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Enrichment of phosphopeptides from lysate using TiO_2

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Protocol status: Working
We use this protocol and it has worked in previous experiments.

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Abstract

This protocol describes the sample preparation for phosphopeptide enrichment from lysed HEK293T cells that were transfected with TurboID, TurboID-LRRK1 or TurboID-LRRK2 and treated with MLi-2, IN04 or DMSO. It is meant to accompany the methods section and manuscript of "Probing the proteome-wide interactomes of Leucine-rich Repeat Kinases 1 and 2 and alterations in their phosphorylation activity".

For details on culturing and treatment of cells as well as lysis of these cells, please refer to Protocol "TurboID-phospho protocol" (doi: 10.17504/protocols.io.ewov1drpyvr2/v1) where these steps are explained in detail. This protocol is only meant to describe the sample processing from a cell lysate to enriched phosphopeptides ready to be measured by DIA-based mass spectrometry.
This protocol starts with the cell lysate obtained in the above mentioned protocol at step 14 and ends with enriched phosphopeptide that can be analysed by mass spectrometry according to steps 45ff of the same protocol.

All steps are described per sample.

Materials

- cOmplete™, EDTA-free protease inhibitor cocktail **Roche** **Catalog #**11873580001
- Tris(2-carboxyethyl)phosphin -hydrochlorid **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**C4706
- Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I1149-5G
- Trypsin/Lys-C Mix, Mass Spec Grade, 5 × 20ug **Promega** **Catalog #**V5073
- Sequencing Grade Modified Trypsin **Promega** **Catalog #**V5113
- HiPPR™ Detergent Removal Spin Column Kit **Thermo Fisher** **Catalog #**88305
- Sep-Pak tC18 1 cc Vac Cartridge 50 mg Sorbent per Cartridge **Waters** **Catalog #**WAT054960
- Titansphere TiO, 5 µm, 500 mg **GL Science** **Catalog #**020-75000
- Glycolic acid **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**124737
- 3M™ Empore™ C18 47 mm Extraction Disc Model 2215 20 pack 3 packs per case **3M corporation** **Catalog #**2215
- Lactic acid solution, 85 % **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**252476

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Safety warnings

After adding acetone to your proteins, always work on ice until you have dried the pellet. If your sample warms up during the precipitation process, you might lose proteins.

After performing the phosphopeptide enrichment, make sure that the exposition to basic pH is as short as possible because this can cause a loss of phosphorylations
Perform the enrichment shortly before measurement to keep native phosphorylation intact.

Before start

You will need 6 volumes of ice-cold acetone for 1 volume of your sample to perform the precipitation. Please calculate the total sample volume before you start and choose the right tube size for the precipitation.

Acetone-Protein precipitation

1 Thaw 200 µL cell lysate in NP-40 buffer ([M] 25 millimolar (mM) Tris-HCl pH 7.4 , [M] 150 millimolar (mM) NaCl, [M] 1 % (v/v) NP-40, [M] 5 millimolar (mM) $MgCl_2$, [M] 1 millimolar (mM) Na_3VO_4, [M] 5 millimolar (mM) NaF, [M] 5 millimolar (mM) β-glycero phosphate, 1x cOmplete™, EDTA-free protease inhibitor cocktail **Roche Catalog #**11873580001 , [M] 1 millimolar (mM) DTT) or other lysis buffers at 0 °C .

2 Add 1 volumes (here 200 µL) of ice-cold acetone and mix sample.

2.1 Add 2 volumes (here 400 µL) of ice-cold acetone and mix sample.

2.2 Add 3 volumes (here 600 µL) of ice-cold acetone and mix sample.

3 Incubate at -80 °C for 00:15:00 , then at -20 °C for 01:30:00 .

4 Centrifuge precipitated samples for 00:10:00 at 16000 x g, 4°C .

4.1 Afterwards, you should be able to see a whitish pellet of precipitated proteins (depending on the protein amount).

4.2

5 Carefully remove supernatant without disturbing the pellet.

5.1 Optionally, wash your pellet with ice-cold acetone one or two more times.
This depends on the downstream processing. Washing of the pellet can for example reduce the amount of detergents in the sample.

6 Dry the pellet at Room temperature with an open lid.

Protein reduction, alkylation and digestion

7 Resuspend protein pellet in 150 µL [M] 8 Mass Percent urea.

7.1 Incubate for 00:15:00 at 37 °C and 800 rpm to resuspend proteins.

8 Add TCEP
Tris(2-carboxyethyl)phosphin -hydrochlorid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C4706
for [M] 3 millimolar (mM) final concentration to reduce cysteines.

8.1 Incubate for 00:30:00 at 37 °C and 650 rpm .

9 Add IAA (
Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I1149-5G) to
[M] 6 millimolar (mM) final concentration to alkylate cysteines.

9.1 Incubate for 00:30:00 at Room temperature and 650 rpm **in the dark**.

10 Dilute urea to [M] 4 Mass Percent using [M] 50 millimolar (mM) ammonium bicarbonate.

11 Add 1 µg
Trypsin/Lys-C Mix, Mass Spec Grade, 5 × 20ug **Promega Catalog #**V5073 to sample.

11.1 Incubate for 03:30:00 at 37 °C and 650 rpm .

12 Dilute urea concentration to [M] 1 Mass Percent using [M] 50 millimolar (mM) ammonium bicarbonate.

13 Add 2 µg Sequencing Grade Modified Trypsin **Promega Catalog #**V5113 .

13.1 Incubate for 18:00:00 at 37 °C and 650 rpm .

14 Remove residual detergents with the
HiPPR™ Detergent Removal Spin Column Kit **Thermo Fisher Catalog #**88305
(200 µL per sample) according to the manufacturer's instructions.

15 Acidify flow-through by adding formic acid to a final concentration of [M] 2 % (v/v) .

15.1 After mixing, check if pH 2 or lower. This is important since acidic pH stops trypsin digestion and is crucial for a successful C18 desalting.

16 Add [M] 1 % (v/v) acetonitrile (ACN) and desalt your sample using
Sep-Pak tC18 1 cc Vac Cartridge 50 mg Sorbent per Cartridge **Waters Catalog #**WAT054960
and a vacuum manifold.

16.1 Wet cartridges with 1 mL pure ACN.

16.2 Equilibrate cartridges with 2 mL [M] 1 % (v/v) ACN, [M] 0.1 % (v/v) formic acid.

16.3 Add sample and apply it slowly to the C18 resin. Do not open valve fully to load sample slowly.

16.4 Wash peptides with 2 mL [M] 1 % (v/v) ACN, [M] 0.1 % (v/v) formic acid.

16.5 Elute peptides into low-binding 1.5 mL tube with 1 mL [M] 50 % (v/v) ACN, [M] 0.1 % (v/v) formic acid.

17 Dry desalted peptides in a vaccuum concentrator.

Phosphopeptide enrichment

18 Weigh 2 mg beads per sample (if ~ 200 µg peptide input) Titansphere TiO, 5 µm, 500 mg **GL Science Catalog** #020-75000 and resuspend in 10 µL per 1 mg beads **Loading buffer 1** ([M] 0.1 Molarity (M) Glycolic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #124737 , [M] 70 % (v/v) ACN, [M] 5 % (v/v) trifluoroacetic acid (TFA)).

18.1 To minimize unspecific binding, incubate TiO_2 beads in **Loading buffer 1** for 00:20:00 at Room temperature and 1200 rpm prior to peptide incubation.

19 Resuspend approx. 200 µg of dried peptides in 200 µL **Loading buffer 1**.

19.1 Incubate for 00:10:00 at 37 °C and 650 rpm for proper resuspension.

20 Add equilibrated beads to peptides at a **peptide:bead ratio of 1:10**.

21 Incubate for 00:20:00 at Room temperature on rolling incubator.

22 Prepare self-made, single-layered C8 Stage tips. See

Citation

Rappsilber J, Mann M, Ishihama Y (2007)
. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips.

https://doi.org/ LINK

for details.
Use

3M™ Empore™ C18 47 mm Extraction Disc Model 2215 20 pack 3 packs per case **3M corporation Catalog** #2215

to make the tips.

23 Equilibrate C8 Stage tips with **Loading buffer 1**.

24 Settle incubated TiO_2 beads by centrifugation 10000 x g for 00:02:00 .

24.1 Transfer 150 µL of supernatant to fresh low-binding tube and keep for second enrichment step, see go to step #31 .

25 Use residual supernatant to transfer beads to equilibrated C8 Stage tips.

25.1 Centrifuge for 00:02:00 at 500 x g and add flow-through to supernatant fraction from step .

26 Wash beads that were retained by C8 Stage tips with 50 µL **Wash buffer 1** ([M] 80 % (v/v) ACN, [M] 1 % (v/v) TFA) and centrifuge at 500 x g for 00:02:00 .

27 Wash beads with 50 µL **Wash buffer 2** ([M] 10 % (v/v) ACN, [M] 0.2 % (v/v) TFA) and centrifuge at 500 x g for 00:02:00 . Transfer tips to new vial.

28 Prepare low-binding tubes with 60 µL [M] 10 % (v/v) formic acid for phosphopeptide elutions to directly acidify them and minimize loss of phosphorlyation by ammonium hydroxide.

29 Elute bound phosphopeptides with 30 µL **Elution buffer 1** ([M] 1 % (v/v) NH_4OH) by centrifugation at 500 x g for 00:02:00 and directly transfer elution to prepared tubes ( ).

30 Elute residual bound phosphopeptides with 30 µL **Elution buffer 2** ([M] 5 % (v/v) NH_4OH, [M] 25 % (v/v) ACN) by centrifugation at 500 x g for 00:02:00 and directly transfer elution to prepared tubes ( ).

31 Repeat phosphopeptide protocol with retained supernatant from and repeat protocol with **Loading buffer 2** ([M] 20 % (v/v)

Lactic acid solution, 85 % **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**252476

, [M] 70 % (v/v) ACN, [M] 5 % (v/v) TFA) instead of **Loading buffer 1**.

This second enrichment step maximizes yield of phosphopeptide by repeating the enrichment and changing the specificity by using lactic acid instead of glycolic acid.

32 Combine phosphopeptide elutions and dry them by vaccuum evaporation.

33 Samples are now ready for mass spectrometric analysis. Please refer to protocol "TurboID-phospho protocol" (dx.doi.org/10.17504/protocols.io.ewov1drpyvr2/v1) for details.

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