**Assessment of PKC-dependent activation of LRRK1 *in vitro***

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**Summary**

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.

*Note: Once the in vitro kinase assay has been performed, we recommend analysing the reaction products by quantitative immunoblotting (as described in* [***XXXXX***](https://dx.doi.org/10.17504/protocols.io.bsgrnbv6)*).*

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

**1) MATERIALS**

**1.1) Reagents:**

1.1.1) Recombinant PKC isoform protein (available from MRC Reagents and Services: <https://mrcppureagents.dundee.ac.uk/>)

1.1.2) Recombinant Rab7A protein (available from MRC Reagents and Services: <https://mrcppureagents.dundee.ac.uk/>)

1.1.3) Recombinant LRRK1 wild type [27-2015] protein

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

1.1.4) Kinase assay buffer: 25 mM HEPES pH 7.5; 0.1% (v/v) 2-mercaptoethanol; 50 mM KCl; 1 mM CaCl2; 10 mM MgCl2; 1 mM ATP

1.1.5) L-α-Phosphatidylserine (Avanti Polar Lipids, resuspended in methanol and chloroform at a 1:1 ratio for long-term storage)

1.1.6) L-α-Diacylglyerol (Avanti Polar Lipids, resuspended in methanol and chloroform at a 1:1 ratio for long-term storage)

1.1.7) 4X Loading buffer: Invitrogen™ NuPAGE™ LDS Sample Buffer, cat no NP0007, or 4X SDS loading buffer: 250mM Tris-HCl, pH6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue.

**1.2) Equipment:**

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

1.2.2) Savant SpeedVac system (Thermo #SPD140DDA, or equivalent)

1.2.3) Thermo mixer (Eppendorf ThermoMixer, or equivalent)

1.2.3) Disposable Glass Culture Tubes (Fisherbrand Round Bottom Disposable Borosilicate Glass Tubes, or equivalent)

**2) METHOD:**

**2.1) Preparation of lipid vesicles for PKC activation**

2.1.1) Clean a disposable glass culture tube by washing three times with 100% methanol. Allow to air-dry.

2.1.2) Pipette 0.5 μl of Diacylglycerol (stock concentration is 10 mg/ml) and 5 μl of 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.*

2.1.3) Vacuum dry lipids using a SpeedVac system for 10 minutes. This should leave a visible, translucent lipid pellet.

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

2.1.4) Resuspend lipids in 50 μl of 25 mM HEPES pH 7.4, 50 mM KCl. Vortex gently until pellet is no longer visible.

**2.2) Kinase Reaction Step 1: Phosphorylation of LRRK1 by PKC**

*Note: If using immunoprecipitated LRRK1 from cells, perform immunoprecipitation and washes (as described in* ***XXXXXX****) before proceeding with Step 2.2.1.*

2.2.1) Prepare a primary “2X master mix” containing 50 mM HEPES pH 7.5, 100 mM KCl, 0.2% (v/v) 2‐Mercaptoethanol, 20 mM MgCl2, 2 mM ATP, 2 mM CaCl2, 200 μg/ml Phosphatidylserine and 20 μg/ml Diacylglycerol.

2.2.2) For each reaction, add 10 μl of the primary “2X master mix” to a clean Eppendorf tube.

2.2.3) Add 5 μl of 200 nM LRRK1 wild type protein (final concentration is 50 nM) to each reaction and allow equilibration on ice for 5 minutes.

*Note: If using LRRK1 immunoprecipitated from cells, add 10 µl of the primary “2X master mix” and 5 ul of 25 mM HEPES pH7.5, 50 mM KCl, 0.1% (v/v) 2‐Mercaptoethanol to each tube containing beads-bound immunoprecipitated LRRK1.*

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

*Note: The final reaction volume should be 20 μl.*

2.2.5) After 30 minutes, transfer the Eppendorf tubes from Step 2.2.4 on ice.

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

2.3.1) Prepare a secondary “master mix” (=Master Mix B) containing 25 mM HEPES pH 7.5, 50 mM KCl, 10 mM MgCl2, 1 mM ATP and 1 μM Rab7A.

2.3.2) Start the second step of the kinase reaction by adding 10 μl Master Mix B to the Eppendorf tubes from Step 2.2.9.

2.3.3) Transferring the Eppendorf tubes to the thermo mixer set at 30oC, 1,000 rpm. Incubate for 45 minutes

2.3.4) Stop the kinase reaction by adding 10 μl of 4X LDS (supplemented with 5% (v/v) 2‐Mercaptoethanol) loading buffer to the reaction mix to a final concentration of 1X.

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

2.3.6) Incubate the samples for 5 min at 70°C on a heat block before proceeding to quantitative immunoblotting analysis.

*Note: If using LRRK1 immunoprecipitated from cells, supplement the samples from Step 2.3.5 with 2-Mercaptoethanol to 1% (v/v) before proceeding to Step 2.3.6.*

**2.4) Analysis of kinase reaction products by quantitative immunoblotting analysis:**

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.

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| --- | --- | --- | --- | --- |
| **­Antibody Target** | **Company** | **Cat. number** | **Host species** | **Dilution** |
| pS72 Rab7A | Abcam Inc. | MJF-38, Clone 1 | Rabbit | 1 μg/ml |
| Rab7A (Total) | Sigma | R8779 | Mouse | 1:2000 |
| LRRK1 (total) (C-terminus) | MRC-PPU Reagents and Services, University of Dundee | S405C | Sheep | 1 μg/ml |
| PKC Alpha | Abcam Inc. | ab11723 | Mouse | 1:2000 |

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**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 30 minutes with excess Mg-ATP. Reactions were subsequently incubated with 1 mM recombinant Rab7A and subjected to a 45-minute 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.