NEUROMICS

mTOR Data Sheet

Catalog Number:	RA25087	**Host:**	Rabbit
Product Type:	Affinity Purified	**Species Reactivity:**	Human, Rat
Immunogen Sequence:	Synthetic peptide (Human) - which represents a portion of human Mammalian Target of Rapamycin encoded in part by exons 5 and 6	**Format:**	Liquid. Tris-citrate/phosphate buffer, pH 7 to 8 and 0.09% Sodium Azide as a preservative. Concentration: 1.0 mg/ml.
Applications:	Immunofluorescence: 1:100-1:400 Immunocytochemistry: 1:100-1:400 Immunoprecipitation: 1-4 ug/mg lysate *Dilutions listed as a recommendation. Optimal dilution should be determined by investigator.		
Storage:	Store frozen. Aliquot as undiluted antisera and immediately place at -20°C. Antisera may have become trapped in top of vial during shipping. Centrifugation of vial is recommended before opening. Stable for at least 6 months at -20°C. Repeated freeze/thaw cycles compromise the integrity of the antiserum.		

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 100ml 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 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**!**

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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 -20OC. 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).

Immunoprecipitation

1. Centrifuge the lysate at 14,000 x g in a precooled centrifuge for 15 minutes. Immediately transfer the supernatant to a fresh centrifuge tube and discard the pellet.
2. To prepare protein A or G agarose/sepharose, wash the beads twice with PBS and restore to a 50% slurry with PBS. It is recommended to cut the tip off of the pipette when manipulating agarose beads to avoid disruption of the beads.
3. Pre-clear the cell lysate by adding 100 ul of either protein A or G agarose/sepharose bead slurry (50%) per 1 ml of cell lysate and incubating at 4 degrees Celsius for 10 minutes on a rocker or orbital shaker. Pre-clearing the lysate will reduce non- specific binding of proteins to the agarose or sepharose when it is used later on in the assay.
4. Remove the protein A or G beads by centrifugation at 14,000 x g at 4 degrees Celsius for 10 minutes. Transfer the supernatant to a fresh centrifuge tube.
5. Determine the protein concentration of the cell lysate, e.g. by performing a Bradford assay. Dilute the cell lysate at least 1:10 before determining the protein concentration because of the interference of the detergents in the lysis buffer with the Coomassie-based reagent.
6. Dilute the cell lysate to approximately 1 ug/ul total cell protein with PBS to reduce the concentration of the detergents in the buffer. A more concentrated cell lysate (i.e., 10 ug/ul) may be necessary to immunoprecipitate a protein, which is found in low levels in a cell model. Add the recommended volume of the immunoprecipitating antibody (see datasheet for detailed information) to 500 ul (i.e., 500 ug) of cell lysate. The optimal amount of antibody that will quantitatively immunoprecipitate the protein of interest should be empirically determined for each cell model.
7. Gently rock the cell lysate/antibody mixture for either 2 hours or overnight at 4 degrees Celsius on a rocker or an orbital shaker. A 2 hour incubation time is recommended for the immunopecipitation of active enzymes for kinase or phosphatase assays.
8. Capture the immunocomplex by adding 100 ul protein A or G agarose/sepharose bead slurry (50 ul packed beads) and gently rocking on either a rocker or orbital shaker for either 1 hour or overnight at 4 degrees Celsius. In many instances, immunocomplex capture can be enhanced by adding 2 ug of a bridging antibody (e.g., rabbit-anti-mouse IgG). This is especially important with antibodies, which bind poorly to protein A, such as mouse IgG1 or antibodies generated in chicken.
9. Collect the agarose/sepharose beads by pulse centrifugation (i.e., 5 seconds in the microcentrifuge at 14,000 rpm). Discard the supernatant and wash the beads 3 times with 800 ul ice-cold modified RIPA buffer. Occasionally, washing with modified RIPA buffer will strip the immunocomplex off of the agarose/sepharose beads. In these cases, washing with the milder PBS is recommended.
10. Resuspend the agarose/sepharose beads in 60 ul 2x sample buffer and mix gently. This will allow for sufficient volume to run three lanes. The agarose/sepharose beads are boiled for 5 minutes to dissociate the immunocomplexes from the beads. The beads are collected by centrifugation and SDS-PAGE is performed with the supernatant. Alternatively, the supernatant can be transferred to a fresh microcentrifuge tube and stored frozen at –20 degrees Celsius for later use. Frozen supernatant should be reboiled for 5 minutes directly prior to loading on a gel.

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Image: mTOR (red) staining of L6 myotubes. Nuclei are stained with nuclear antigen antibody (green).

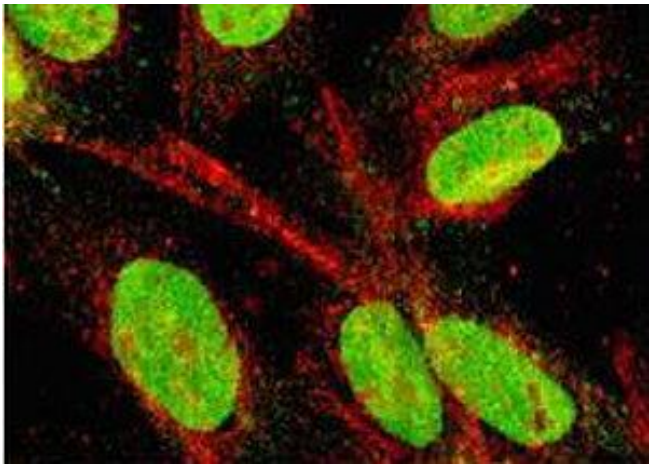
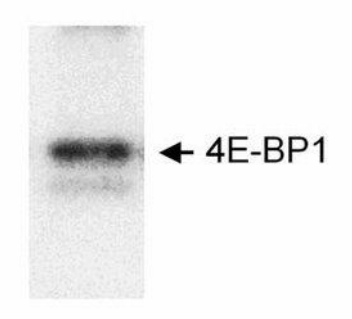


Image: mTOR protein kinase assay: mTOR (dilution -1:1000) immunoprecipitates were incubated at 30°C with recombinant 4E-BP1 and 32P-gATP. Autoradiograph shows 32P incorporated into 4E-BP1.



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