Western Blot Protocol

PromoKine

Materials

- Gel running apparatus
- Transfer apparatus
- Power supply
- SDS-PAGE mini gel
- Nitrocellulose membrane cut to size of gel
- Filter Paper (Whatman) cut to size of gel
- Tweezers
- X-ray film
- X-ray processor
- Gel loading pipette tips
- Pipettor, small volumes

Buffer Formulations

SDS/Running Buffer:
- 25 mM Tris
- 192 mM Glycine
- 0.1% SDS

Transfer Buffer:
- 20 mM Tris
- 150 mM Glycine
- 20% methanol
- 0.038% SDS

Blocking Buffer
- 5% non-fat dry milk
- TBS

Wash Buffer (TBST)
- 125 mM NaCl
- 25 mM Tris pH 8.0
- 0.1% Tween-20

Procedures

Running Protein Samples onto a Gel

1. Cast a mini SDS-PAGE gel per your labs standard protocols or purchase pre-made gels.
2. Clamp the gel to the apparatus with per manufacturer directions. Add running buffer.
3. Remove the comb gently so as to not disturb the wells.
4. Add 6 µL of your select marker to a well.
5. Add 7.5 µL of lysate per well (2 mg/mL for 15µg per lane).
6. Apply the anode and cathode wires to the appropriate poles and cover.
7. Run at 130V for 2 hours (or until the dye front is close to the bottom).

Transfer of Proteins onto Membrane

8. Assemble the “sandwich” for the transfer apparatus per the diagram below.
    a. Note: Handle gel and membrane with tweezers – do not touch!
9. Pre-wet sponges and filter paper in transfer buffer.

a. Note: Filter papers and membrane should be same size as the gel.

10. Insert the “sandwich” and insert into the transfer apparatus, making sure the gel is on the cathode (-) and the membrane is on the anode (+) side of the apparatus.
11. Transfer the proteins to the membrane at 250mA in transfer buffer for 2 hours
12. Remove the cassette from the apparatus, discard the gel, and place membrane on paper towels to dry. (Wash the membrane in 1xTBST to remove any leftover gel.)

Blocking

13. Incubate the blot with blocking buffer overnight at 4˚C or 2 hours at room temperature with gentle agitation.
14. Remove blot from blocking solution.

Primary Antibody Incubation

15. Mix antibody with peptide in a 1:1 ratio (e.g. 1µg antibody and 1µg peptide) to ensure a molar excess of peptide and incubate for 30 minutes at 37˚C.
16. Incubate the blot with the primary antibody for one hour at room temperature or overnight at 4˚C.
17. Wash the blot three (3) times 10 minutes each in washing buffer with gentle agitation.

Secondary Antibody Incubation

18. Dilute 1 µL anti-rabbit IgG-HRP conjugated secondary (or other appropriate secondary) in 10 mL of blocking buffer to make a 1:10000 dilution

    a. Note: working dilution of secondary can vary from 1:2000 to 1:10000.

19. Incubate blot with secondary antibody for one (1) hour at room temperature.
20. Wash three (3) times for 10 minutes each in washing buffer with gentle agitation.

Development

21. Drain wash buffer
22. Add ECL solution (Amersham) per manufacturer directions and develop for 1 minute.
23. Drain the fluid.
24. Cover the blot in plastic wrap.
25. Expose the blot to X-ray film for 1 minute in a dark room.

    b. If there is no banding, expose the film for 5 minutes, then 30 minutes and up to overnight if the signal is weak.

    c. If the signal is strong, expose the film for 30 seconds or less.

26. Develop the film in an X-ray processor.

Notes

27. Optimal dilutions should be determined by each laboratory for each antibody.

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