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Western blotting of XK and VPS13A V.1

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We use this protocol and it's working

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Abstract

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

Troubleshooting

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 DMSO to a final concentration of [M] 30 micromolar (µM) for 48:00:00 . 2d

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 pellet in PBS, centrifuging and repeating for a total of 3 times. 5m

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 3×10s pulses with Virsonic 550 (Virtis).

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

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, Licor) in 5% BSA in TBST for 01:00:00 at Room temperature . 1h

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.