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Protocol status: Working
 We use this protocol and it's working

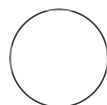
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In vitro LRRK2 kinase activity assay using mass-spectrometry as readout

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ABSTRACT

This protocol can be used in this form or with small adjustments regarding concentration and reaction time to determine in vitro substrate phosphorylation for any purified kinase substrate pair.

It can be used to determine inhibition curves by addition of concentration series of kinase inhibitors.

Reaction is carried out in 50 µL reaction mix containing final concentration of 50 nM kinase and 5 mM substrate.

MATERIALS

purified protein kinase
 purified kinase substrate
 buffer: 20 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, 20 µM GDP, 2.5 mM MgCl₂
 100 mM ATP

Step-by-Step Protocol

- 1 Prepare 50 μ L reaction mix I: 50 nM purified LRRK2 and 5 μ M Rab8 substrate in buffer containing 20 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, 20 μ M GDP, 2.5 mM $MgCl_2$. Aliquot 25 μ L per tube.

Note

If you want to run multiple reactions prepare a master mix and aliquot 25 μ L per tube.

- 2 Prepare 25 μ L reaction mix II: 2 mM ATP + 2.5 mM $MgCl_2$ in buffer containing 20 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, 20 μ M GDP, 2.5 mM $MgCl_2$.

Note

Multiply if you want to run more than one reaction.

- 3 Prepare 25 μ L reaction mix III: same as reaction mix II but no ATP as negative control.

- 4 Start reaction by adding 25 μ L reaction II to reaction mix I.
Note: for negative control add 25 μ L reaction mix III to second aliquot of reaction mix I.

- 5 Mix by vortexing and brief centrifugation for 30 sec with 500xg.

- 6 Incubate reaction for 3 h at room temperature.
Note: depending on kinase this may be shorter or longer.
- 7 Stop reaction by adding 50 µL mass spec buffer (dH₂O+0.1% formic acid).
- 8 Store at -80°C or proceed directly with mass spectrometry analysis.

Note

For determination of inhibition curves, prepare a concentration series of inhibitor in DMSO. Prepare a master mix of kinase and substrate pair and aliquot 25 µL per tube. Add the desired amount of inhibitor to each of the tube and incubate all reactions at room temperature.

Note

For analysis: substrate phosphorylation/turnover can be determined as ratio between phosphorylated and unphosphorylated peak intensity for each sample analyzed.