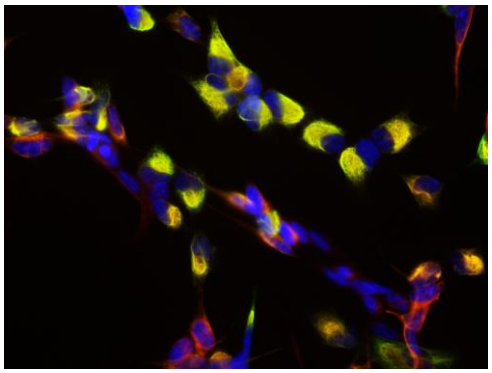
NEUROMICS

Nestin Data Sheet

Catalog Number:	RA22125	**Host:**	Rabbit
Product Type:	Polyclonal Whole Serum Antibody	**Species Reactivity:**	Human. (does not react with rodent Nestin)
Immunogen Sequence:	Amino acids 315-630 of the human protein HGNC name: NES	**Format:**	Liquid, 100 ul aliquot
Applications:	Immunofluorescence/Immunocytochemistry: 1:1000-5,000 Immunohistochemistry: 1:1000-5,000 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.		

Application Notes

Description/Data:



The protein proved to be an unusual member of the intermediate filament family, containing an α-helical region homologous to that found in keratin and neurofilament subunits. The Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC) name for Nestin is NES.

Image: SH-SY5Y neuroblastoma cells were stained with Nestin (RA221025) at 1:5,000 (red) and CH22108 against vimentin (green) at 1: 10,000. Vimentin is main partner of nestin forming heterdimers and further polymerizing to form intermediate filaments. Co-localization of vimentin and nestin in cells was shown in yellow. Blue is DAPI staining of DNA.

Nestin is relatively poorly conserved in protein sequence across species boundaries, so that the mouse and human proteins have an overall identity of only 62%. As a result antibodies to the human protein often fail to recognize the rodent homologue and vice versa. Antibodies to nestin are widely used to identify neural stem cells. This antibody was raised in rabbit against recombinant human nestin from aa 315-630, which has only 55% identity to homologous mouse sequence. Nestin (RA22125) therefore recognizes nestin protein in human, but not in rodent.

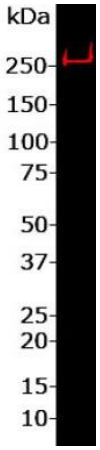


Image: Western blot of SH-SY5Y homogenate probed with Nestin (RA22125) at 1:2,000. A single strong band running at ~260 kDa corresponding to full length nestin

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Immunostaining of cells in tissue culture:

The purpose of fixation is denature the components of cells enough so that they stay on the dish and can be bound by antibodies, hopefully without destroying cellular morphology. Fixatives such as formalin, paraformaldehyde and glutaraldehyde chemically cross-link proteins, by binding to amino acid side chains, generally the most chemically reactive ones like amines (Lysine, Arginine, Glutamine and Asparagine). This chemical modification can also have the consequence of blocking antibody binding sites. Substances such as acetone and methanol are not true fixatives but are denaturants, which precipitate proteins without covalently modifying them. We routinely use a combination of mild formalin fixation followed by cold methanol for neurons, mixed neuron/glial cultures and most of the widely used human and rodent cell lines. The formalin preserves the cellular morphology quite well, while the methanol further denatures the proteins of the cells and helps keep what is left of the cell adherent to the dish. For soluble proteins it may be necessary to miss the methanol step, but in this case you have to be very careful with the washing steps, as the cells tend to wash off the dish. Certain antibodies may be very sensitive to formalin fixation, so you may have to experiment a little, perhaps missing out that step. The following procedure works for antibodies to most cytoskeletal and signaling molecules. This procedure is good for cells in 6 well tissue culture plates or in 35mm tissue culture dishes. These are just big enough that you can look from above with a typical immunofluorescence microscope through a glass coverslip. This allows you to see the specimens very well and take very high quality pictures. (However note that it's a pain to changes lenses on the microscope if you use the 6 well dishes, since you have to rack the lens right the way up to do this, and you have to take out the two neighboring lenses in the turret since they will hit the other wells of the dish! It's less of a problem with 35mm dishes but still a pain. No procedure is perfect….).

1. Draw of culture medium with aspirator and add 1 mL of 3.7 % formalin in PBS solution to the dish. (make up from 10 mLs 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 ~10 µL (=1%) of goat serum (Sigma), and can incubate for 30 minutes. Can then add antibody reagents. Typically 100 µL of hybridoma tissue culture supernatent or 1ml of mouse ascites fluid 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.).
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 µLs of secondary antibody. These are fluorescently labeled Goat anti mouse or rabbit antibodies and are conjugated to ALEXA dyes and were originally marketed by Molecular Probes(Eugene Oregon, the ALEXA dyes are sulphonated rhodamine compounds and are much more stable to UV than FITC, TRITC, Texas red etc. Molecular Probes was bought by Invitrogen, which now markets these reagents). 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 22 mm square coverslip. View in the microscope!

Immunostaining of Tissue Specimens:

Here are two alternative procedures, both of which work well. The more well established method is to perfuse the animal with fixative while under anesthetic. This requires some surgical skills, since the perfusion has to done in the aorta, which can only be reached by opening the chest cavity. Obviously the animal has to be fully anesthetized, and is in most cases a small rodent such as a rat. You will also need to obtain approval from your local animal care authorities to do this. It is a lot easier to sacrifice the animal first, dissect out the tissues you want and just drop them into fixative. This works fine, and can be performed on animals killed at slaughterhouses or for other purposes.

Fixed tissues are then impregnated with sucrose, which acts as a cryoprotectant, preventing ice crystal formation during freezing. Specimens are then rapidly frozen and sectioned on a cryostat.

A. Perfusion.

1. Perfuse animal with heparinized normal saline after anesthetizing with pentabarbitol.
2. When blood has been washed out, perfuse in 4% paraformaldehyde fixative freshly made up in PBS (best), 4% paraformaldehyde in PBS not so fresh (O.K.) or 1+9 solution of Fisher Formalin in PBS (also O.K. for most purposes). Dissect out tissues of interest and put into fixative solution.
3. The time the tissue spends in fixative has an impact on which antibodies will work; many antibodies bind epitopes that are destroyed by fixatives, so we recommend starting with a very mild fixation. Accordingly, we let tissues sit only 1-2 hours in fixative at 4°C and then wash 3 times in PBS with mild shaking. After this procede to C below.

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B. No Perfusion.

1. Dissect out tissues and drop into fixative at room temperature. Fixative is 4% paraformaldehyde fixative freshly made up in PBS (best), 4% paraformaldehyde in PBS not so fresh (O.K.) or 1+9 solution of Fisher Formalin in PBS (also O.K. for most purposes). To aid in the penetration of the fixative cut tissues down to steaks of no more than 0.5 cm thickness. Also its good to gently shake specimens on a rocker platform to allow fixative to penetrate tissues. For pilot experiments with antibodies you have n't tried before 1-2 hour fixation is enough. Can leave longer or overnight at 4°C if you know the particular antigen/antibody reaction can stand this.
2. Move specimens to PBS and proceed to C below.

C. Sucrose impregnation and rapid freezing.

1. Transfer the tissue to 20% sucrose in PBS, leave overnight at 4°C.
2. Transfer the tissue to 30% sucrose in PBS, leave at 4°C to impregnate fully. Typically, when the tissue sinks, it is fully impregnated, which usually take 2-3 days.
3. Rapidly freeze in isopentane (a.k.a. 2-methylbutane) cooled with liquid nitrogen. To do this put 5-10 mls of isopentane in a small polystyrene or other plastic container; a 35mm film container or a 50 mL beaker is fine for this purpose. Then put that in a larger plastic container, such as a 500 mL polystyrene or polypropylene beaker in which you put ~100 mL liquid nitrogen. Let the liquid nitrogen cool the isopentane til you can see it start to freeze, which starts at -160°C. The isopentane freezes from the bottom of the container, forming round white pearl like objects. When this happens drop the specimen directly into the bottom of the container. Take specimen out using plastic or plastic coated forceps (they tend to stick to metal forceps) and either store them at -70°C or proceed immediately to sectioning. Tissue should now not be allowed to thaw out until after sectioning.
4. Mount on cryostat and cut 5 to 20mm thick sections. Cut at -25°C and mount sections on "+" marked slides or subbed slides. Store at -20°C or -70°C or proceed to immunostaining right away.

D. Immunostaining with fluorescent secondary antibodies

1. Optional; Wash sections for 30 seconds in cold Acetone (-20°C). This step allows better antibody penetration, but may wash out your antigen, especially in the case of cytosolic proteins. Let section dry before next step.
2. Optional; block non-specific binding by incubation of section with 1% goat serum in PBS for 1 hour at 37°C.
3. Draw circle round dry section with PAP pen to prevent loss of antibody. Add primary antibodies; Typically dilute pure primaries to ~1 microgram/ml and make up in PBS plus 0.1% goat serum or BSA. Can apply rabbit and mouse antibodies at same time for double label immunocytochemistry. Typically incubate for 1 hour at 37°C or 2 hours at room temperature or overnight at 4°C.
4. Wash sections at least 3 times, at least 5 minutes each time, in PBS. To reduce background can include 0.1% Tween 20 in PBS.
5. Apply secondary antibodies. Best are goat anti-mouse and goat anti-rabbit antibodies from Molecular Probes, and best fluorochromes are the ALEXA conjugates; ALEXA 488 and ALEXA 594. The 488 is very similar in spectral properties to FITC, and the 594 is similar to rhodamine and Texas red. Both are resistant to bleaching and work at 1 microgram/mL dilution, which is 1:2,000 from the 2 milligram/mL material obtained from Molecular Probes. Incubate with secondaries in PBS plus 0.1% goat serum. Typically incubate for 1hr at 37°C or 2 hours at room temperature or overnight at 4°C.
6. Wash sections at least 3 times, at least 5 minutes each time, in PBS. To reduce background can include 0.1% Tween 20 in PBS.
7. Mount in mounting medium, a useful one being Vectasheild mounting medium with DAPI, which allows you to stain nuclei with the DNA intercalating fluorescent dye DAPI, which you can see on a fluorescence microscope fitted with appropriate blue filters.
8. View on fluorescence microscope.

Western Blotting:

The ability to transfer proteins from SDS-PAGE gels to nitrocellulose or PVDF membranes has become routine in most laboratories. An important early paper was that of Towbin et al. (Towbin et al 1979). Later studies used other kinds of membranes, notably the nylon like material PVDF, which allowed proteins transferred from SDS-PAGE gels to be subjected to direct peptide sequencing (Matsudaira, 1975). We are assuming you ran a regular SDS-PAGE slab gel. Nitrocellulose and PVDF both work well for blotting; nitrocellulose is more fragile, being somewhat brittle, and is also highly flammable, in fact explosive (another name for nitrocellulose is gun cotton, put a match to a small piece if you don't believe us - we cannot be held responsible if you burn your lab down). Here is a basic nitrocellulose/PVDF protocol.

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

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liter). The gel is put onto the filters and a prewetted nitrocellulose filter is put ontop 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 ontop 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.

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 transer 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).
2. Wash membranes in TBS (10mM Tris, 154mM NaCl, pH=7.5 plus 0.1% Tween 20) for 3 times at least five minutes each time with extensive agitation.
3. Incubate in second antibody (peroxidase-conjugate, phosphatase conjugate or radioactive). Add BSA or NFM carrier as before if necessary. Incubate for at least one hour at room temperature or 37°C can also do overnight at 4°C with shaking as before.
4. Wash membranes in TBS (10mM Tris, 154mM NaCl, pH=7.5 plus 0.1% Tween 20) for 3 times at least five minutes each time with extensive agitation.

A. Alkaline Phosphatase Blot System

1. Incubate in alkaline phosphatase conjugated antibody against the primary antibody (e.g. Goat anti-mouse, rabbit or chicken; buy from Sigma or some other trusted source). Typical concentration is 1:1,000 in TBS (10mM Tris/HCl, 154mM NaCl, pH=7.5). Add a small amount of BSA or NFM to act as carrier. Incubate for 1 hour at room temperature (or 37°C) with shaking.
2. Wash in TBS three times 5 minutes each. (N.B. the alkaline phosphatase enzyme is inhibited by EDTA, which chelates zinc and magnesium, and by phosphate, which inhibits forward reaction. Make sure therefore you use TBS which is EDTA and phosphate free- Don't make up developer in PBS!)
3. Put into developer. Buffer is 100mM Tris/HCl, 100mM NaCl, 5mM $MgCl_2$ pH=9.5. To 10ml of this add 33µl BCIP-T (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt, make up 50mg/ml in water or Dimethyl formamide; in water makes a yellow suspension) and 33µl of NBT (Nitro Blue Tetrazolium, also 50mg/ml in water). Can store these solutions at -20°C. Can buy this solution made up already from Sigma. Reaction product is purple, and appears in a few minutes; can incubate for up to an hour if the signal is weak. Watch development of reaction and stop with water. Some of background disappears on drying.

B. Horse Radish Peroxidase Staining

After washing of blots in TBS or PBS (must not have azide in wash buffer! This inhibits the peroxidase enzyme) add reaction mixture. This is; 20 mls 0.1M Tris/HCl pH=7.2 (Vecta stain buffer). 200 µl NiCl (80 mg/ml), 6 µl 30% hydrogen peroxide, 1ml of 5mgs/ml diaminobenzidine. (Wear gloves, DAB is carcinogenic). Alternate protocol; Make 20 mls ammonium acetate buffer (50mM, pH=5.0). Add 1 ml of 10mg/ml Diaminobenzidine, 40µl 30% hydrogen peroxide. Brown reaction product is seen in 1-10 minutes, not quite so nice as above method.

C. Chemiluminescence Staining

Chemiluminescence has an advantage of perhaps an order of magnitude greater sensitivity than the dye based methods above. In addition, several films may be exposed from a single blot, giving an advantage in interpretation of weak and strong signals on the same membrane. However, it requires a darkroom to perform and is more expensive in reagents. Reagents are generally bought in a kit, and we recommend simply following the kit instructions.

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