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Western blot protocol for detecting ATP10B in mouse/rat brain

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

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Abstract

Protocol for detection of ATP10B in rat and mouse brain tissue by Western blotting

Materials

Recipe for 100 mL 1x RIPA buffer :

- 5 mL Tris pH 7.4
- 1 mL Triton x100
- 1 g Deoxycholate
- 3 mL 5M NaCl
- 200 µL 0.5M EDTA (pH 8.0)
- 1 mL 10% SDS

Add water until 100 mL (MilliQ water)
Add protease and phosphatase inhibitors (Pi + PPi) right before use

Reagents used during Western blotting :

- 6X SDS buffer
- PageRuler™ Plus Prestained Protein Ladder
- 3-8% NuPAGE™ Tris-Acetate gel
- NuPAGE™ Tris-Acetate SDS Running Buffer
- Trans-Blot® TurboTM PVDF Membrane
- 5% milk powder dissolved in PBS-T 0.1% (PBS + 0.1% Triton-X100)
- ATP10B HPA034574 (Sigma) primary antibody
- Goat anti-rabbit HRP secondary antibody (Dako)
- ECL Prime chemiluminescence kit (GE Healthcare)

Recipe for 50 mL 6x SDS buffer (160 mM TrisHCl pH 6.8, 2% SDS, 200 mM DTT, 40% glycerol, bromophenol blue):

- 5 mL 1.6M TrisHCl pH 6.8
- 1 g SDS
- 1.54 g DTT

Add water until 25 mL (MilliQ water) and dissolve

- 20 mL glycerol

Add water until 50 mL (MilliQ water)
Add spatula tip of bromophenol blue

Before start

Perform protein extraction from snap-frozen brain tissue:

- weigh tissue
- add RIPA buffer (see Materials) : 10 X of the weight (40 mg = 400 µL)
- homogenize samples using sample homogenizer
- sonicate samples at 4 degrees C, 3 times 15 seconds (keep the samples on ice between each sonication)
- centrifuge samples at 6000 g for 10 minutes at 4 degrees C
- collect supernatant and measure protein concentration
- aliquot protein extracts and store at -20 degrees C

Day 1

4h 5m

1 Prepare samples for Western blotting : 30m
- 30 µg proteins in 12 µL of volume (adjust with milliQ water)
- 2.4 µL of 6X SDS buffer + 10% of β-mercaptoethanol

2 Vortex samples and spin down

3 Boil the samples for 00:10:00 at 98 °C 10m

4 Load samples, together with 7 µL of mass marker (PageRuler™ Plus Prestained Protein Ladder) on a 3-8% NuPAGE™ Tris-Acetate gel. Use NuPAGE™ Tris-Acetate SDS Running Buffer for migration. 30m

5 Run the gel at 80V for 00:10:00 10m

6 Run the gel at 150V for another 00:45:00 45m

7 Transfer the proteins on PVDF membrane (Trans-Blot® TurboTM PVDF Membrane) using Trans-Blot Turbo Transfer System (Bio-Rad), using the pre-programmed protocol STANDARD SD: 00:30:00 , up to 1.0 A, 25 V. 30m

8 Block membranes for 01:00:00 using 5% milk dissolved in PBS-T 0,1% at Room temperature 1h

9 Incubate membranes Overnight in ATP10B HPA034574 (Sigma) diluted 1/500 in 5% milk PBS-T 0,1% at 4 °C 30m

Day 2

3h 10m

10 Wash membranes with PBS-T 0,1% for 10 minutes, 5 times at Room temperature : 50m
- PBS-T 00:10:00
- PBS-T 00:10:00
- PBS-T 00:10:00

- PBS-T 00:10:00
- PBS-T 00:10:00

11 Incubate membranes for 02:00:00 in secondary antibody solution Goat anti-rabbit HRP (Dako) diluted 1/10000 in 5% milk PBS-T 0,1% at Room temperature 2h

12 Wash membranes with PBS-T 0,1% for 10 minutes, 5 times at Room temperature : 50m

- PBS-T 00:10:00
- PBS-T 00:10:00
- PBS-T 00:10:00
- PBS-T 00:10:00
- PBS-T 00:10:00

13 Develop membranes using ECL Prime (GE Healthcare) for at least 00:10:00 10m