

Western Blotting

1. Mix the samples (organ membranes: 50 µg/lane; transfected cells: 500,000 cells/lane) with sample-buffer X 2, and heat 10 min at 70°C. 5-50 microliter of the samples applied to Minigel lane (0.75-1.5 mm width) and run at standard conditions. We run 5-15% acrylamide (37.5:1
2. acrylamide: bisacrylamide) minigel (1.5 mm width) at 30 mA/gel ~1-1.5h.

We use the semi dry transfer method:

3. Before beginning the transfer, wet both the blotting paper and the nitrocellulose (we use pure cellulose nitrate 0.45 µm) with the buffer solution as described below.
 1. Put four sheets of blotting paper on to the semi dry system.
 2. Lay on the nitrocellulose
 3. Lay the gel on top.
 4. Four sheets of blotting paper on top of the gel.

Important: Make sure there are no air bubbles between the layers.
5. The transfer is performed at 100 mA/minigel for 3 hr.

	Concentration	For 100 ml
Tris Base	48 mM	5.8 g
Glycine	39 mM	2.9 g
SDS	0.037%	3.7 ml of 10%
Methanol	20%	200 ml
Distilled water		Up to 1000 ml

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4. Blocking with 5% non-fat dry milk (Marvel or Carnation) in PBS, and 0.025 % Na azide, overnight at 4°C.
 The non-fat milk should be dissolved freshly, centrifuged 10000 rpm 10 min, and filtered through glass filter (Gelman Acrodisc).
5. Incubation with first antibody 2 h at room temperature in blocking solution:
 We first incubate the Ab and Ab-Ag (for negative control) into 0.5 ml PBS-1%BSA for 1 h. The antibody preparation should be centrifuged before use (10000xg 5 min.) to get rid of possible aggregates. After centrifugation, we mix the Ab solution with 4.5 ml 5% non fat dry milk in PBS centrifuged and filtrated.
6. From this stage, Na azide should be omitted.
 Wash 4 x 10 min. with PBS-0.1% tween 20.
 2 liters of washing buffer are needed for the two sets of washing.
7. Incubation with the secondary antibody into 5% non-fat dry milk in PBS (**without Na azide**) (HRP-conjugated goat anti-rabbit antibody, Jackson Laboratories, 1:20000-1:6000) 1 h at room temperature.
 Wash 4 x 10 min. with PBS-0.1% tween 20.
 Perform ECL with commercial kits.