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Assessment of PKC-dependent activation of LRRK1 in vitro

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We describe a non-radioactive assay that we deploy for analysing the kinase activity of recombinant LRRK1 following *in vitro* activation by Protein kinase C (PKC) isoforms. This assay can also be used to analyse the effect of PKC on LRRK1 immunoprecipitated from cells.

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PKC activation, lipid vesicles, Phosphorylation of LRRK1, immunoblotting analysis, ASAPCRN

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Note: *Once the in vitro kinase assay has been performed, we recommend analysing the reaction products by quantitative immunoblotting (as described in **XXXXX**).*

Note: *This protocol can be adapted to analyse activation of LRRK1 that has been immunoprecipitated from cells (as described in **XXXXXX**).*

Reagents:

1. Recombinant PKC isoform protein (available from MRC Reagents and Services: https://mrcppureagents.dundee.ac.uk/)
2. Recombinant Rab7A protein (available from MRC Reagents and Services: https://mrcppureagents.dundee.ac.uk/)
3. Recombinant LRRK1 wild type [27-2015] protein

Note: Recombinant LRRK1 protein is expressed and purified by following the protocol described in: **XXXXX**

4. Kinase assay buffer:

A	B
HEPES pH 7.5	25 mM
2-mercaptoethanol	0.1% (v/v)
KCl	50 mM
CaCl2	1 mM
MgCl2	10 mM
ATP	1 mM

1. L-α-Phosphatidylserine (Avanti Polar Lipids, resuspended in methanol and chloroform at a 1:1 ratio for long-term storage)
2. L-α-Diacylglyerol (Avanti Polar Lipids, resuspended in methanol and chloroform at a 1:1 ratio for long-term storage)
3. **4X Loading buffer**:

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

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Anti-Rab7 antibody Mouse monoclonal **Sigma Aldrich Catalog #R8779**

Recombinant Anti-PKC alpha antibody **Abcam Catalog #ab11723**

Equipment:

- Refrigerated bench-top centrifuge (Eppendorf microcentrifuge 5417R, or equivalent).

Refrigerated Centrifuge
Centrifuge
Eppendorf **EP-5417R**

- Savant SpeedVac system (Thermo #SPD140DDA, or equivalent)
- Thermo mixer (Eppendorf ThermoMixer, or equivalent)
- Disposable Glass Culture Tubes (Fisherbrand Round Bottom Disposable Borosilicate Glass Tubes, or equivalent)

Preparation of lipid vesicles for PKC activation

1

Clean a disposable glass culture tube by washing. Allow to air-dry.

1.1 Clean a disposable glass culture tube by washing with 100% methanol. (1/3)

1.2 Clean a disposable glass culture tube by washing with 100% methanol. (2/3)

1.3 Clean a disposable glass culture tube by washing with 100% methanol. (3/3)

2

Pipette **0.5 µL** of Diacylglycerol (stock concentration is **10 mg/mL**) and **5 µL** of

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Phosphatidylserine (stock concentration is **10 mg/mL**) into the cleaned and dried glass tube.

> **Note**: These quantities will provide sufficient lipid vesicles for 25 reactions at a volume of **20 µL** per reaction.

3 Vacuum dry lipids using a SpeedVac system for **00:10:00** . This should leave a visible, translucent lipid pellet. 10m

> **Note**: Ensure that lipids are completely dried as any residual chloroform or methanol will inhibit the kinase reaction.

4 Resuspend lipids in **50 µL** of **25 millimolar (mM)** HEPES **7.4**, **50 millimolar (mM)** KCl. Vortex gently until pellet is no longer visible.

Kinase Reaction Step 1: Phosphorylation of LRRK1 by PKC

5

> **Note:** *If using immunoprecipitated LRRK1 from cells, perform immunoprecipitation and washes (as described in **XXXXXX**) before proceeding with Step 5.*

Prepare a primary “2X master mix” containing

A	B
HEPES pH 7.5	50 mM
KCl	100 mM
2-Mercaptoethanol	0.2% (v/v)
MgCl2	20 mM
ATP	2 mM
CaCl2	2 mM
Phosphatidylserine	200 µg/ml
Diacylglycerol	20 µg/ml

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6

For each reaction, add **10 µL** of the primary "2X master mix" to a clean Eppendorf tube.

7 5m

Add **5 µL** of **200 nanomolar (nM)** LRRK1 wild type protein (final concentration is **50 nanomolar (nM)**) to each reaction and allow equilibration **On ice** for **00:05:00** .

> **Note**: If using LRRK1 immunoprecipitated from cells, add **10 µL** of the primary "2X master mix" and **5 µL** of **25 millimolar (mM)** HEPES **pH 7.5**, **50 millimolar (mM)** KCl, 0.1% (v/v) 2-Mercaptoethanol to each tube containing beads-bound immunoprecipitated LRRK1.

8

Start the kinase reaction by adding **5 µL** of **400 nanomolar (nM)** PKC Alpha protein (final concentration is **100 nanomolar (nM)**).

> **Note**: The final reaction volume should be **20 µL** .

9 30m

After **00:30:00** , transfer the Eppendorf tubes from Step 8 **On ice** .

Kinase Reaction Step 2: Phosphorylation of Rab7A by PKC-activated LRRK1

10 Prepare a secondary "master mix" (=Master Mix B) containing

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A	B
HEPES pH 7.5	25 mM
KCl	50 mM
MgCl2	10 mM
ATP	1 mM
Rab7A	1 µM

11 

Start the second step of the kinase reaction by adding **10 µL** Master Mix B to the Eppendorf tubes from Step 5.

12 

45m

Transferring the Eppendorf tubes to the thermo mixer set at **30 °C** , 1,000 rpm. Incubate for **00:45:00** .

13 

Stop the kinase reaction by adding **10 µL** of 4× LDS (supplemented with 5% (v/v) 2-Mercaptoethanol) loading buffer to the reaction mix to a final concentration of 1×.

14 

10m

If using LRRK1 immunoprecipitated from cells, stop the kinase reaction by adding **30 µL** of 4× LDS loading buffer to the reaction mix to a final concentration of 2×, incubate the mixture at **70 °C** on a heat block for **00:10:00** to elute LRRK1 from the resin, and collect the eluent by centrifugation through a 0.22-µm-pore-size Spinex column.

15 

5m

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

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Note: If using LRRK1 immunoprecipitated from cells, supplement the samples from Step 14 with 2-Mercaptoethanol to 1% (v/v) before proceeding to Step 15.

Analysis of kinase reaction products by quantitative immunoblotting analysis: 1h 15m

16

The reaction products can be analysed by quantitative immunoblotting analysis (as described in XXXX). **Table 1** lists the primary antibodies that we recommend using, which include antibodies to detect Rab7A phosphorylation at Serine-72.

A	B	C	D	E
Antibody Target	**Company**	**Cat. number**	**Host species**	**Dilution**
pS72 Rab7A	Abcam Inc.	MJF-38, Clone 1	Rabbit	1 mg/ml
Rab7A (Total)	Sigma	R8779	Mouse	1.430556
LRRK1 (total) (C-terminus)	MRC-PPU Reagents and Services, University of Dundee	S405C	Sheep	1 mg/ml
PKC Alpha	Abcam Inc.	ab11723	Mouse	1.430556

17 1h 15m

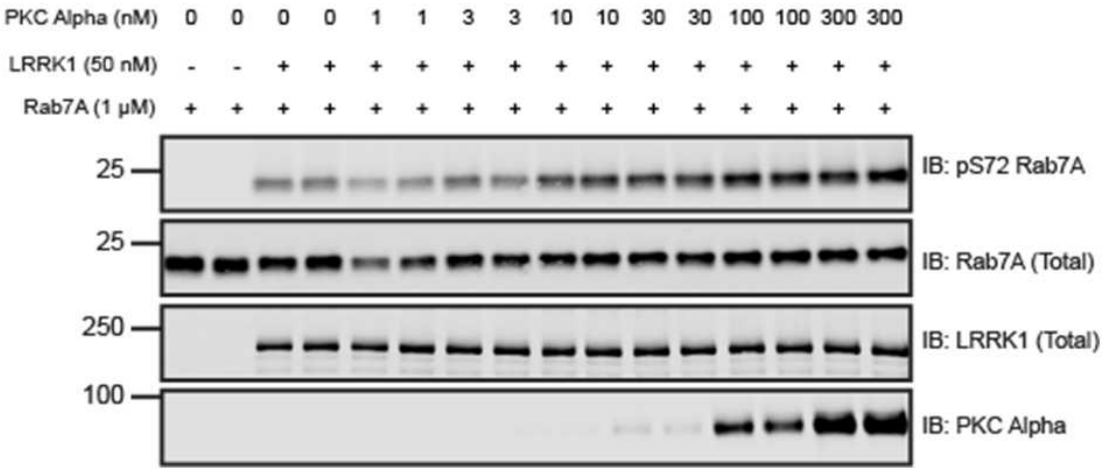

Figure 1: PKC alpha dose-dependent activation of recombinant LRRK1 in vitro. Recombinant LRRK1 wild type [27-2015] was incubated with increasing concentrations of PKC Alpha (1 to 300 nM) at **30 °C** for **00:30:00** with excess Mg-ATP. Reactions were subsequently incubated with **1 micromolar (µM)** recombinant Rab7A and subjected to a

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00:45:00 kinase reaction at **30 °C** in the presence of excess Mg-ATP. 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.

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