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Western blotting

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We use this protocol and it's working

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Abstract

Sampson/Caudle Lab Western blot protocol.

Troubleshooting

Sample Prep & Running the Gel

1 Sample preparation:
From your protein assay, determine amount of sample to be used to obtain a loading concentration of 20 µg total protein per lane.

In a separate tube for each sample, add the amount of protein from sample tube and appropriate amount of buffer to give a volume of 20 µl. Add 6.6 µl of 4X loading dye containing DTT. Total volume should be 26.6 µl. Vortex and let stand at room temperature while you set up your gel box.

2 Wearing gloves, open the plastic around the gel, remove the white tape from the bottom and dispose of packaging in hazardous waste.

3 Remove the plastic comb from the lanes and rinse the cassette and lanes with water.

3.1 Label the gel cassette if needed.

4 Insert the electrode plate into the gel box. If running one gel, place the gel cassette in front of the electrode plate with the low side of the cassette facing inward. Use a filler plate from the western blot drawer as a place holder on the back side of the electrode plate. If running two gels, place the second gel on the back side of the electrode plate, also with its lower side facing inward.

5 Secure gels in place using the gray lever insert.

6 Fill the chamber of the gel box with 1X Running Buffer until all of the wells are covered and the outside chamber is filled to the black line.

7 Using gel loading pipet tips, fill one lane with ladder (5 µL, stored in -20°C freezer). This will allow you to measure the molecular weight of your protein of interest.

8 Fill the rest of the lanes with your premixed samples - 20 uL. Be sure to vortex samples well before loading.

9 Once all samples are loaded, attach the top of the gel box, making sure that the electrodes are properly in contact with the lid.

10 Plug lid into the power supply on the western station (red to red, black to black).

11 Turn power supply on and run the gel at 150 V for 60-70 min. Check on your gel periodically to see how far the samples have run to ensure separation of bands at the molecular weight of your protein of interest. You will know the gel is running when you see a wall of bubbles rising from the wire on the electrode plate.

Gel Transfer

12 Place 6-8 blotting sponges into the bottom of a glass Pyrex dish and pour 50-100 mL of transfer buffer onto the sponges to fully soak them. Remove any air bubbles in the sponges with the white roller at the western station.

13 Place two sheets of filter paper into the transfer buffer as well.

14 Place pre-cut PVDF membrane into a small dish of methanol and let it shake for 1 min. The methanol step activates the membrane and allows proteins to stick to it. DO NOT SKIP THIS STEP.

15 After the gel has finished running, shut off the power supply and disassemble the gel box. Take out the gel cassette.

16 Using a spatula, crack open the gel cassette.

17 Keep the gel stuck to the taller side of the gel cassette and throw away the short side.

18 Cut off the well tabs at the top of the gel with a spatula and throw them away.

19 Take one sheet of the soaked filter paper and place it against the gel.

20 Flip the cassette over.

21 Push the spatula through the slit at the bottom of the cassette (where the white tape used to be). This will push the gel onto your filter paper.

22 Build the transfer sandwich in the transfer box. All pieces of the sandwich should be thoroughly soaked with transfer buffer and rolled with the white roller to remove air bubbles. The order you should arrange the transfer stack in is:

**BOTTOM/back of holder
2-4 sponges
Filter paper
Gel
PVDF Membrane (activated with methanol)
Filter paper
2-4 sponges
**TOP

23 Roll with white roller between layers as you arrange the stack to remove excess bubbles. Push together both sides of the box (liquid should run out as you squeeze the top and bottom together) and place into the gel box for the transfer.

24 Once the transfer box is securely in the gel box with the metal pieces touching, fill the center compartment (where your sandwich is) with transfer buffer.

25 Fill the outside compartment with transfer buffer for cooling to 1-2 inches from the top of the chamber.

26 Attach the box lid, plug electrodes into the power supply and transfer at 10 V overnight (in the cold room) OR at 30V for 90 minutes (room temp – edit: better results if you transfer in cold room).

Blocking

27 Make 5% BSA solution

28 After the transfer has finished, shut off the power supply.

29 Remove the membrane from the transfer box and place in a small tray.

30 Pour blocking solution (5% BSA in TBST) over it and shake for 1 hour at room temperature

31 Throw gel away in biohazardous waste.

32 Optional: You can stain your gel with Coomassie staining for protein loading and transfer efficiency. Alternatively, you can stain your membrane with Ponceau S staining (red) to check for transfer.

Primary Antibody Incubation

33 In a 15mL conical, make 10 mL of primary solution using 5% BSA in TBST (can use same liquid from blocking buffer)

34 Common Primary Dilutions:
Millipore Rat anti-DAT 1:1,000 (10 uL in 10 mL)
Millipore Rabbit anti-TH 1:1,000 (10 uL in 10 mL)
Sigma Mouse anti-actin 1:5,000 (2 uL in 10 mL)
Rabbit anti –VMAT2 (polyclonal) 1:5,000
Rabbit anti-SV2C in glycerol 1:1,000 (1:2500 if straight serum)

35 Vortex well.

36 Place the blot into a plastic bag or container. Pour primary solution into the bag/container until membrane is fully covered.

37 Incubate on the shaker in the 4°C fridge overnight.

Secondary Antibody Incubation

38 Remove blot from the container and perform 3 washes of 1X TBST for 10 min each (3 × 10 min), at room temperature.

39 Make secondary antibody. In 50 mL conical, mix 50 mL of 1X TTBS and secondary antibody. Secondary antibody is commonly used at 1:1000, 1:5,000 or 1:10,000. Can be reused ~10 times if stored in 4C.

40 Secondaries stored in -20∗C freezer with primary antibodies
NOTE: Secondary antibody should be made against the host animal for the primary.

41 Vortex well.

42 Pour off final wash and incubate blot in secondary solution for 1-2 hours at room temperature with shaking.

43 After secondary incubation, rinse 3 × 10 min in 1X TBST at room temperature with shaking. If you're having trouble with dirty blots, leave membrane in final TBST rinse for 30+mins.

Imaging

44 After the final wash, transfer blot to a clean tip lid. Mix 500 µL of each SuperSignal™ West Femto Maximum Sensitivity Substrate reagent into the dish.
NOTE: These reagents react with each other, so you must switch tips when switching between the bottles.

45 Pipet repeatedly over the membrane for 2 min.

46 Place blot into plastic bag and image on the Azure Biosystems imager.

47 Save all images to the data drive.

48 CLEAN THE GLASS SURFACE IN THE IMAGER with DI water and a kimwipe after you're done.

Protein Quantification

49 Drag Western Blot jpg file in Image J

50 Click on 'Image' tab → 'Transform' → 'Rotate' and adjust image to make sure the lanes are horizontal.

51 Click on the 'rectangle tool' and select the bands you want to analyze.

52 Click on the 'Analyze' tab →'Gels' → 'Select First Lane' (or Control 1) → 'Plot Lanes' (or Control 3).

53 Click on ‘Straight’ tool to divide the peaks. Draw lines to divide peaks. Hold ‘Shift’ while drawing lines for a straight line.

54 Click on ‘Wand (tracing) tool’ and select each individual peak. A new window should pop up for the area under each peak.

55 Once you have all the areas (ID/integrated densities) for each peak (band). Copy and paste the values into excel corresponding to their ID from the gel layout.

56 Do the same for the control protein.

57 Then divide the ID of your target protein/ID ctl.

58 Graph and do stats on these values in GraphPad.