Immuno-Blotting (Western Blotting): Enhanced Chemiluminescent Detection using Peroxidase Labelled Primary Antibody Conjugate

Solutions Required

- **Electroblot Transfer Buffer:** 0.025M Tris, 0.192M Glycine, 20% (v/v) Methanol, pH 8.3. Add 18.2 g Tris, 86.4 g Glycine, 1200 ml Methanol, up to a total volume of 6 litres.
- **PBS:** 8.0g NaCl and 1.15g Na_2HPO_4, 0.2g of KH_2PO_4, and 0.2g of KCl, up to 1 litre. Adjust pH to 7.4 if necessary.
- **PBS-Tween (0.1% (v/v) Tween 20):** 0.5 ml Tween 20 to 500 ml PBS.
- **PBS-2% (w/v) BSA (Blocker):** 10 g BSA to 500 ml PBS, adjust pH to 7.4 with 1 M NaOH. Freeze in 50 ml tubes.
- **Probing Buffer:** 5% (w/v) Carnation Skim Milk Powder in PBS + 0.1% (w/v) Tween 20. Adjust pH to 6.5 with 1 M Phosphoric Acid. Centrifuge at 3500 X g for 30 minutes just before addition of antibody.
- **Bromophenol Blue:** 0.1% (w/v) Bromophenol blue in water.
- **Amido Black:** 100 mg Amido Black, 45 ml Methanol, 10 ml Acetic Acid, 45 ml water.
- **Destain Solution:** 10% (w/v) Acetic Acid, 25% (w/v) Methanol in water.
- **ECL Western Blotting Detection System:** Amersham #RPN-2106

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Method

A. SDS PAGE:

For precast 10 x 8 cm gels: Prepare samples and load 0.5 ul plasma or 50 ng purified protein per well. Electrophorese until the dye front is at the edge of the gel.

B. Electroblotting onto membrane:

1. When tracking dye has reached bottom of gel, turn off power supply.
2. Hydrate membrane (nitrocellulose or Immobilon-P PVDF) as per manufacturer's instructions. For Immobilon this consists of a 10-second wash in 100% MeOH followed by 60 seconds in distilled water then several minutes in the transfer buffer. The cassette is then assembled with sponge on bottom, followed by 3MM filter paper, gel, membrane, filter paper and top sponge. Each item should be soaked by dipping in transfer buffer prior to assembly of the cassette, and kept wet during the assembly. Do not allow air bubbles to become trapped between the gel and membrane. The cassette is closed and placed in the Transphor unit with the membrane on the cathode side (red) of the gel.
3. Transfer of proteins is performed at 500 mAmps for 1 hour at RT.

C. Probing and detection:

1. Turn off power and remove the cassette from the Transphor unit. Trim the membrane to the same size as gel. Put a small nick in the membrane to mark the point of application of the first sample. Cut off molecular weight standard lane and stain in Amido Black for 5 minutes, and then destain. Place remaining membrane in a plastic dish containing 50 ml of PBS-2% (w/v) BSA (blocking solution).
2. Block membrane for 2 hours at ambient temp., or overnight at 4°C on a shaking platform.
3. Rinse briefly with PBS-Tween.
4. Incubate membrane with 50 ml probing buffer containing peroxidase conjugated antibody for 2 hours at ambient temperature on a rocker. The appropriate dilution for each species should be determined by titration.
5. Wash membrane with PBS-Tween three times, 15 minutes each.
6. Develop as per instructions for the ECL Western Blotting Detection System, starting at step #12 on page #12 in the ECL Instruction Manual. Exposure times using Kodak XAR-5 film is typically between 2 and 20 minutes at room temperature.

Reference

Towbin, H., Gordon, J.: Immunoblotting and Dot Immunobinding - Current Status and Outlook. J. Immunol. Meth., 72:313 (1984).

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