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Western blot - alpha-synuclein

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We use this protocol and it's working

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Abstract

This protocol describes how to detect alpha-synuclein in protein derived from STC-1 cells by western blot using DAB/peroxidase or ECL to visualise the bands.

Materials

RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate)

Protease inhibitors (0.8 µM Aprotinin, 40 µM Bestatin, 140 µM E-64 at, Leupeptin at 20 µM and Pepstatin A at 15 µM, 1mM phenylmethanesulfonyl fluoride)

Towbin transfer buffer: 25 mM Tris, 192 mM glycine, pH 8.3, with 20% methanol (vol/vol).
To prepare 1 L of buffer:
- 800 mL distilled H2O
- 200 mL methanol
- 3.03 g Tris base
- 14.4 g Glycine

Sample preparation

1d 4h 16m

1 *Sample preparation using either protein pellet from an extraction (step 1.1)* ***or*** *direct lysis (step 1.2)*

1.1 Dissolve protein pellets from the TRI Reagent® extraction in 2% SDS containing 8 M urea.
Vortex 00:00:30 twice, then orbital shaker 260 rpm 00:30:00
30m 30s

1.2 Dissolve cells in RIPA buffer containing protease inhibitors (see Materials) On ice 00:30:00
30m

2 Mix samples with 4x NuPAGE™ LDS sample buffer containing 50 mM dithiothreitol
Vortex 00:00:20 twice, then orbital shaker 260 rpm 00:15:00
15m 20s

3 Heat samples 70 °C 00:10:00
10m

4 Centrifuge samples 00:00:30 to collect condensation from tube lid. Vortex 00:00:20 then centrifuge again 00:01:00
1m 50s

Electrophoresis and transfer

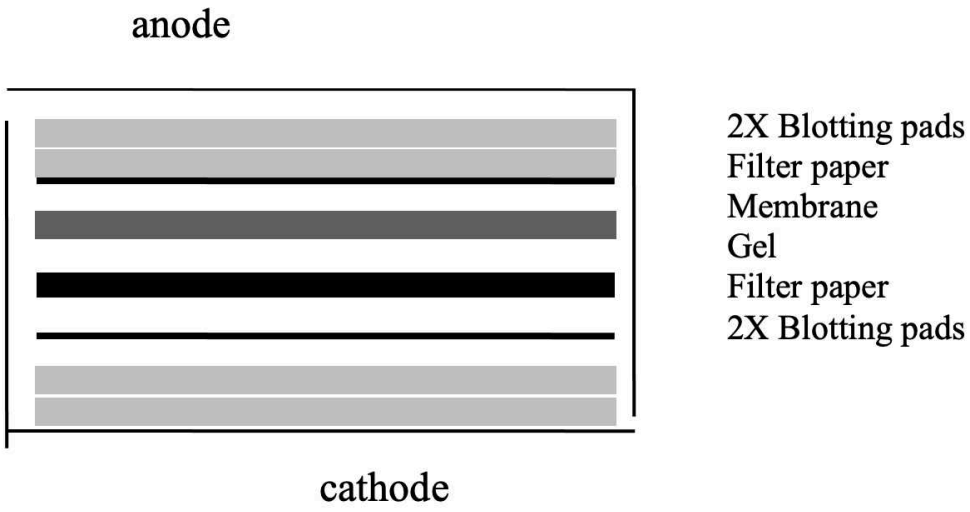
1d 4h 16m

5 Separate samples (~ 10 ug total protein) by polyacrylamide gel electrophoresis using precast Bolt™ or NuPAGE™ 12%, Bis-Tris, 1.0 mm, Mini Protein Gels at 180 V 00:45:00 or until dye front reaches the bottom of the gel. Run with pre-stained or biotinylated size markers
45m

6 Wet PVDF membrane with methanol and then equilibrate in Towbin buffer (see materials)

7 Soak transfer sandwich components (4 sheets of filter paper and 5 blotting pads) in Towbin transfer buffer and assemble in the transfer cassette in the following order:
Starting with the cathode plate
2 x blotting pads
2 x filter paper

gel
PVDF
2 x filter paper
3 x pad (use 3 because there is less chance of the gel slipping)
Use a roller to remove any air bubbles



8 Place cassette in transfer tank and transfer protein onto 0.2 mm PVDF (polyvinylidene difluoride) membranes (30 V, 01:00:00 in Towbin buffer — 1h

9 Rinse membranes with PBS 00:00:30 — 30s

Immunodectection of protein bands — 1d 1h 5m 30s

10 Fix proteins to membrane for 00:20:00 with 4% paraformaldehyde — 20m

11 Wash with PBST (PBS containing 0.01% (v/v) Tween™-20) 4 x 00:05:00 — 5m

12 Block non-specific binding sites with block solution (PBST containing 2% (w/v) BSA and 0.005 % (w/v) thiomersal) for 01:00:00 — 1h

13 Incubate membranes in block solution containing rabbit monoclonal antibody against α-synuclein (1:1250) (ab212184) and β-actin (1:5000) (ab241153) 20:00:00 Room temperature — 20h

14 Wash with PBST 4 x 00:05:00 — 5m

15 *Incubate membrane with secondary antibody in block solution (step 15.1* ***or*** *step 15.2)*

15.1 Incubate membrane with biotinylated goat anti-rabbit IgG (1:1000) (Stratech) 01:15:00 and go to **step 16** — 1h 15m

15.2 **OR** incubate membrane with goat anti-rabbit conjugated to peroxidase (1:25,000) (Stratech) 01:15:00 and go to **step 18** — 1h 15m

16 Wash with PBST 4 x 00:05:00 — 5m

17 incubated with peroxidase conjugated streptavidin (1:1000) (Roche) 00:45:00 — 45m

18 Wash with PBST **4** x 00:05:00 — 5m

19 Wash with PBS **2** x 00:05:00 — 5m

20 *Visualise bands with DAB if biotinylated secondary and streptavidin-peroxidase were used (step 20.1)* ***or*** *use ECL plus (Amersham) if anti-rabbit peroxidase secondary was used (step 20.4)*

20.1 **For DAB detection** incubate in PBS containing 0.05 % (w/v) 3,3′-diaminobenzidine, 0.015 % (v/v) H_2O_2 and 0.05 % (w/v) nickel ammonium sulphate for ≤ 00:00:30 — 30s

20.2 Rinse in running tap water > 00:05:00 — 5m

20.3 Dry membrane 60ºC 1 hour

20.4 **For chemiluminescent detection** use an Amersham ECL plus kit according to the manufacturer's instructions

21 Visualize ECL or digitize DAB stained membrane using a ChemiDoc™ MP imaging system (Biorad)

22 ECL reagents can be washed off the membrane and the membrane then processed for DAB staining if desired