Western blotting

This protocol may be used to perform Western blot (protein immunoblot) to detect specific proteins.

Keywords: protein electrophoresis blot

Expand

Reagents, supplies, and equipment:

Buffers

1. Tris-Glycine electrophoresis buffer:

   0.25 M Tris
   1.92 M glycine
   1% SDS

   5X stock:
   15.1 g Tirs
   72 g glycine
   50 ml 10% SDS
   q.s. 1 L H_2O

2. 1X Electrophoresis buffer - prepare fresh

   100 ml 5X stock
   400 ml DI H_2O

3. 4X SDS gel-loading buffer w/o DTT (aliquots stored at -20°C)

   25 mM Tris-HCl (pH 6.8 at 25°C)
   8% w/v SDS
   40% glycerol
   0.04% w/v bromophenol blue or phenol red

   For 100 ml:
   Add 25 ml 1M Tris HCl to 40ml glycerol and stir
   While stirring, add 8 g SDS until clear
   Add 10 ml 0.4% bromophenol blue (0.4% w/v bromophenol blue stir O/N and filter)
   q.s. with DI H_2O

4. 10X transfer buffer w/o methanol (RT)

   30.3 g (0.25 M) Tris base
   144 g (1.92 M) glycine
   q.s. 1 L DDI H_2O

5. 1X transfer buffer w/ methanol (may be used twice; store at 4°C)

   100 ml 10X transfer buffer
   800 ml DIH_2O
   200 ml methanol

6. 10X TBS**:** (stable at 4°C for several months)

   24.2 g Tris base (100mM)
   80 g NaCl (0.9%)
   Adjust pH to 7.6 with HCl
   q.s. 1 L with DDI H_2O
   (Add 1 ml Tween-20 to 1 L 1xTBS as working solution store at 4°C ≤1week)

7. Blocking buffer: 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk

   Mix 10ml 10X TBS in 90 ml water
   Add 7.5 g nonfat dry milk (Bio-Radad: #170-6404) and mix well.
   While stirring, add 0.15 ml Tween-20

8. Antibody dilution buffer: X TBS, 0.1% Tween-20 with 5% BSA

   (For some antibodies indicated in vendor manual)
   Mix 2 ml 10X TBS to 18 ml water
   Add 1.0 g BSA and mix well
   While stirring, add 20 µl Tween-20 (100%)

9. Ponceau S solution (Sigma #P7170)

10. DTT reducing buffer (Invitrogen #NP0004)

Protein Standards

1. Pre-stain dual color protein standard (Bio-Rad: #161-0374, store at -20°C)
2. Biotin linked ladder detection pack (Cell signal: #7727 store at-20°C)10X

Gel: 4-12% Tris-HCL Ready Gel 10 well (Biorad #1611105)

Apparatus: Bio-Rad MP3 (Run at 100V 1h 15 min)

Other equipment/tools:

1. Extra-thin loading pipette tips
2. Ice bucket
3. PCR tubes
4. 3MM Whatman paper
5. Sponges
6. Paper-cutter
7. Trans-Blot transfer medium pure nitrocellulose membrane 0.45 µm. (Biorad: #162-0145)
8. Flat head forceps
9. Scissors
10. Plastic tank
11. Western lightening chemiluminescence reagent plus (Pierce #NEL103)
12. Film (Kodak, #NEF586 Blue XB-1)
13. Restore Western blot stripping buffer (Pierce #021059)
14. PCR machine and microcentrifuge

Procedure

1. Prepare 500 ml 1X electrophoresis buffer in H_2O and ice bucket. Turn on PCR machine to pre-warm.
2. Aliquot protein samples to PCR tubes (~25 µl/sample), add 1/3 volume of loading buffer and 1/10 volume of DTT; mix by pipetting up and down. Keep on ice! Include a negative control (if necessary) and two protein ladders (pre-stained and biotin linked).

3. Heat the samples and the biotin linked protein ladder to 95 - 100°C in PCR machine for 5 min. Do NOT heat the prestained ladder.
4. Immediately remove tubes from the PCR machine and place on ice for 3 min. Caution - This is HOT! Place gloved hands over the lids of tubes to prevent them from popping off.
5. Microcentrifuge samples at 12,000 rpm, at 4°C for 5 min.
6. Set up the gel and tank system while the centrifuge is spinning. (Slowly remove comb from gel).
7. Slowly load 10 - 20 µl of sample onto gel using extra thin pipette tips.
8. Run at 100 V for 1.5 hr or until the loading dye reaches the end of the gel.
9. While the gel is running, prepare the prewet membrane as follows:
   a. Prepare 1X transfer buffer
   b. Remove the precut nitrocellulose membrane and cut 6 sheets (3 for each side) of 3 MM Whatman filter paper to the same size as the precut membrane (usually 3" x 4").
   c. Soak the transfer membrane, filter paper, and sponges in the transfer buffer at least 30 min before gel is finished. There are 2 sponges, 6 pieces of filter paper, and 1 nitrocellulose membrane per gel. Use forceps to manipulate everything into the transfer buffer.
10. Just prior to completion of the gel run, prepare a plastic box by pouring in enough 1X transfer buffer to cover gel.
11. When gel is finished:
    a. Cut a corner on the bottom right of gel. Also remove the top wells.
    b. Transfer gel to plastic box and soak gel in transfer buffer for 30 min. Keep transfer buffer on ice!
12. Place the tank for transfer in a plastic tub and pack with ice.
13. Take transfer apparatus to the cold room and perform the following:
    a. Pour all of available transfer buffer into the tank (including all the buffer used previously).
    b. Assemble "sandwich" for Bio-Rad Transblot using the porous plastic sandwich apparatus. (membrane size should be 1 - 2 mm larger than gel). The black part of the sandwich apparatus faces the cathode (negative) and the white part faces the anode (positive).
    c. Assemble the "sandwich" in the following order: black side of porous plastic sandwich, sponge, 3 pieces of 3MM Whatman filter paper, gel, membrane, 3 pieces of 3MM Whatman filter paper, sponge and white side of porous plastic sandwich. Use forceps to handle everything except the gel. DO NOT LET MEMBRANE DRY.
    d. RRemove bubbles between the sandwich and clamp the gel holder.
    e. Place gel inside the red and black apparatus; black side of the sandwich facing the black part of the apparatus.
    f. Cover the lid and immerse the entire unit in ice, burying the lid.
14. Transfer the Mini-Transblot at 100 V for 1 hr.
15. While transfer is running, prepare 1X TBS with Tween-20 (This buffer may be used multiple times).
16. Upon completion cut the membrane at the same corner as the gel corner cut and rinse the membrane with DI H_2O.
17. Wash membrane in H_2O by shaking for 5 min. Drain H_2O.
18. Pour Ponceau Stain onto membrane:
    a. Stain for 1 min. Pour off the stain (return to tube) and rinse several times with DI H_2O until protein bands are visible.
    b. According to the size of the protein, mark the place you want to split the membrane. Cut the membrane in sections that contain the specific proteins.
    c. Cut the bottom right corners of the membrane sections and place them carefully in size matched boxes (small to small, large to large) containing TBS.
19. Wash with 1X TBS 3 times for 5 min and shake at RT.
20. Incubate in blocking buffer for 1 hr at RT, shaking at 50 rpm.
21. While blocking, prepare the primary antibody at appropriate dilutions in blocking buffer (i.e. GAP-DH at 1:8,000).
22. Incubate membrane in appropriate volume of primary antibody + buffer; gently agitate at 50 rpm O/N, at 4°C (cold room).
23. The next day, wash 3X for 10 min each with TBS/T.
24. While washing, prepare secondary antibodies. Prepare HRP-conjugated secondary antibody (1:10,000, specific antigen detection) in appropriate volume of blocking solution the as well as HRP-conjugated anti-biotin antibody (1:6,000, detection of biotinylated protein marker).
25. Incubate membrane by gently shaking at RT for 1 hr with respective secondary antibody.
26. Wash 3X for 10 min each with TBS/T.
27. While washing, prepare for chemiluminescence as follows:
    a. Open the film cassette.

b. Lay a large piece of plastic wrap on top of the bottom of the intensifying screens and smooth to remove wrinkles.
c. Mix 1 ml oxidizing reagent with 1 ml luminol reagent.

28. Using forceps, arrange the membranes (protein side up) on the plastic wrap (note order).
29. Pipette 2 ml of the mixed reagents onto the membrane surface.
30. Fold the plastic wrap over the membrane to seal and close the cassette.
31. Take the following to the dark room: scissors, forceps, film in black bag, film cassette, pen, and timer.
32. To develop:
    a. Open the film cassette and place film on top of the plastic wrapped membrane.
    b. Conduct multiple exposures ranging between 2 sec to 20 min, depending on the strength of the signals. (Cut film corner for orientation).
    d. Develop film (Small pieces of film will jam the machine; 8 X 10 cm is the minimum size).
33. After development, rinse membrane 2 - 3 times in fresh PBS or TBS solution.
34. To strip and re-blot:
    a. Wash membrane in Western blotting stripping solution, RT for 20 min.
    b. Perform three 5 min washes in 1X TBS.
    c. If necessary, re-stain with Ponceau beginning with step 22 or 23, depending on antibody species.

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