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

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Western Blotting

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ABSTRACT

We got a great result to detect alpha-synuclein and GCase protein using this WB protocol

ATTACHMENTS

Western blot.pdf

PROTOCOL integer ID:
85741

Keywords: ASAPCRN, Western blot

MATERIALS

RIPA-protein lysates
Precision Plus Protein Dual Color Standards
2-Mercaptoethanol
4X Leammli loading buffer (Biorad)
10X Tri Glycine SDS Buffer (Biorad)
4-20% TGX precast gel (Biorad)
Gel tank and power supply
Trans-Blot Turbo Midi or Mini 0.2 um PVDF Transfer Packs
Trans-Blot Turbo Transfer System
Methanol
Paraformaldehyde and glutaraldehyde
Ponceau-S solution (sigma)
Intercept blocking buffer (LICOR)
Anitibody dilution buffer (LICOR)
Skim milk powder
SuperSiganal West Pico PLUS, Peroxide solution (Thermo Fisher Scientific)
TBS or PBS buffer

- TBST (PBST): TBS (TBS) + 0.05% of Tween20

-Antibodies

	A	B	C
	Product	Cat#	Company
	Purified Mouse Anti-α-Synuclein Clone 42/α-Synuclein (RUO)	610787	BD Biosciences
	Anti-Glucocerebrosidase (C-terminal)	G4171	Sigma-Aldrich

1 Prepare loading buffer as a 1:9 ratio of 2-Mercaptoethanol (4 °C) to 4x Leammli sample loading buffer (ie., 50 mL 2-Mercaptoethanol + 450 mL Leammli buffe).

2 Mix samples with loading buffer as a 3:1 raitio to dilute proteins out to be the same protein amount (protein quantification is needed. Usually, protein per each loading is 15-30 µg, mini gel max capacity: 30-35 µL per well, midi gel: 20-30 µL per well)

3 Denature protein by boiling at 99 °C for 00:05:00 in the Thermocycler. 5m

4 Prepare running buffer by diluting out 10X Tri Glycine SDS Buffer (Biorad).

5 Select right concentration of TGX precast gel (Biorad) to run and remove comb from the casing by seesawing under running RO water) until it gently pops out. Remove the bottom strip.

6 Prepare tank and gel inserted in it. Then fill the compartment surrounding the gels and loading area with running buffer. Lift your gel gasket briefly to remove any bubbles at the bottom.

7 Examine your gel to see if any wells are distorted or bent out of shape. If so, take a pipette tip and gently nudge the wells back into place. Remove bubbles from wells.

8 Load your samples and 10 mL of protein ladder. Empty wells get loading buffer.

9 Affix the lid and run at 80 V for 00:10:00. Check to see if the gel is running properly, then increase voltage to 120 V. Run until the loading buffer reaches the bottom of the gel. Approx. time: 1~1.5 hours depending on size of proteins. 10m

10 Pry gel out of plastic cast by separating both sides at the margins. Use a tool to cut out the wells and the peripheries of the gel. Then slightly wash with RO.

11 Open a tray of the Turbo-Blot transfer machine (Biorad). Place the upper layer of the Trans-Blot Turbo mini or midi transfer pack (Biorad) in the tray with upward membrane direction. Remove any bubbles with a roller.

12 Lifting from the denser bottom, place the gel onto the membrane. Place the remaining layer of the Blot membrane to sandwich the gel. Remove any bubbles with a roller.

13 Close the lid and insert the tray back into the Turbo-Blot transfer machine.

14 Turbo -> size of gel (mini, midi, 2 mini)-> A or B -> run. Midi gel: 00:07:00 . 7m

15 Remove from Turbo-Blot transfer machine, cut the membrane down to size, and cut a corner of the membrane to denote its orientation. Use a pencil to mark the membrane.

16 Dry the membrane for 00:30:00 at Room temperature sandwiched between two blotting papers. 1h 10m 20s
PVDF membrane: Reactivate with 100% methanol for 00:00:20 , then wash with TBS or PBS for 5 min (alpha synuclein antibody: instead of drying and MeOH activation, incubate the membrane in fixation solution (4% paraformaldehyde + 0.01% glutaraldehyde) for 00:30:00 . after fixation, washing with TBS or PBS for 00:10:00 three times)

17 (optional) After rinsing with RO water, Stain membrane by pouring Ponceau S solution covering the membrane and incubate for 00:05:00 . Bands of protein should become visible. Pour the solution back into its original bottle, and rinse with RO water three times until the background 15m

stain disappears. After imaging, wash with TBS or PBS for 00:10:00 .

18 Block the membrane in 50% Intercept blocking buffer (LI-COR, dilute with 1X TBS or PBS) with [M] 2.5 Mass / % volume skim milk for 02:00:00 by shaking incubation 2h

19 Prepare your 1°antibody solution by diluting your 1°antibody in its required dilution (42/α-Synuclein
BD science: 1000X) in Intercept antibody diluent solution(LI-COR or the same Intercept blocking buffer containing 0.2% tween 20) with the same composition used in blocking solution.

20 Discard the blocking solution from your membrane and add the antibody solution. Incubate in the cold room Overnight on a shaker. 2h

21 The next day, either discard the 1°antibody solution or save it by pouring it into a tube (stable for two weeks)(unless it is milk, in which you should discard it before it spoils).

22 Wash three times for 00:10:00 each with wash buffer (TBST or PBST). 10m

23 Create 2° antibody solution by diluting 2° antibody of your choice and of corresponding species with Intercept antibody diluent with the same composition used in blocking solution, incubating for no more than 01:00:00 at Room temperature on the shaker. (2° antibody dilutions are generally 1:10,000. 1h

24 Wash three times for 00:10:00 each with wash buffer (TBST or PBST). 10m

25 Stain the membrane with ECL solution (SuperSiganal West Pico PLUS, Peroxide solution : enhanced solution = 1:1) for 00:05:00 and take image with Chemidoc MP (Biorad) 5m