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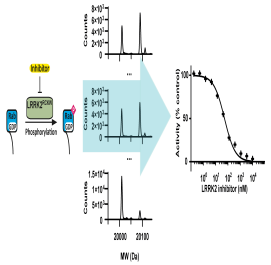
In vitro LRRK2 kinase activity assay using mass-spectrometry as readout V.2



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

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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 µM substrate.

Materials

Purified proteins

kinase: $LRRK2^{RCKW}$
protein sequence:
KKAVPYNRMKLMIVGNTGSGKTTLLQQLMKTKKSDLGMQSATVGIDVKDWPIQIRDKRKRDLVLNVWDFAGREEFYSTHPH
FMTQRALYLAVYDLSKGQAEVDAMKPWLFNIKARASSSPVILVGTHLDVSDEKQRKACMSKITKELLNKRGFPAIRDYHFVN
ATEESDALAKLRKTIINESLNFKIRDQLVVGQLIPDCYVELEKIILSERKNVPIEFPVIDRKRLLQLVRENQLQLDENELPHAVHFL
NESGVLLHFQDPALQLSDLYFVEPKWLCKIMAQILTVKVEGCPKHPKGIISRRDVEKFLSKKRKFPKNYMSQYFKLLEKFQIAL
PIGEEYLLVPSSLSDHRPVIELPHCENSEIIIRLYEMPYFPMGFWSRLINRLLEISPYMLSGRERALRPNRMYWRQGIYLNWSPE
AYCLVGSEVLDNHPESFLKITVPSCRKGCILLGQVVDHIDSLMEEWFPGLLEIDICGEGETLLKKWALYSFNDGEEHQKILLDD
LMKKAEEGDLLVNPDQPRLTIPISQIAPDLILADLPRNIMLNNDELEFEQAPEFLLGDGSFGSVYRAAYEGEEVAVKIFNKHTSL
RLLRQELVVLCHLHHPSLISLLAAGIRPRMLVMELASKGSLDRLLQQDKASLTRTLQHRIALHVADGLRYLHSAMIIYRDLKPH
NVLLFTLYPNAAIIAKIADYGIAQYCCRMGIKTSEGTPGFRAPEVARGNVIYNQQADVYSFGLLLYDILTTGGRIVEGLKFPNEF
DELEIQGKLPDPVKEYGCAPWPMVEKLIKQCLKENPQERPTSAQVFDILNSAELVCLTRRILLPKNVIVECMVATHHNSRNASI
WLGCGHTDRGQLSFLDLNTEGYTSEEVADSRILCLALVHLPVEKESWIVSGTQSGTLLVINTEDGKKRHTLEKMTDSVTCLY
CNSFSKQSKQKNFLLVGTADGKLAIFEDKTVKLKGAAPLKILNIGNVSTPLMCLSESTNSTERNVMWGGCGTKIFSFSNDFTI
QKLIETRTSQLFSYAAFSDSNIITVVVDTALYIAKQNSPVVEVWDKKTEKLCGLIDCVHFLREVMVKENKESKHKMSYSGRVK
TLCLQKNTALWIGTGGGHILLLDLSTRRLIRVIYNFCNSVRVMMTAQLGSLKNVMLVLGYNRKNTEGTQKQKEIQSCLTVWDI
NLPHEVQNLEKHIEVRKELAEKMRRTSVE

substrate: Rab8A
protein sequence:
GHMDYLFKLLLIGDSGVGKTCVLFRFSEDAFNSTFISTIGIDFKIRTIELDGKRIKLQIWDTAGQERFRTITTAYYRGAMGIMLVY
DITNEKSFDNIRNWIRNIEEHASADVEKMILGNKCDVNDKRQVSKERGEKLALDYGIKFMETSAKANINVENAFFTLARDIKAK
MDKK

Buffers and Reagents

reaction buffer: 20 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, 20 µM GDP, 2.5 mM $MgCl_2$

ATP 100 mM stock in dH_2O

mass spec buffer: dH_2O + 0.1% formic acid

Troubleshooting

Step-by-Step Protocol

1 Prepare 50 µL reaction mix I: 50 nM purified $LRRK2^{RCKW}$ and 5 µM Rab8 substrate in buffer containing 20 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, 20 µM GDP, 2.5 mM $MgCl_2$.
Aliquot 25 µL per tube.

> Note
>
> If you want to run multiple reactions prepare a master mix and aliquot 25 µL per tube.

2 Prepare 25 µL 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 µM GDP, 2.5 mM $MgCl_2$.

> Note
>
> Multiply if you want to run more than one reaction.

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

4 Start reaction by adding 25 µL reaction II to reaction mix I.
Note: for negative control add 25 µL 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: depeding on kinase this may be shorter or longer.

7 Stopp reaction by adding 50 µL mass spec buffer (dH_2O+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.