitro kinase assay using LRRK2 immunoprecipitated from cells

39

The amount of kinase and that of substrate, as well as the duration of the reaction should be optimised to ensure that the measured activity of LRRK2 is in the linear range. We recommend to perform a pilot study to establish the optimal parameters to use for the kinase assay under the experimental conditions of choice. Figure 1 shows the immunoblotting data for an in vitro kinase assay using LRRK2 immunoprecipitated from increasing amounts of cell lysates.

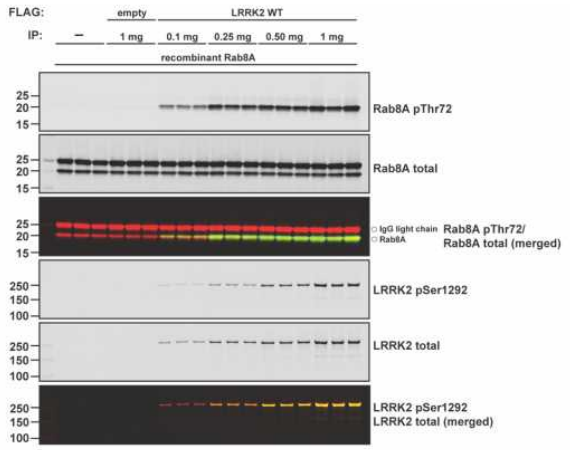

Figure 1: *In vitro* phosphorylation of recombinant Rab8A by immunoprecipitated FLAG-LRRK2 WT. HEK293 cells were transfected with FLAG-empty or FLAG-LRRK2 WT. The cells were lysed 24 h post transfection and the indicated amounts of cell extract were subjected to a FLAG immunoprecipitation, followed by a 45 minute kinase reaction at 30°C in the presence of 5 µg recombinant Rab8A and excess Mg-ATP. 20% of FLAG-empty and FLAG-LRRK2 WT kinase reactions were subjected to immunoblot analysis with the indicated antibodies and the membranes were developed using the Odyssey CLx scan Western Blot imaging system.

Prepare a "master mix" containing **50 Milimolar (mM)** Tris-HCl **pH 7.5** , **10 Milimolar (mM)** MgCl2, **1 Milimolar (mM)** ATP, and recombinant Rab protein.

40 Start the kinase reaction by adding the master mix to the immunoprecipitated kinase and transferring the Eppendorf tubes to the thermo mixer set at **30 °C** , **1000 rpm** .

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41 Stop the kinase reaction by adding 4X LDS loading buffer to the reaction mix to a final concentration of 2X.

42

Incubate the mixture at **70 °C** on a heat block for **00:10:00** to elute LRRK2 from the resin.

43

Collect the eluent by centrifugation through a **0.22 µm** -pore-size Spinex column.

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

45

Incubate the samples for **00:05:00** at **70 °C** on a heat block before proceeding to quantitative immunoblotting analysis.

Analysis of kinase reaction products by quantitative immunoblotting analysis

46 Once the in vitro kinase assay has been performed, we recommend analysing the reaction products by quantitative immunoblotting (as described in dx.doi.org/10.17504/protocols.io.bsgrnbv6).

The reaction products can be analysed by quantitative immunoblotting analysis (as described in dx.doi.org/10.17504/protocols.io.bsgrnbv6). Table 1 lists the primary antibodies that we recommend using, which include antibodies to detect Rab phosphorylation, as well as LRRK2 auto-phosphorylation sites.

Table 1:

Antibody Target	Company	Cat. number	Host species	Dilution
Rab8A pThr72**	Abcam Inc.	ab230260	Rabbit	0.5 µg/ml
Rab8A total	Sigma	WH0004218M2	Mouse	1 µg/ml
Rab10 pThr73	Abcam Inc.	ab230261	Rabbit	1 µg/ml
Rab10 total	Nanotools	0680–100/Rab10-605B11	Mouse	1 µg/ml
LRRK2 pThr1357	Abcam Inc.	ab270606	Rabbit	1:1,000
LRRK2 pThr1503	Abcam Inc.	ab154423	Rabbit	1:1,000
LRRK2 pS1292	Abcam Inc.	ab203181	Rabbit	1:2,000
LRRK2 total (C-ter)	Antibodies Inc./NeuroMab	75-253	Mouse	1 µg/ml

**This antibody cross-reacts with phosphorylated Rab3A, Rab10, Rab35 and Rab43.

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Figure 2: *In vitro* phosphorylation of recombinant Rab10 by LRRK2 immunoprecipitated from mouse embryonic fibroblasts (MEFs). Endogenous LRRK2 was immunoprecipitated from 4 mg cell extracts derived from littermate matched wild-type and LRRK2$^{-/-}$ (knock-out), or littermate matched wild-type and LRRK2$^{[G2019S]}$ KI MEFs. The immunoprecipitates were subjected to an *in vitro* kinase reaction at 30°C in the presence of 1 mg recombinant Rab10 and excess Mg-ATP, with or without 1μM LRRK2 inhibitor MLi-2. The reactions were terminated after 1 hour and analyzed by immunoblotting with the indicated antibodies. The membranes were developed using the Odyssey CLx scan Western Blot imaging system.

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