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Western Blot Protocol

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Protein lysis

1 Made lysis buffer.
- 1:100 DF PPI enzyme in RIPA buffer

2 Placed brains on metal tube holder, which is on ice, and added 500µL lysis buffer. Transferred brain and lysis buffer to thehomogenizer, then homogenized with equipment on Minqing's/Tobias's bench.

3 Transferred homogenate back to the original tube. Then, added 500 uL lysis buffer to clean the glass tube and placed that 500µL to the tube with the brain homogenate (1,000µL total). Mixed homogenate by inverting the tubes.

4 Sonicated 3 cycles of 10 pulses while samples are on ice. Between each cycle, mix tube by inverting.

5 Used these samples for the ThermoFisher BCA assay protocol at 1:5 DF, 1:10 DF, and 1:20 DF.

Thermo BCA assay

6 Prepare standards according to the table below using BSA from one ampule in the kit. The diluent should be the same lysis buffer used to prepare protein samples.

Tube	Diluent Volume (µL)	Volume/Source of BSA (µL)	Final [BSA] (µg/mL)
1	0	300 from stock BSA	2000
2	125	375 from stock BSA	1500
3	325	325 from stock BSA	1000
4	175	175 from tube 2	750
5	325	325 from tube 3	500
6	325	325 from tube 5	250
7	325	325 from tube 6	125
8	400	100 from tube 7	25
9	400	0	0

NOTE: I typically will load these standards in the plate in reverse order.

7 Calculate reagent A and reagent B volumes according to equations below.

Reagent A volume = (# of standards + # of samples) x (# of replicates) x (200µL)

Reagent B volume = (Reagent A volume)/50

8 Mix reagent A with reagent B and vortex to make BCA working reagent.

9 Add 2.5 µL sample + 22.5 µL lysis buffer or 25 µL standard to individual wells of a 96 well flat-bottom plate.

10 Add 200 µL BCA working reagent to each well.

11 Place lid on plate and mix for 30 seconds using plate reader.

12 Incubate at 37C for 30 minutes.

13 Read absorbances using plate reader.

14 Calculate concentrations using BCA Assay spreadsheet.

Western blot (day 1)

15 Boil proteins for 10 minutes at 95°C, 65°C, or RT.Cool to room temperature.
- The boiling temperature is dependent on the type of protein.

16 Spin down samples in microcentrifuge.

17 Assemble vertical gel apparatus.
Remove gel from package and rinse with distilled water.
Remove green strip from bottom of gel.
Gently remove comb. Straighten wells, if necessary.

18 Pour running buffer over wells of gel to rinse out storage buffer. Make sure internal chamber is completely full.
- Running buffer = 400mL dH_20 + 100mL 5X Running Buffer

19 Add 5µL Precision Plus Protein Kaleidoscope Prestained Protein Standard to appropriate wells.
NOTE: If using a small number of samples, avoid edge wells and load protein standard on both sides of experimental samples.

20 Load gel slowly using gel-loading tips.

21 Run gel at 100V for 10 minutes.

22 Increase voltage to 200V and run until dye front runs off of gel. (~20-30 min)

23 Prepare blotting items. Rinse pads in dH_2O. Equilibrate all transfer components in transfer buffer. Equilibrate nitrocellulose membrane in sterile dH_2O first and then transfer buffer.
- Transfer buffer = 200mL Methanol + 200mL 5X Transfer buffer + 600mL dH_20

24 Place clear side of transfer apparatus in reservoir of transfer buffer.

25 Stack transfer items in the following order in transfer buffer:
i. Clear side
ii. 1 sponge pad
iii. 2 pieces of filter paper
iv. Nitrocellulose (notch at top right)
v. Gel (lane 1 on left)
vi. 2 pieces of filter paper
    1. Roll in all directions to remove bubbles
vii. Sponge pad
viii. Black side

26 Clamp shut and place in unit with black to black.

27 Place transfer apparatus in tank.

28 Add ice pack to the tank and fill to thevery top with transfer buffer.

29 Place tank in secondary container and surround with ice.

30 Running conditions…
i. For proteins <200kDa, transfer at 100V for 1 hour in the cold room.
ii. For proteins >200kDa, transfer at 20V overnight in the cold room.
** Minqing's suggestion: run the transfer for 2h at 110V.

31 Disassemble transfer apparatus and trim/cut membrane.

32 Place membrane in blocking buffer for 1 hour at RT on orbital shaker.
- Block buffer = 5% dry milk in TBST
- TBST = 500µL 10% Tween-20 + 100mL 10X TBS + fill up to 1,000mL dH_20

33 Aspirate or pour off blocking buffer.

34 Incubate overnight in blocking buffer + primary antibody at appropriated dilution at 4°C (cold room) on an orbital shaker.

Western blot Continued (day 2)

35 Aspirate and save antibody solution.
i. Can reuse antibody 3-5 times but must store in final concentration 0.02% sodium azide to prevent fungal contamination.
ii. Menglong also told me that you can freeze the antibody blots at -20C. However, do no use it more than 5 times.

36 Quickly wash 3 times with 1X TBST, pouring off immediately.

37 Wash 3 times for 15 minutes each at RT with 1X TBST on orbital shaker.

38 Add appropriate secondary Ab diluted in blocking buffer and incubate at RT while rocking for 1-2 hours.
a. Use the buffer made by the company (Odyssey TBS-based blocking buffer, which is located in the deli fridge by Tobias's/Minqing's lab bench)
b. ***This and all remaining steps should be done in the dark to avoid photobleaching.***

39 Quickly wash 3 times with 1X TBST, pouring off immediately.

40 Wash 3 times for 15 minutes at RT with 1X TBST while shaking.

41 Image membrane with Odyssey CLx Infrared Imager.