http://www.chemicon.com/techsupp/Protocol/WBMembrane.asp

Suggested Western Blot Protocol: (Membrane bound proteins)

1. Mix samples (1:1) (organ membranes: 50 µg/lane; transfected cells: 500,000 cells/lane) with 2X sample-buffer, and heat 10 min at 70°C. A protocol for organ membrane preparation is available at: http://www.chemicon.com/techsupp/membrane.asp. We always recommend the use of proteinase inhibitors (for instance APMSF, leupeptin (Chemicon Catalog Number EI8), pepstatin A (Chemicon Catalog Number **EI9**), 1-10µM/mL. Typically 1 gram of tissue for every 10 mL of buffer is needed, but the amount can be adjusted depending upon the amount of tissue processed. Protein extracts should not be too dilute to avoid loss of protein due to adsorption to the wall of the vessel (glass or plastic). The minimum protein concentration should not be less than 0.1 mg/mL, and optimum concentration is 1-5 mg/mL. Tissue is homogenized by physical disruption and/or sonication. Generally all steps are performed with pre-chilled buffers. Following crude homogenization, cleared lysates are centrifuged at 12,000 x g for 10-15 minutes and protein concentrations are measured using a protein compatible kit test kit. Typically 20-75 µg/lane is used.

2. 5-50 µL applied to Minigel lane (0.75-1.5 mm width) and run at standard conditions. (60 mA for 2 1.5 mm Minigel gels, 1.4 h). It is suggested that you run 5-15% acrylamide (37.5:1 acrylamide:bisacrysmide) minigel (1.5 mm width) at 30 mA/gel ~1-1.5 hours. Note that for protein standards, Chemicon Catalog Number 2230 is an excellent and universal molecular weight marker system for SDS gels of all kinds.

3. Transfer in wet (Tobin buffers) or semi-dry systems under standard conditions (3 h 100 mA for two minigel gels, or overnight at 100 mA for complete transfer).

4. Stain the transferred bands with CHEMICON® BLOT-FastStain™ (Catalog Number 2076).

5. Destain with deionized water until clear, and proceed with blocking.

6. Block with CHEMICON® QuickBlocker™ (2080) or new ChemiBLOCKER™ (2170) at a 5% solution or use a purified casein protein diluted in PBS for 3 hours at room temperature or overnight at 2-8°C. **If using commercial non-fat milk (not recommended), it should be dissolved freshly, centrifuged 10,000 rpm for 10 min, and filtered to reduce background.**

7. Incubation with first antibody 2 h at room temperature or overnight at 4°C in 1% blocking solution or PBS/TBS only (we prefer very simple antibody solutions

because it prevents possible absorption of the antibody, increases primary antibody reactivity, and simplifies troubleshooting). The antibody preparation should be centrifuged before use (10,000 g 5 min.) to remove any precipitates, and it can be slowly filtered through a 0.45um filter by hand if necessary but care should be taken not to filter the solution too quickly or denaturation of the antibody may occur. Optimal working dilutions and incubation time will need to be determined by the end user. Typical dilutions with membrane preparations are 1:500-1:3000 usually.

8. Wash 4 x 10 min. with 50 mL of PBS-0.1% tween 20. Typically the blot should float in the wash solution, and often rocking the blot and wash solution on a shaker helps in reducing non-specific backgrounds. DO NOT LET THE BLOT DRY OUT. If one has to stop the western blot procedure one can leave the blot in wash solution overnight at 4°C, but this is not the optimal procedure.

9. Incubation with the secondary antibody (HRP-conjugated goat anti-rabbit antibody, for example CHEMICON® Catalog Number AP132P,) diluted appropriately (usually 1:10,000-1:50,000) 45-1h at room temperature. Remember that secondary antibody concentration determines the sensitivity of the signal; thus optimization of its final concentration can greatly influence the ultimate signal to noise ratio observed. Many of today's high-grade HRP conjugated secondaries can be diluted much more than HRP antibodies of just a few years ago. If a number of background bands is observed, one troubleshooting point is to reduce the amount of secondary antibody added, as this can lessen the number of non-specific primary antibodies detected.

10. Wash 4 x 10 min. with PBS-0.1% tween 20 as in step 8, do not let the blot dry out.

11. Perform ECL with commercial kits as directed (ChemiLUCENT, CHEMICON® Catalog Number 2600).

12. After development, rinse membrane 2-3X in fresh PBS/TBS solution. The blot can be stripped and reused with a new primary antibody with Chemicon's Reblot Plus (2500) or Reblot (2060) blot recovery systems.

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7. Incubation with first antibody 2 h at room temperature or overnight at 4°C in 1% blocking solution or PBS/TBS only (we prefer very simple antibody solutions because it prevents possible absorption of the antibody, increases primary antibody reactivity, and simplifies troubleshooting). The antibody preparation should be centrifuged before use (10,000 g 5 min.) to remove any precipitates, and it can be slowly filtered through a 0.45um filter by hand if necessary but care should be taken not to filter the solution too quickly or denaturation of the antibody may occur. Optimal working dilutions and incubation time will need to be determined by the end user. Typical dilutions with membrane preparations are 1:500-1:3000 usually.

8. Wash 4 x 10 min. with 50 mL of PBS-0.1% tween 20. Typically the blot should float in the wash solution, and often rocking the blot and wash solution on a shaker helps in reducing non-specific backgrounds. DO NOT LET THE BLOT DRY OUT. If one has to stop the western blot procedure one can leave the blot in wash solution overnight at 4°C, but this is not the optimal procedure.

9. Incubation with the secondary antibody (HRP-conjugated goat anti-rabbit antibody, for example CHEMICON® Catalog Number **AP132P**,) diluted appropriately (usually 1:10,000-1:50,000) 45-1h at room temperature. Remember that secondary antibody concentration determines the sensitivity of the signal; thus optimization of its final concentration can greatly influence the ultimate signal to noise ratio observed. Many of today's high-grade

Antibodies in the News
Introduction to Antibodies

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10. Wash 4 x 10 min. with PBS-0.1% tween 20 as in step 8, do not let the blot dry out.
11. Perform ECL with commercial kits as directed (ChemiLUCENT, CHEMICON® Catalog Number **2600**).
12. After development, rinse membrane 2-3X in fresh PBS/TBS solution. The blot can be stripped and reused with a new primary antibody with Chemicon's Reblot Plus (**2500**) or Reblot (**2060**) blot recovery systems.

General Methods and Application Protocols

Molecular Biology Protocols & Buffers

Current List of CE Marked Products

Reference Charts, Maps, Sequences & Lists

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CHEMICON International, Inc - 28820 Single Oak Drive - Temecula, CA 92590 - Ph: 951-676-8080