Western Blot Protocol

1. Prepare loading buffer as a 1:9 ratio of 2-Mercaptoethanol (4°C) to 4x Leammli sample loading buffer (ie., 50 μL 2-Mercaptoethanol + 450 μL 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, 15-30 ug protein per each loading, mini gel max capacity: 30-35μL per well, midi gel: 20-30μL per well)
3. Denature protein by boiling at 99°C for 5 minutes in the Thermocycler.
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 μl of protein ladder. Empty wells get loading buffer.
9. Affix the lid and run at 80 V for 10 minutes. 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.
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: 7 minutes.
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 30 min at RT sandwiched between two blotting papers.
    PVDF membrane.: Reactivate with 100% methanol for 20 seconds, 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 30 min. after fixation, washing with TBS or PBS for 10 min three time)
17. (optional) After rinsing with RO water, Stain membrane by pouring Ponceau S solution covering the membrane and incubate for 5 min. 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 10 min.
18. Block the membrane in 50% Intercept blocking buffer (LI-COR, dilute with 1X TBS or PBS) with 2.5% skim milk for 2 hours by shaking incubation
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.
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 3x for 10 minutes each with wash buffer (TBST or PBST).
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 1 hour at RT on the shaker. (2° antibody dilutions are generally 1:10,000.
24. Wash 3x for 10 minutes each with wash buffer (TBST or PBST).
25. Stain the membrane with ECL solution (SuperSiganal West Pico PLUS, Peroxide solution : enhanced solution = 1:1) for 5 min and take image with Chemidoc MP (Biorad)

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