In vitro kinase assay: phosphorylation of PI3Kc1 by TBK1and ULK1

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MAR 31, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.j8nlkwr51l5r/v1

Protocol Citation: Elias Adriaenssens 2023. In vitro kinase assay: phosphorylation of PI3Kc1 by TBK1and ULK1. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlkwr51l5r/v1

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

Created: Feb 28, 2023

Last Modified: Mar 31, 2023

PROTOCOL integer ID: 77801

Keywords: ASAPCRN, ULK1 complex, TBK1, PI3Kc1, ATG14L, kinase assay, phosphorylation

ABSTRACT

This protocol describes in vitro kinase assay, in which PI3Kc is tested for phosphorylation by TBK1 and ULK1 complex either by western blotting (S29 on ATG14L subunit) or mass spectrometry analysis.

ATTACHMENTS

kinase assay ULK1c or TBK1 on PI3Kc.png

MATERIALS

Plasmids for expressing and purifying human ULK1c, TBK1 and PI3Kc1:
ULK1c: pCAG-MBP-TSF-TEV-ULK1 Addgene ID: 171416
pCAG-GST-TEV-FIP200-MBP Addgene ID: 171410
pCAG-EGFP-ATG13 Addgene ID: 171413
pCAG-ATG101 Addgene ID: 189590

PI3Kc1: pGBdest-GST-PI3Kc1 Addgene ID: 187992
TBK1: TBK1 (GFP-TBK1 can also be used Addgene ID: 187830)

Kinase buffer without ATP:
20 mM TRIS-Cl pH 7.4
150 mM NaCl
1 mM DTT

Kinase Buffer with 2xATP/MgCl2
20 mM TRIS-Cl pH 7.4
150 mM NaCl
1 mM DTT
20 mM MgCl2 (final in the reaction 10 mM)
0.2 mM ATP (final in the reaction 0.1 mM)

Electrophoresis:
SDS-PAGE 4-12% NuPAGE gel

SDS-PAGE running buffer (25 mM TRIS pH 8.8, 190 mM Glycine, 0.1% SDS)
Coomassie staining solution
Destaining solution: (40% ethanol, 10% acetic acid)

Transfer/Western Blotting:
Nitrocellulose Blotting Membrane Amersham Protran 0.2 µM NC Cat# 10600001(Cytiva)
Transfer Buffer (125 mM TRIS pH 8.0, 960 mM Glycine, 10 % Ethanol)
Washing Buffer (PBST: PBS + 0.1% Tween20)
Blocking Buffer (5% BSA in PBST)

Antibodies:
Rabbit anti p-ATG14 (S29): Cell Signaling Cat# 13155 (used 1: 1000 dilution in blocking buffer)
Rabbit anti ATG14: Cell Signaling Cat# 5504 (used 1: 1000 dilution in blocking buffer)
Peroxidase-conjugated goat anti rabbit IgG: Jackson ImmunoResearch Cat# 111-035-003 (used 1: 10 000 dilution in blocking buffer)

Signal developing:
SuperSignal™ West Femto Maximum Sensitivity Substrate ThermoFischer Scientific Cat# 34094

In vitro kinase assay: phosphorylation of ATG14 by TBK1

1 Prepare Kinase Buffer without ATP and MgCl2.

2 Prepare 100 µL of Master Mix I containing TBK1 at the final concentration of 50 nM and PI3Kc1 at the final concentration of 800 nM in the Kinase Buffer without ATP and MgCl2.

3 Prepare 100 µL of Master Mix II containing ULK1c at the final concentration of 200 nM and PI3Kc1 at the final concentration of 800 nM Kinase Buffer without ATP and MgCl2.

4 Distribute 25 µL of each of the Master Mixes to new Eppendorf tubes.

5 To start the reaction add 25 µL of Kinase Buffer with 2xATP/MgCl2 to three tubes containing the Master Mixes (time points 5, 10 and 30 min) and 25 µL of Kinase Buffer without ATP to one of the tubes (time point 0). To control for potential protein instability, all samples should be kept at room temperature for the same period of time. Therefore start pipetting the Kinase Buffer with 2xATP/MgCl2 to the 30 min sample, after 20 min move on to the 10 min sample and after additional 5 min to the 5 min sample, meanwhile keeping all of them in RT.

6 To stop the reactions add 6x Protein Loading Buffer and boil the samples for 5 min at 95 °C.

7 Separate samples via SDS-PAGE using 4-12% NuPAGE polyacrylamide gel and perform transfer (100 V for 1h at 4 °C) to a nitrocellulose membrane for western blot analysis . For Coomassie staining, incubate the SDS-PAGE gel in Coomassie stain for 10 min gently rocking at RT, destain for 10 min, and incubate in ddH_2O overnight. For Mass Spectrometry analysis excise the bands the next morning with a sharp and clean scalpel.

8 For western blot analysis continue by blocking the membrane for 1 h in Blocking Buffer gently rocking at RT.

9 Incubate with Primary Antibodies (diluted 1:1000 in Blocking Buffer) overnight at 4 °C gently rocking.

10 Wash the membrane 3x10 min with PBST.

11 Incubate for 1 h with Secondary Antibodies (diluted 1:10 000 in Blocking Buffer) gently rocking at RT.

12 Wash the membrane 3x10 min with PBST.

13 Develop the Blot using Femto Maximum Sensitivity Substrate (ThermoFischer Scientific).