OriGene Technologies Inc.

9620 Medical Center Drive, Ste 200
Rockville, MD 20850
UNITED STATES
Phone: +1-858-888-7900
Fax: +1-858-888-7904
US-info@acris-antibodies.com

OriGene EU

Acris Antibodies GmbH
Schillerstr. 5
32052 Herford
GERMANY
Phone: +49-5221-34606-0
Fax: +49-5221-34606-11
info@acris-antibodies.com

Polyclonal Antibody to p70 S6 Kinase (p70S6K) pThr229 - Aff - Purified

Catalog No.: BP7138

Quantity: 0.1 ml

Background: Ribosomal Protein S6 Kinase with a molecular mass of 70 kDa (p70-S6K or p70-S6Kβ1) is a member of a serine/threonine kinase family that phosphorylates the 40S ribosomal protein S6, thereby modulating the translation of ribosomal proteins and translation elongation factors. p70-S6K is activated in response to the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, and is required for progression through the cell cycle and for cell growth. Activation of p70-S6K is regulated by phosphorylation of seven different residues distributed throughout the protein, with the critical sites being threonines 229, 389, 421 and serine 424. Phosphorylation is progressive, culminating in phosphoregulation of threonine 229 within the activation loop.

Host: Rabbit

Immunogen: A chemically synthesized phosphopeptide derived from the region of human p70S6K that contains threonine 229.
Remarks: The sequence is conserved in rat.

Format: **State:** Liquid purified Ig fraction
Purification: Sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated p70-S6K. The final product is generated by affinity chromatography using a p70S6K-derived peptide that is phosphorylated at threonine 229.
Buffer System: Dulbecco's phosphate buffered saline (without Mg2+ and Ca2+), pH 7.3 (+/- 0.1), 50% Glycerol with 1.0 mg/ml BSA (IgG, protease free) as a carrier and 0.05% Sodium Azide as a preservative.

Applications: Western blotting: use the antibody at a 1/1000 starting dilution.
Positive control used: Human Jurkat cells; Hek293 cells +/- EGF; truncated p70 S6 Kinase protein, an N-terminus His-tagged fusion protein corresponding to amino acids 1-421 of human p70 S6 Kinase containing a T412E mutation to become constitutively active.
Other applications not tested. Optimal dilutions are dependent on conditions and should be determined by the user.

Specificity: Reacts with p70S6K.
p70S6Kβ2 is 80% homologous in the area of threonine 229.
Species: Human and Rat.
Other species not tested.

Storage:

Store at -20°C. We recommend a brief centrifugation before opening to settle vial contents. Then, apportion into working aliquots and store at -20°C.
For short-term storage (up to one week), 2-8°C is sufficient.
Avoid repeated freezing and thawing.
Shelf life: one year from despatch.

General Readings:

1. Shah OJ, Hunter T. Critical role of T-loop and H-motif phosphorylation in the regulation of S6 kinase 1 by the tuberous sclerosis complex. J Biol Chem. 2004 May 14;279(20):20816-23. Epub 2004 Mar 1. PubMed PMID: 14993219.
2. Lekmine F, Uddin S, Sassano A, Parmar S, Brachmann SM, Majchrzak B, et al. Activation of the p70 S6 kinase and phosphorylation of the 4E-BP1 repressor of mRNA translation by type I interferons. J Biol Chem. 2003 Jul 25;278(30):27772-80. Epub 2003 May 20. PubMed PMID: 12759354.
3. Gonzalez-Garcia A, Garrido E, Hernandez C, Alvarez B, Jimenez C, Cantrell DA, et al. A new role for the p85-phosphatidylinositol 3-kinase regulatory subunit linking FRAP to p70 S6 kinase activation. J Biol Chem. 2002 Jan 11;277(2):1500-8. Epub 2001 Oct 29. PubMed PMID: 11684675.
4. Tu VC, Bahl JJ, Chen QM. Signals of oxidant-induced cardiomyocyte hypertrophy: key activation of p70 S6 kinase-1 and phosphoinositide 3-kinase. J Pharmacol Exp Ther. 2002 Mar;300(3):1101-10. PubMed PMID: 11861821.
5. Adi S, Wu NY, Rosenthal SM. Growth factor-stimulated phosphorylation of Akt and p70(S6K) is differentially inhibited by LY294002 and Wortmannin. Endocrinology. 2001 Jan;142(1):498-501. PubMed PMID: 11145615.
6. Zhang Y, Dong Z, Bode AM, Ma WY, Chen N, Dong Z. Induction of EGFR-dependent and EGFR-independent signaling pathways by ultraviolet A irradiation. DNA Cell Biol. 2001 Dec;20(12):769-79. PubMed PMID: 11879570.
7. Hillier T, Long W, Jahn L, Wei L, Barrett EJ. Physiological hyperinsulinemia stimulates p70(S6k) phosphorylation in human skeletal muscle. J Clin Endocrinol Metab. 2000 Dec;85(12):4900-4. PubMed PMID: 11134159.

Protocols:

Western Blotting Procedure

1. Lyse approximately 10e7 cells in 0.5 mL of ice cold Cell Lysis Buffer (formulation provided below). This buffer, a modified RIPA buffer, is suitable for recovery of most proteins, including membrane receptors, cytoskeletal-associated proteins, and soluble proteins. Other
cell lysis buffer formulations, such as Laemmli sample buffer and Triton-X 100 buffer, are also compatible with this procedure. Additional optimization of the cell stimulation protocol
and cell lysis procedure may be required for each specific application.
2. Remove the cellular debris by centrifuging the lysates at 14,000 x g for 10 minutes. Alternatively, lysates may be ultracentrifuged at 100,000 x g for 30 minutes for greater clarification.
3. Carefully decant the clarified cell lysates into clean tubes and determine the protein concentration using a suitable method, such as the Bradford assay. Polypropylene tubes are
recommended for storing cell lysates.
4. React an aliquot of the lysate with an equal volume of 2x Laemmli Sample Buffer (125 mM
Tris, pH 6.8, 10% glycerol, 10% SDS, 0.006% bromophenol blue, and 130 mM dithiothreitol [DTT]) and boil the mixture for 90 seconds at 100°C.
5. Load 10-30 µg of the cell lysate into the wells of an appropriate single percentage or gradient minigel and resolve the proteins by SDS-PAGE.
6. In preparation for the Western transfer, cut a piece of PVDF membrane slightly larger than

the gel. Soak the membrane in methanol for 1 minute, then rinse with ddH2O for 5 minutes. Alternatively, nitrocellulose may be used.
7. Soak the membrane, 2 pieces of Whatman paper, and Western apparatus sponges in transfer buffer (formulation provided below) for 2 minutes.
8. Assemble the gel and membrane into the sandwich apparatus.
9. Transfer the proteins at 140 mA for 60-90 minutes at room temperature.
10. Following the transfer, rinse the membrane with Tris buffered saline for 2 minutes.
11. Block the membrane with blocking buffer (formulation provided below) for one hour at room temperature or overnight at 4°C.
12. Incubate the blocked blot with primary antibody at a 1:1000 dilution in Tris buffered saline supplemented with 3% Ig-free BSA and 0.1% Tween 20 overnight at 4°C or for 2 hours at room temperature.
13. Wash the blot with several changes of Tris buffered saline supplemented with 0.1% Tween 20.
14. Detect the antibody band using an appropriate secondary antibody, such as goat F(ab')2
anti-rabbit IgG alkaline phosphatase conjugate or goat F(ab')2 anti-rabbit IgG horseradish peroxidase conjugate in conjunction with your chemiluminescence reagents and instrumentation.

Cell Lysis Buffer Formulation:
10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM Na4P2O7
2 mM Na3VO4
0.1% SDS
0.5% sodium deoxycholate
1% Triton-X 100
10% glycerol
1 mM PMSF (made from a 0.3 M stock in DMSO)
or 1 mM AEBSF (water soluble version of PMSF)
60 µg/mL aprotinin
10 µg/mL leupeptin
1 µg/mL pepstatin
(alternatively, protease inhibitor cocktail such as Sigma Cat. # P2714 may be used)

Transfer Buffer Formulation:
2.4 gm Tris base
14.2 gm glycine
200 mL methanol Q.S. to 1 liter,
then add 1 mL 10% SDS.
Cool to 4°C prior to use.

Tris Buffered Saline Formulation:
20 mM Tris-HCl, pH 7.4
0.9% NaCl

Blocking Buffer Formulation:

100 mL Tris buffered saline
5 gm BSA
0.1 mL Tween 20

Peptide Competition Experiment
The specificity of a Phosphorylation Site Specific Antibody (PSSA) in each experimental system can be confirmed through peptide competition. In this technique, aliquots of antibody are pre-incubated with peptide containing the sequence of the phosphopeptide immunogen used to raise the PSSA and the corresponding non-phosphopeptide. Following preincubation with the peptide, each antibody preparation is then used as a probe in antibody-based detection methods, such as Western blotting, immunocytochemistry, flow cytometry, or
ELISA. With a PSSA specific for the phosphorylated target protein, pre-incubation with an excess of peptide containing the sequence of the phosphopeptide immunogen will block all
antigen binding sites, while pre-incubation with the corresponding non-phosphopeptide will
not affect the antibody.
In performing the Peptide Competition Experiment, it is important to note that the optimal dilutions of both antibody and peptide should be determined empirically for each specific application. The optimal dilution of antibody in these procedures is below saturating, as determined by previous experiments in your system.
The optimal dilution of peptide used in these procedures will depend on the overall affinity or
avidity of the antibody, as well as the quantity of the target antigen. A 50-150 fold molar excess of peptide to antibody is found to be effective for most peptide competition experiments.
In the example presented below, the PSSA is used as a dilution of 1:1000 and the peptides are used at a concentration of 333 nM. The total volume of the phosphopeptide and nonphosphopeptide-pre-incubated antibody preparations is 2 mL, sufficient for probing Western blot strips, as well as for use in other antibody-based detection methods. Under these conditions, the molar excess of peptide to antibody is › / = 50.
Procedure:
1. Prepare three identical test samples, such as identical PVDF or nitrocellulose strips to which the protein of interest has been transferred. The test samples should be blocked using a blocking buffer, such as Tris buffered saline supplemented with 0.1% Tween 20, and either
5% BSA or 5% non-fat dried milk.
2. Prepare 6.5 mL of working antibody stock solution (1:1000 in this example) by adding 6.5 µL of antibody stock solution to 6.5 mL of buffer containing blocking protein, such as TBS supplemented with 0.1% Tween 20, and either 3% BSA or 3% non-fat dried milk.
3. Apportion the unused PSSA into working aliquots and store at at -20°C for future use (the stock PSSA contains 50% glycerol and will not freeze at this temperature).
4. Allow the lyophilized control peptides to reach room temperature, ideally under desiccation.
5. Reconstitute each of the control peptides to a concentration of 66.7 µM with nanopure water. (i.e. for a peptide with a molecular mass of 1500, reconstitution with 1 mL water yields
a solution with a concentration of 66.7 µM).
6. Apportion the unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.

7. Label 3 test tubes as follows:

- tube 1: water only no peptide control
- tube 2: phosphopeptide
- tube 3: non-phosphopeptide

8. Into each tube, pipette the following components:
- tube 1: 2 mL diluted PSSA solution plus 10 uL nanopure water
- tube 2: 2 mL diluted PSSA solution plus 10 uL phosphopeptide
- tube 3: 2 mL diluted PSSA solution plus 10 uL non-phosphopeptide

9. Incubate the three tubes for 30 minutes at room temperature with gentle rocking. During this incubation, the peptides have the chance to bind to the combining site of the antibody.
10. At the end of the incubation step, transfer the contents of each of the three tubes to clean
reaction vessels containing one of the three identical test samples.
For Western blotting strips:
Incubate the strips with the pre-incubated antibody preparations for 1 hour at room temperature or overnight at 4°C.
Wash each strip four times, five minutes each, to remove unbound antibody.
Transfer each strip to a new solution containing a labeled secondary antibody [e.g., goat F(ab')2 anti-rabbit IgG alkaline phosphatase conjugate or goat F(ab')2 anti-rabbit IgG horseradish peroxidase conjugate.
Remove unbound secondary antibody by thorough washing, and develop the signal using your chemiluminescent reagents and instrumentation.
The signal obtained with antibody incubated with the "Water Only, No Peptide Control" (Tube
1), represents the maximum signal in the assay. This signal should be eliminated by preincubation with the "Phosphopeptide" (Tube 2), while pre-incubation with the "Non-Phosphopeptide" (Tube 3) should not impact the signal. If the "Phosphopeptide" only partially
eliminates the signal, repeat the procedure using twice the volume of water or peptide solutions listed in Step 8. If partial competition is seen following pre-incubation with the "Non-Phosphopeptide", repeat the procedure using half the volumes of water or peptide solutions listed in Step 8.

Pictures:

Peptide Competition and Phosphatase Stripping: Extracts of Jurkat cells were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was either untreated (1-4) or treated with lambda phosphatase (5), blocked with a 5% BSA-TBST buffer for one hour at room temperature, then incubated with the p70-S6K [pT229] antibody for two hours at room temperature in a 3% BSA-TBST buffer, following its prior incubation with: no peptide (1,5), the non-phosphorylated peptide corresponding to the phosphopeptide immunogen (2), a generic phosphothreonine-containing peptide (3), or the phosphopeptide immunogen (4). After washing, the membrane was incubated with goat F(ab')2 anti-rabbit IgG HRP conjugate and signals were detected using the Pierce SuperSignal(TM) method. The data show that only the phosphopeptide corresponding to p70S6K [pT229] blocks the antibody signal, demonstrating the specificity of the antibody. The data also show that phosphatase stripping eliminates the signal, further verifying that the antibody is phospho-specific.