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Western blotting

 Forked from [Western blotting to detect ATP13A2 and ATP13A3](#)

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Protocol status: Working

We use this protocol and it's working

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ASAP

Abstract

Protocol to detect proteins via Western Blotting.

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).