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Western Blotting

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Protocol status: Working

We use this protocol and it's working

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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](#)

187KB

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
SuperSignal 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

Troubleshooting



- 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 buffer).
- 2 Mix samples with loading buffer as a 3:1 ratio 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 stain disappears. After imaging, wash with TBS or PBS for ⌚ 00:10:00 . 15m
- 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 (SuperSignal West Pico PLUS, Peroxide solution : enhanced solution = 1:1) for 00:05:00 and take image with Chemidoc MP (Biorad) 5m