Western blotting of XK and VPS13A V.1

Chase Amos[1], Pietro De Camilli[1]

[1]1Departments of Neuroscience and of Cell Biology, Howard Hughes Medical Institute, Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, Connecticut 06510, USA 2Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815

Chase Amos

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

ABSTRACT

This protocol describes collection of protein from cultured cells and immunoblotting.

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Cell culture and treatments

2d 0h 5m

1 Culture K562 or COS-7 (ATCC) at 37 °C and 5% CO2, using RPMI for K562 or DMEM for COS-7 containing 10% FBS, [M] 1 millimolar (mM) sodium pyruvate, [M] 100 Mass Percent penicillin, [M] 100 Mass Percent streptomycin, [M] 2 millimolar (mM) L-glutamine, [M] 1 Mass Percent non-essential amino acids, (all from Gibco) and [M] 2.5 Mass Percent plasmocin (InvivoGen).

2 For K562 cells treated with hemin, supplement the media with hemin (Sigma Aldrich) dissolved in DMS 2d to a final concentration of [M] 30 micromolar (µM) for 48:00:00 .

Cell lysis and sample preparation

2d 0h 5m

3 Prior to K562 lysis, pellet the cells by centrifuging at 1100 rpm, 4°C, 00:05:00 . Resuspend the pe 5m in PBS, centrifuging and repeating for a total of 3 times.

3.1 Resuspend in PBS and centrifuge 1100 rpm, 4°C, 00:05:00 (1/3). 5m

3.2 Resuspend in PBS and centrifuge 1100 rpm, 4°C, 00:05:00 (2/3). 5m

3.3 Resuspend in PBS and centrifuge 1100 rpm, 4°C, 00:05:00 (3/3). 5m

4 Prior to lysis of confluent COS-7 cells, aspirate media and wash with PBS 3 times.

5 Lyse cells with 2% SDS by either resuspending (K562) or adding to culture dish and scraping using a Corning cell-lifter (COS-7). Sonicate lysates using 3x10s pulses with Virsonic 550 (Virtis).

6 Centrifuge 13300 rpm, Room temperature, 00:10:00 and collect the post-nuclear supernatant in 10m new Eppendorf tube.

7 Determine protein concentration in sample using Pierce BCA assay (ThermoFisher).

8 Prepare samples at desired concentration and add SDS loading buffer to reach a final concentration of [M] 50 millimolar (mM) pH 06.8 Tris, [M] 2 Mass / % volume SDS, [M] 0.1 Mass / % volume bromophenol blue, [M] 10 % (v/v) glycerol, and [M] 1 % (v/v) beta-mercaptoethanol.

9 Boil 95 °C for 00:10:00 . 10m

Gel electrophoresis and immunoblotting 1h 15m

10 Prepare gel apparatus with 4-12% Tris Glycine gels (Invitrogen) and Tris-Glycine SDS running buffer.

11 Load samples into gel and run until dye front reaches bottom (120-150 V).

12 Remove gel and set up transfer cassette with nitrocellulose membrane.

13 Transfer at 30 V Overnight at 4 °C in NuPage transfer buffer (Invitrogen)

14 Remove nitrocellulose membrane and block membrane with 5% BSA in TBST for 01:00:00 at Room temperature . 1h

15 Add primary antibodies at desired concentration in 5% BSA in TBS-T, incubate Overnight at 4 °C .

16 Wash membrane with TBST. Repeat a total of 3 times.

16.1 Wash membrane for 00:05:00 with TBST (1/3). 5m

16.2 Wash membrane for 00:05:00 with TBST (2/3). 5m

16.3 Wash membrane for 00:05:00 with TBST (3/3). 5m

17 Incubate membrane with secondary antibodies conjugated to IRdye 800CW or IRdye 680CW (1:10,000 1h

Licor) in 5% BSA in TBST for 01:00:00 at Room temperature .

18 Wash membrane with TBST. Repeat a total of 3 times.

18.1 Wash membrane for 00:05:00 with TBST (1/3). 5m

18.2 Wash membrane for 00:05:00 with TBST (2/3). 5m

18.3 Wash membrane for 00:05:00 with TBST (3/3). 5m

19 Image membranes using a Licor Odyssey Infrared Imager.