

Method for Use of Novocastra Antibodies in Western Blotting

SAFETY NOTE: LATEX GLOVES SHOULD BE WORN AT ALL STAGES TO AVOID CONTAMINATION OF THE NITROCELLULOSE AND TO PROVIDE PROTECTION AGAINST HAZARDOUS REAGENTS

REAGENTS

1. Appropriate cell lysate in 1x SDS sample buffer.
2. Buffers for electrophoresis and transfer.
3. Primary antibody.
4. Alkaline phosphatase conjugated secondary antibody.
5. Substrate solutions.

EQUIPMENT

1. SDA gel electrophoresis system, eg. BioRad Mini-Protean II.
2. Semi-dry Western blot apparatus, eg. Hoefer SemiPhor.
3. Tube roller/shaking platform.
4. Plastic universal tubes etc.

PROCEDURES

Electrophoresis and Transfer:

1. Prepare and run appropriate strength SDS polyacrylamide gels. Stop electrophoresis just before dye front migrates off the end of the gel, thus allowing orientation after removal of the stacking gel.
2. Equilibrate gel in ice-cold transfer buffer for 5 minutes.
3. Prepare blot sandwich with three pieces of blotting paper (Sigma, P4556) each slightly larger than the gel soaked in transfer buffer and laid on top of one another. Place a piece of nitrocellulose (eg Amershan Hybond C extra) of similar size onto the filter paper; the nitrocellulose should have a blue biro line ruled along one of the long edges and be prewetted with transfer buffer.
4. Carefully place the gel onto the nitrocellulose, taking care to avoid trapping air bubbles. Align the dye front with the pen line on the nitrocellulose. Complete the sandwich with a further three sheets of soaked blotting paper. Run the transfer at a constant current of approximately 60 mA per minigel for 30 minutes.

Blocking:

1. Disassemble sandwich and remove excess nitrocellulose. Remove strip of nitrocellulose containing standards and stain as desired for protein. Place rest of sheet in block buffer (10% (w/v) non-fat dried milk in rinse buffer (RB) for 60 minutes at 25°C or overnight at 4°C.
2. Discard block buffer and rinse sheet briefly with RB.

Primary Antibody Incubation:

1. Make appropriate dilutions of antibody in antibody dilution buffer (10% (v/v) FCS in RB).
2. Place nitrocellulose in antibody dilution and incubate at 25°C for 60 minutes with gentle agitation. This may conveniently be performed in a disposable 30ml universal tube on a tube roller.
3. Rinse nitrocellulose in RB for 3 x 5 minutes.

Secondary Antibody Incubation:

1. Dilute appropriate conjugated secondary antibody in antibody dilution buffer. Alkaline phosphatase conjugates are recommended

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and described. Alternative conjugates and methods, eg HRP, biotin, ECL should be performed according to manufacturers instructions.

2. Incubate nitrocellulose with diluted secondary for 60 minutes at 25°C with gentle agitation as above.
3. Rinse nitrocellulose 3 x 5 minutes with RB.
4. Rinse nitrocellulose 2 x 5 minutes with alkaline phosphatase buffer (APB).

Visualisation:

1. Place 10ml APB in a disposable 30ml universal tube. Add 33µl BCIP solution and 66µl NBT solution (see SOLUTIONS section below) and mix by gentle inversion.
2. Place nitrocellulose in a clean plastic tray. Pour developer solution over nitrocellulose and leave until bands have developed. This may take from a few seconds to several minutes.
3. Stop the reaction by rinsing the nitrocellulose in 10% (v/v) acetic acid and allow to air dry at 25°C prior to storage/photography.

SOLUTIONS

<u>Transfer buffer (store at 4°C)</u>	g/l
25 mM Tris base	3.03
192 mM Glycine	14.4
0.1% Sodium dodecyl sulphate	1.0
20% Methanol	

Make up to 1 litre with deionized water.

Rinse buffer (1x)
Phosphate buffered saline plus 0.05% (v/v) Tween 20

<u>Alkaline Phosphatase buffer (1x)</u>	g/l
100 mM Sodium chloride	5.85
100 mM diethanolamine	10.7 ml
5 mM MgCl ₂	1.02

Prepare as 10x stock: dissolve MgCl₂ and NaCl in water, prior to addition of diethanolamine. pH to 9.5 with HCl.

BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate (p-toluidine salt)). Store at -20°C in small aliquots as a 50 mg ml⁻¹ solution in 100% dimethyl formamide.

NBT (Nitro-blue Tetrazolium). Store at -20°C in small aliquots as 50 mg ml⁻¹ solution in 70% (v/v) dimethyl formamide in dH₂O.