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Polyclonal Antibody to c-Raf [pS621]

Catalog No.: BP7037

Quantity: 0.1 ml

Concentration: 0.5 mg/ml

Host: Rabbit

Immunogen: The antiserum was produced against a chemically synthesized phosphopeptide derived from a region of human c-Raf that contains serine 621. The sequence is conserved in mouse and rat.

Applications: The antibody has been used for Western blotting applications. For Western blotting applications, we recommend using the antibody at 0.1-1.0 µg/mL. At 0.50 µg/mL, the dilution provides 100 mL working solution, which at 10 mL/blot allows 10 blots to be performed. Positive control used: Immunoprecipitates or cell lysates from EGF-stimulated Hek293 cells transfected with c-Raf. Other applications not tested. Optimal dilutions of this antibody are dependent on conditions and should be determined by the user.
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Specificity: The Raf family of serine/threonine-specific kinases is comprised of three members (A-Raf, B-Raf, and c-Raf) that play a critical role in regulating cell growth and differentiation, and couple growth factor receptor stimulation to nuclear transcription factors via the Ras/mitogen-activated protein kinase (MAPK) pathway. c-Raf kinase (also known as Raf-1) is a key 74 kDa signal transducer of multiple extracellular stimuli that is regulated by several pathways, and that once activated phosphorylates MEK which in turn phosphorylates ERK. Together with serine 43 and serine 259, serine 621 is one of the three constitutive phosphorylation sites of c-Raf in resting cells. Phosphorylation of serine 621 has been shown to positively or negatively regulate c-Raf kinase activity, depending on the system. Human c-Raf. Mouse and rat (100% homologous) c-Raf have not been tested, but are expected to react. A-Raf and B-Raf (89%) have not been tested, but may cross-react in cells expressing high levels of these proteins.

Add. Information: BP7037/ME0905
Western Blotting Procedure
1. Lyse approximately 107 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. This cell lysis buffer formulation is available as a separate product which requires supplementation with protease inhibitors immediately prior to use. 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 100oC.
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.
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) overnight at 4oC.
12. Incubate the blocked blot with primary antibody at a concentration of 0.1-1.0 _g/mL in Tris buffered saline supplemented with 3% BSA and 0.1% Tween 20 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 _g/mL aprotinin
10 _g/mL leupeptin
1 _g/mL pepstatin
(alternatively, protease inhibitor
cocktail such as Sigma catalog
number 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 4oC 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
To demonstrate the specificity of a Phosphorylation Site Specific Antibody, we recommend the following peptide competition experiment which uses our control peptides. These control peptides have the sequences of the phosphopeptide immunogen used to raise the antibody and the corresponding non-phosphorylated peptide. In the competition experiment, 200-500 fold molar excess of the phosphorylated and non-phosphorylated peptides are pre-incubated with aliquots of the antibody prior to use in immunoassay procedures.
A sample calculation for the determination of the 200 fold molar excess of peptide to antibody is presented below. The following assumptions have been made:
â?˜ The molecular mass of an IgG molecule is 150,000 daltons.
â?˜ Each mole of antibody binds two moles of peptide.
â?˜ The Phosphorylation Site Specific Antibody is used at a concentration of 0.5 µg/mL.
The optimal antibody concentration for use in peptide competition experiments is below saturating as determined by previous experiments in your system. If an optimal concentration has not been determined, it is suggested that the concentration provided on the antibody Product Analysis Sheet be used. A final antibody concentration of 0.5 µg/mL is satisfactory for most applications.
The molarity of the 0.5 µg/mL antibody solution is:
(0.5 µg/mL)(1000 mL/L)/(150,000 µg/µmole) = 0.00333 µM.
Because each mole of antibody binds two moles of peptide, 0.5 µg/mL antibody can bind 0.00667 µM of peptide.
A 200 fold molar excess of peptide is (200)(0.00667 µM) = 1.334 µM.
The following procedure describes peptide competition experiments using antibody at a concentration of 0.5 µg/mL and a 200 fold molar excess of peptides based on the calculation above, in a total volume of 2 mL.

Storage:

Store at -80oC. Upon initial thawing, apportion into working aliquots and store at -80oC. Avoid repeated freeze-thaw cycles to prevent denaturing the antibody.

General Readings:

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2. Dumaz N, Light Y, Marais R. Cyclic AMP blocks cell growth through Raf-1-dependent and Raf-1-independent mechanisms. Mol Cell Biol. 2002 Jun;22(11):3717-28. PubMed PMID: 11997508.
3. Zhang BH, Guan KL. Regulation of the Raf kinase by phosphorylation. Exp Lung Res. 2001 Apr-May;27(3):269-95. PubMed PMID: 11293329.

4. Gu M, Lynch J, Brecher P. Nitric oxide increases p21(Waf1/Cip1) expression by a cGMP-dependent pathway that includes activation of extracellular signal-regulated kinase and p70(S6k). J Biol Chem. 2000 Apr 14;275(15):11389-96. PubMed PMID: 10753954.
5. Thorson JA, Yu LW, Hsu AL, Shih NY, Graves PR, Tanner JW, et al. 14-3-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. Mol Cell Biol. 1998 Sep;18(9):5229-38. PubMed PMID: 9710607.
6. Mischak H, Seitz T, Janosch P, Eulitz M, Steen H, Schellerer M, et al. Negative regulation of Raf-1 by phosphorylation of serine 621. Mol Cell Biol. 1996 Oct;16(10):5409-18. PubMed PMID: 8816453.
7. Morrison DK, Heidecker G, Rapp UR, Copeland TD. Identification of the major phosphorylation sites of the Raf-1 kinase. J Biol Chem. 1993 Aug 15;268(23):17309-16. PubMed PMID: 8349614.

Protocols:

1. Prepare three identical test samples, such as identical nitrocellulose or PVDF strips with transferred protein. The test samples should be blocked with BSA or non-fat dried milk in a buffer compatible with an antibody based detection method, such as Tris buffered saline or phosphate buffered saline.
2. Slowly thaw the Phosphorylation Site Specific Antibody on ice.
3. Prepare 3 mL of a 2x (1 µg/mL) antibody stock solution in a buffer appropriate for the application. Suggested buffer formulations are TBS or PBS supplemented with blocking protein such as BSA or non-fat dried milk.
4. Apportion the unused Phosphorylation Site Specific Antibody into working aliquots and store at -80°C for future use.
5. The lyophilized control peptides should be warmed to room temperature, ideally under desiccation.
6. Reconstitute each of the control peptides to a concentration of 100 µM using nanopure water at room temperature. As indicated on the peptide labels, each vial contains 0.1 mg. For a peptide with a molecular mass of 1500, reconstitution with 0.67 mL water yields a solution with a concentration of 100 µM.
7. Allow the peptides to dissolve at room temperature, then gently triturate several times using a pipette. Avoid introducing air bubbles.
8. Label 3 test tubes as follows:
â?˘ tube 1: water only no peptide control
â?˘ tube 2: phosphopeptide
â?˘ tube 3: non-phosphopeptide
9. Prepare 2x peptide stock solutions (2.66 µM) or water control by pipetting the following:
â?˘ tube 1: water control stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL water.
â?˘ tube 2: phosphopeptide 2x stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL reconstituted (100 µM)
phosphopeptide.
â?˘ tube 3: non-phosphopeptide 2x stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL reconstituted (100 µM) non-phosphopeptide.
10. Apportion unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.
11. Pipette 1 mL of the 2x antibody stock into each of the tubes marked 1, 2, and 3. The tubes should be incubated for 30 minutes at room temperature with gentle rocking.
12. The pre-incubated antibody in each of the three tubes is then ready for use. Pipette the contents of each tube onto the three identical test samples.
For Western blotting strips:
â?¦ Incubate these strips for 2 hours at room temperature, followed by several washes to remove unbound antibody.
â?¦ Transfer each strip to a new solution containing a labeled secondary antibody (example

goat anti-rabbit IgG-alkaline phosphatase conjugate).
â?¦ Remove unbound secondary antibody by thorough washing and develop bands.
The signals obtained with antibody incubated with “(1) water only no peptide control”, which represents the maximum signal, and the signals obtained with “(2) phosphopeptide and “(3) non-phosphopeptide” are readily compared under these conditions.