



Catalog Number:	RA22105	Host:	Rabbit
Product Type:	Whole Serum	Species	Human, Mouse, Rat, Cow,
		Reactivity:	Chicken
Immunogen Sequence:	Recombinant fusion protein containing the extreme C-terminal segment of rat NF-M	Format:	Liquid.
Applications:	Immunocytochemistry: 1:1,000-2500 (ABC or other enzyme linked procedure) Immunohistochemistry: 1:1000-2,500 Western Blot: 1:1,000-5,000 Dilutions listed as a recommendation. Optimal dilution should be determined by investigator.		
Storage:	Antibody can also be aliquotted and stored frozen at -20° C to -70° C in a manual defrost freezer for six months without detectable loss of activity. The antibody can be stored at 2° -8° C for 1 month without detectable loss of activity. Avoid repeated freeze-thaw cycles		
References:	1. Harris, J., Ayyub, C. and Shaw G. A molecular dissection of the carboxyterminal tails of the major neurofilament subunits NF-M and NF-H. J Neurosci Res 30:47-62 1991. 2. Wang, Y., Zhang, J., Mori, S. and Nathans. J. Axonal Growth and Guidance Defects in Frizzled3 Knock-Out Mice: A Comparison of Diffusion Tensor Magnetic Resonance Imaging, Neurofilament Staining, and Genetically Directed Cell Labeling. J. Neurosci. 26:355-364 2006.		

Application Notes

Immunostaining Cell Cultures

1. Draw of culture medium with aspirator and add 1 ml of 3.7 % formalin in PBS solution to the dish. (make up from 10mls Fisher 37% formalin plus 90mls PBS, the Fisher formalin contains 37% formaldehyde plus about 1% methanol which may be relevant sometimes). Let sit at room temp for 1 minute. (can add 0.1% Tween 20 to PBS used here and all subsequent steps to reduce background; probably best not to do this first time round though as it may extract your antigen or help wash your cells off the dish).
2. Take off the formalin/PBS and add 1ml of cold methanol (-20°C, kept in well sealed bottle in fridge). Let sit for no more than 1 minute.
3. Take off methanol and add 1ml of PBS, not letting the specimen dry out. To block nonspecific antibody binding can add ~10ml (=1%) of goat serum (Sigma), and can incubate for 30 minutes. Can then add antibody reagents. Typically hybridoma tissue culture supernatant, mouse ascites fluid, affinity purified or crude serum. Incubate for 1 hour at room temp. (or can go at 37°C for 30 minutes to 1 hour, or can do 4°C overnight, exact time not too critical). Can do very gentle shaking for well adherent cell lines (3T3, Hek293 etc.).

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4. Remove primary antibody and replace with 1 ml of PBS. Let sit for 5-10 minutes, replace PBS and repeat twice, to give three washes in PBS.
5. Add 0.5 mls of secondary antibody. These are fluorescently labeled Goat anti mouse or rabbit antibodies and are conjugated to ALEXA dyes and are from Molecular probes (Eugene Oregon, the ALEXA dyes are sulphonated rhodamine compounds and are much more stable to UV than FITC, TRITC, Texas red etc.). Typically make 1:2,000 dilutions of these secondaries in PBS plus 1% goat serum, BSA or non fat milk carrier. Incubate for 1 hour at room temp. (or can go at 37°C for 30 minutes to 1 hour, or can do 4°C overnight). Can do gentle shaking for well adherent cell lines (3T3, HEK293 etc.).
6. Remove secondary antibody and replace with 1 ml of PBS. Let sit for 5-10 minutes, replace PBS and repeat twice, to give three washes in PBS.
7. Drop on one drop of Fisher mounting medium onto dish and apply 22mm square coverslip. View in the microscope!

Immunostaining Tissue

Solutions

PBS - sodium phosphate-buffered (100 mM; pH 7.2) isotonic (0.9% NaCl, w/v) saline Antibody dilution buffer (PBS with 0.1% non-ionic detergent, such as Triton X-100 or Tween-20) fluorescein anti-fading reagent -- Make up a 2 mg/ml phenylene diamine solution in PBS (phenylene diamine requires extensive vortexing to put it into solution). Once the phenylene diamine is completely dissolved, add an equal volume of glycerol and mix. This reagent will last about a week at -20°C. Discard this reagent when it starts to turn dark brown.

Other Reagents

Fluorescein-labeled goat anti-rabbit IgG

1. Prepare your tissue sections or cultured cells as you normally would. Wash your sections or cells for 1 min with PBS at room temperature.
2. Incubate your sections or cells with your chicken primary antibodies (diluted in "antibody dilution buffer") for at least 1 hour at room temperature. The concentration of your antibody may be anywhere from 1:50-1:150 depending on the titre of the antibody and the concentration of your antigen.
3. Wash your sections or cells over a 10 minute period at room temperature (with two changes of PBS).
4. Incubate your sections or cells with fluorescein-labeled goat anti-rabbit IgG (1:500 dilution in "antibody dilution buffer" for 1 hour at room temperature. Be sure to keep these slides or culture dishes in subdued light (e.g., in a drawer) to avoid bleaching of the fluorescein dye.
5. Repeat step #4
6. Add a drop of "fluorescence anti-fading reagent" (i-BRITE Plus) to your sections or cells. Place a coverslip over the section. If you want to reduce messiness, you may also seal the coverslip by painting the edges with nail polish.
7. Store the slides or culture dishes in the refrigerator (in the dark).

Western Blotting

1. Run gel as usual. Take gel out of electrophoresis apparatus. Cut into segments as required; Part of gel can be stained directly in Coomassie brilliant blue R-250 (2.5 g Coomassie Brilliant Blue R-250, 450 mls methanol, 100 mls glacial acetic acid, water to 1 liter). Part to be used for electroblotting is put into tap water on shaker, after first having marked it unambiguously to identify top/bottom, left and right etc.
2. Leave in water on shaker for 5 minutes. This step can be substituted by washing the gel in electro-transfer buffer (see below) for 5 minutes.
3. We use a semidry blotter, which we have found to be quicker, more economical and easier than fully submerged blotting methods. We cut Whatman 3M filter papers to the size of our gels, and place three of these onto the semi dry blotter. These are then wet with transfer buffer (we routinely use 3.03 g Tris base, 14.4 g Glycine, 10% Methanol per liter). The gel is put onto the filters and a prewetted nitrocellulose filter is put on top of the gel. Alternately put a PVDF membrane on top; if you are using PVDF remember it is essential to prewet the PVDF in 100% methanol. Great care should be taken to ensure that no air bubbles are anywhere in this stack of membranes. Then three more wetted Whatman 3M filters should be placed on top of the pile, again taking great care not to have any bubbles in pile. Put the top onto the apparatus and screw it down. Proteins in transfer buffer are negative in charge mostly due to residual SDS and they therefore move from -ve to +ve pole. So the +ve electrode is above the nitrocellulose and the -ve side is below the gel.

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4. Run for 30 minutes to 1 hour at ~100mA. The most reliable way of doing this is to use a powerful power supply 200-500mA and put it on constant voltage, with a setting of 5 to 10 Volts. Low molecular weight proteins (20kDa or less) will transfer in 30 minutes at 5 Volts, while higher molecular weight (150kDa or more) transfer in 60 minutes at 10 Volts.

5. After running disassemble the apparatus and remove nitrocellulose filter. Stain this for 5 minutes on shaker in Ponceau reagent (0.25% Ponceau S in 40% methanol and 15% acetic acid). Destain with regular SDS-PAGE gel destain solution (7.5% methanol, 10% acetic acid). If you transferred efficiently, the proteins can be seen as pale pink bands. This tells you whether the transfer was O.K. or not and also exactly where the bands are. You can photograph, photocopy or mark the position of the bands directly with a pencil. If you can't see any bands at this stage, it's probably smart to try to optimize steps 3 and 4. The gel may be discarded or may be stained as usual in coomassie, to see how much protein is left behind.

6. After Ponceau staining put the nitrocellulose filter into blocking solution, such as 1% bovine serum albumin (BSA) or 1% Carnation non fat milk (NFM), for 20 minutes to 1 hr at RT or 37°C. Since the NFM works just as well as BSA but is much cheaper, there is really no good reason to use BSA. Ponceau staining will fade to become completely invisible. Carry on with antibody incubations etc.

Antibody Incubations:

1. Put in antibody solutions. Volume should be enough to cover blot and allow it to float freely when you agitate. In initial experiments, antibody concentration should generally be about 1:100 - 1:1,000 for ascites, CL350 tissue culture supernatant or antiserum, undiluted to 1:10 for monoclonal supernatant, and about 1-10µg/ml for a pure IgG. If dilution brings antibody concentration to less than 50 µgs/ml, add some BSA or NFM to act as carrier protein (e.g. make BSA or NFM concentration 1mg/ml). Incubate for at least 1 hour with shaking (can be room temperature or at 37°C, can also do overnight at 4°C).

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