Protein extraction and Western Blot*

Protocol graciously provided by the Institution for Clinical Science, Department of Ophthalmology, Lund University.

Pre-preparation:

Tris-Buffered Saline (TBS) pH 7.6
Mix 4.84gTRIS BASE (20mM),
16 g NaCl,
10 mL HCL (1M),
and add Millipore-H20 to 2L.

Tris-Buffered Saline Tween (TBS-T)
1 L TBS + Tween 1 ml.

Non-Fat Dry Milk (NFDM)
100 mL TBST and add 5 g of Non-fat dried milk (NFDM).

Running Buffer
Add 50 µL of NuPage stock with distilled water to 1 L.

Running Buffer + Anti-oxidants
200 µL of Running Buffer with 50 µL anti-oxidants.

Procedure for Lysis of Scaffold-cultured Mammalian Cells

1. Carefully remove decant culture medium from adherent wells.
2. Wash all cells twice with cold PBS.
3. Add ice-cold RIPA buffer (with inhibitors) to the cell wells. Use ~600 µL of buffer per well (12 well plates), it is important that the fibers are covered in buffer.
4. Keep on ice for 20 minutes, swirling the plate occasionally for uniform spreading.
5. Collect the lysate and transfer to a cold microcentrifuge tube. Centrifuge samples at 4◦C at 14 000 x g for 15 minutes to pellet the cell debris.
   Note: To increase yield, sonicate the pellet for 30 seconds with 50% pulse.
6. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice.

*Suggested procedure, please adjust according to your experimental needs.

Bradford, 5.2 Microplate Assay Protocol

Preparation of working reagent.

1. Mix 20 µL of reagent S and 1 mL of reagent A in an eppendorf tube (working solution).
2. Prepare 7 Eppendorf tubes with 1, 2,3,4,6,8,10 µg/µL BSA in dH2O. (standard solution).
3. Add 5 µL of standard solution to each of the 96 well plate (3 wells/eppendorf and 3 for the cell sample.
4. Add three wells with only RIPA buffer.
5. Add 25 µL of working solution to each well.
6. Add 200 µL of reagent B to each well and put on shaker for 15 min. Cover the well plate with aluminium foil.
7. Run the well plate in the Spectrophotometer. Measure absorbance in the spectrophotometer and provide the range of the excitation and emission wavelength.

Preparation

1. Turn on water bath to 95˚C.
2. Put samples on ice. Fill in experiment protocol.
3. Calculate the volume of protein you want to load (ex. 15 µg). Use loading buffer (4x laemmlie) to dilute the samples. Each well for the electrophoresis need 15 µL.
4. Mix and centrifuge samples, place tubes in floating holder and heat in water bath 95˚C for 5 minutes. Remove from bath (turn off) and put samples on ice. When cool, centrifuge again. If samples are on ice, mix and vortex before loading gel.

*Suggested procedure, please adjust according to your experimental needs.

Running gel

1. Prepare 1 x Running Buffer (NuPAGE). Mix 50 mL stock with distilled water to make 1 L total.

2. Prepare running chamber (XCell SureLock, Invitrogen).
    a) Open gel pouch (refrigerator, Novex NuPAGE) and remove cassette. Rinse with distilled water.
    b) Peel off tape covering slot on cassette and remove comb.
    c) Gently flush wells with running buffer twice.
    d) Fill wells with running buffer- Be careful and avoid trapping bubbles.
    e) Lower Buffer Core into holder so that negative electrode fits into slot.
    f) Insert tension wedge behind Buffer Core- unlocked.
    g) Insert gel cassettes (if only one, use Buffer Dam in place of rear gel). Well side (shorter wall) should face in towards electrode wire.
    h) Pull tension wedge lever towards front. See pages 5-7 of SureLock manual for pictures.

3. Mix 200 mL of running buffer with 500 µL antioxidant. Fill inner core chamber (small chamber) with small amount of this buffer and check to be sure it is not leaking. If not, continue to fill core so that surface is well above top of wells.

4. Load samples according to experiment protocol (15µL/well). For ladder, load 5 µL of Page Ruler (in WB Freezer) in one well. Note on protocol if something goes wrong.

5. Fill outer chamber with 600 mL running buffer (no antioxidant). Secure lid.

6. Plug electrodes into power supply (Biorad Power Pack 200), pushing firmly to ensure contact. Turn on power supply and run gel at 150 V (constant) for 90-100 minutes. Make sure it is running by checking for small bubbles from electrode wire in inner chamber.

7. Make sure you have TBS-T and blocking buffer.

8. When done (colored front should be near edge or have just run out), shut off power supply and remove lid.

*Suggested procedure, please adjust according to your experimental needs.

Blotting

9. Open Invitrogen iBlot device. Using gloves, take one anode stack, one cathode stack, one sponge and one filter paper from boxes (Invitrogen/Novex, WB lab, shelf above iBlot device). Put filter paper in tray with destilled water.

10. See iBlot instruction sheet if you need pictures. Unseal anode stack, keep in plastic tray. Place stack (with tray) on blot device. Align with Gel Barriers on right. Copper strip will point to the left.

11 Using gloves, remove gels from chamber. Use gel knife (Invitrogen, black handle) to pry plates of one gel apart, being careful when removing top plate (short side) so that gel stays on bottom plate.

12. Cut off wells and bulge at bottom and throw away in acrylamide waste.

13. Remove gel from electrophoresis plate by placing it in a tray with running buffer. Ease off of plate and place gel on top of anode stack, starting with one side and easing down while remaining centered and not introducing bubbles.

14. Place the wet filter paper on top of gel, run roller over to remove bubbles.

15. Unseal cathode stack, throw plastic tray away. Place stack on top of filter paper so that electrode side (copper mesh) is facing up.

16. Make sure all sides are aligned, remove bubbles with roller.

17. Place sponge in lid of device with the metal contact on the upper right corner.

18. Close lid, secure latch and check for red light which confirms that the circuit is closed. Set for program 3 and press Start button.

19. When done, press Stop button. Using gloves, open lid and carefully lift stack to be sure protein has transferred to membrane under gel (no color left in gel). Discard sponge. Remove top half of stack and discard gel in acrylamide waste.

20. Lift membrane and clip off upper right hand corner, so you know where the beginning is, and cut to several pieces if necessary. Wet in bath with TBS-T so it doesn't dry out while handling. Place in 50 mL tube, being careful to not let membrane overlap itself.

21. Fill with 10 mL TBS-T, seal tube and run on roller for 10 minutes.

22. If you have 2 gels, blot the second one now (step 15).

23. Pour out TBS-T and fill with 10 mL blocking buffer (5 % dry milk in TBS-T). Seal tube and run on roller for 2-2½ hours.

*Suggested procedure, please adjust according to your experimental needs.

Incubation and development

24. Add primary antibody directly to 5 mL blocking buffer (e.g. Nestin #801, Tubulin #705, Synaptophysin #804, GADPH #803, GFAP #287).

25. Seal tube and run on roller in cold room overnight.

26. Book Developing machine.

27. Discard antibody solution. Rinse 3x15 minutes with TBS-T (10-20 mL) at room temperature.

28. Remove last rinse, add at least 4 mL of secondary antibody and blocking solution (NFDM solution). Run on roller for 1 hour.
    Anti-rabbit: #HAF008, dilute 1:2000
    Goat Anti-mouse: #741, dilute 1:40 000

29. Discard antibody solution. Rinse 3 x 15 minutes with TBS-T (10-20 mL) at room temperature.

30. Check to be sure developing machine is available. Just prior to development, with lights off, mix Millipore Immobilon Western chemiluminescent HRP substrate: 1 mL of solution A with 1 mL of solution B. Cover tube with aluminium foil.

31. Take tube with membrane, 2 disposable transfer pipettes and the chemiluminescent solution to the developing machine. Open drawer of machine and roll membrane onto surface. Use pipette to add a few drops of TBS-T from tube under membrane so that no bubbles are trapped under it. Make sure membrane is centered on surface.

32. Remove excess TBS-T if any. Use second pipette to drip chemiluminescent solution on top of membrane. Use side of pipette to make sure it is evenly distributed on the membrane and do not let the solution run off the sides. Close drawer.

33. Log onto computer and open program- .

34. Select new protocol, choose “chemiluminescent”.

35. Select membrane size. Set exposure time: take the longest first (600 seconds).

36. Click to see membrane, change zoom to fit whole membrane.

37. Click run. Adjust exposure time as needed, run again.

38. Save pictures to USB, select “export” and choose picture format.

39. Log off, put membrane back in tube, clean machine surface.

40. Run on roller 10 minutes, then remove, place in plastic wrap, label and store in refrigerator.

*Suggested procedure, please adjust according to your experimental needs.

Stripping protocol

1. Put the membrane in stripping buffer 4 mL/membrane in a 50 mL tube.
2. Incubate membrane for 5-10 min in room temperature on a roller.
3. Discard the buffer and refill with 4ml stripping buffer on roller for 10 min.
4. Wash with 5-10 mL PBS for 2 x 10 minutes on a roller.
5. Wash with 10 mL TBS-T for 2 x 5min on roller.
6. Incubate with 20 mL blocking buffer (TBS-T + drymilk) for 2 h or overnight.
7. Continue with blocking protocol.

*Suggested procedure, please adjust according to your experimental needs.