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Western blotting

Forked from Western blotting to detect ATP13A2 and ATP13A3

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ABSTRACT

Protocol to detect proteins via Western Blotting.

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

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MATERIALS

- **Antibodies**:

- Goat anti-mouse IgG (H+L) secondary antibody HRP conjugated: Thermo Scientific, 31430
- Goat anti-rabbit IgG (H+L) secondary antibody HRP conjugated: Thermo Scientific, 31460
- Mouse monoclonal anti-GAPDH (lot #067M4785V, dilution 1:5,000): Sigma, G8795.
- Rabbit anti-ATP13A2 antibodies (lot #0000102992, dilution 1:1,000): Sigma, A3361.
- Rabbit anti-ATP13A3 antibody (lot # 000035781, dilution 1:2,000): Atlas Antibodies, HPA029471.

- 0.25% Trypsin-EDTA: Gibco, 25200056
- Dulbecco's Phosphate Buffered saline modified without calcium chloride and magnesium chloride (DPBS): Gibco, D8537
- Micro-BCA Protein Assay Kit: Pierce BCA Protein Assay Kit, Thermo Scientific, 23225
- NuPAGE LDS sample buffer: Invitrogen, NP0007
- Ponceau staining: Sigma, P7170
- Pre-cast 4-12% Bis-Tris gels: Invitrogen, NP0321BOX
- PVDF membranes: Thermo Scientific, 88518
- RIPA Lysis and Extraction Buffer: Invitrogen: 89900
- SIGMAFAST Protease Inhibitor Cocktail Tablets, EDTA-Free: Sigma: S8830
- SuperSignal West Pico PLUS chemiluminescent Substrate: Thermo Scientific, 34095

Harvesting cells

1 collect the cells by scrapping them with a scrapper in Dulbecco's Phosphate Buffered saline modified without calcium chloride and magnesium chloride (DPBS) or, using 0.25% Trypsin-EDTA for which stop enzymatic reaction by adding culture medium.

2 Centrifuge cell suspensions at 450 g, 4°C for 00:05:00. 5m

3 Resuspend cell pellets with DPBS and centrifuge following the same indications as in **2**. Repeat once.

4 Discard supernatants and keep cell pellets on ice.

Cell lysis and protein concentration determination

5 Resuspend cell pellets in RIPA buffer (RIPA Lysis and Extraction Buffer supplemented with protease cocktail inhibitors.

6 Vortex 00:00:30 and keep on ice for 00:30:00. 30m 30s

7 Centrifuge at 20,000g, 4°C for 00:30:00. 30m

8 Keep supernatants on ice to proceed with protein concentration determination using the micro-BCA Protein Assay Kit.

SDS-PAGE 7h 10m

9 Loading

9.1 Mix 20 µg of protein with NuPAGE LDS sample buffer and 5% β-mercaptoethanol final.

9.2 Load protein on pre-cast 4-12% Bis-Tris gels. Include at least one lane with a protein ladder.

10 Running

10.1 Run for 00:10:00 at 100V and 01:30:00 at 110-130V. 1h 40m

11 Transfer

11.1 Transfer onto PVDF membranes using a liquid transfer and following settings: 100V, 01:15:00, 4°C. 1h 15m

11.2 Scan membrane if necessary.

12 Blocking

12.1 01:00:00 Block membranes with blocking buffer (5% milk powder in 1X TBS and 0.1% Tween20 (REF)) for 01:00:00 at room temperature, 19 rpm. 2h

13 Primary antibodies

13.1 Incubate membrane with primary antibodies in solution (1% bovine serum albumin in 1X TBS-Tween20 (TBS-T) buffer), Overnight at 4°C, 19 rpm. 1h

13.2 Wash membrane three times for 00:05:00 in TBS-T, 19 rpm. 5m

14 Secondary antibodies

14.1 Incubate membrane with peroxidase-conjugated secondary antibodies in solution (1% milk powder in 1X TBS-T) for 01:00:00 at room temperature and, 19 rpm. 1h

14.2 Wash membrane five times for 00:05:00 in TBS-T, 19 rpm. 5m

15 Detection

16 Use a chemiluminescence reagent to detect signal and acquire with a Biorad Camera (Vilber Lourmat) and its software (ImageLab).