

Polyclonal Antibody to Raf (c-Raf) pSer259 - Aff - Purified

Catalog No.:	BP7035
Quantity:	0.1 ml
Background:	<p>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. Serine 259 is one of the three constitutive phosphorylation sites of c-Raf in resting cells together with serine 43 and serine 621. Serine 259 is phosphorylated by PKA and is the main mediator of PKA-induced inhibition of c-Raf kinase activity. Phosphorylation of serine 259 blocks the membrane localization of c-Raf.</p>
Host:	Rabbit
Immunogen:	<p>Chemically synthesized phosphopeptide derived from a region of human c-Raf that contains serine 259.</p> <p>Remarks: The sequence is conserved in mouse and rat.</p>
Format:	<p>State: Liquid Ig fraction</p> <p>Purification: Immunoaffinity purified. 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 c-Raf. The final product is generated by affinity chromatography using a c-Raf-derived peptide that is phosphorylated at serine 259.</p> <p>Buffer System: Dulbecco's phosphate buffered saline (without Mg²⁺ and Ca²⁺), pH 7.3 (+/- 0.1), with 1.0 mg/mL BSA (IgG, protease free) as a carrier, containing 0.05 % sodium azide as preservative</p>
Applications:	<p>Western blot (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.</p> <p>Positive control: Immunoprecipitates of EGF-stimulated Hek293 cells transfected with c-Raf, and HCT-8 cells.</p> <p>Other applications not tested. Optimal dilutions are dependent on conditions and should be determined by the user.</p>
Specificity:	<p>This antibody detects c-Raf.</p> <p>Species: Human, mouse, rat.</p> <p>Other species not tested.</p>
Add. Information:	A-Raf (90%) was also shown to react in HCT-8 cells, a human cancer cell line expressing high levels of A-Raf.

Storage: Store at -80 °C. Upon initial thawing, aliquot and store at -80 °C. Avoid repeated freezing and thawing.
Shelf life: one year from despatch.

General Readings: Dhillon, A.S., et al. (2002) Cyclic AMP-dependent protein kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. *Mol. Cell. Biol.* 22(10):3237-3246.
Dumaz, N., et al. (2002) Cyclic AMP blocks cell growth through Raf-1 dependent and Raf-1-independent mechanisms. *Mol. Cell. Biol.* 22(11):3717-3728.
Zhang, B.H. and K.L. Guan (2001) Regulation of the Raf kinase by phosphorylation. *Exp. Lung Res.* 27(3):269-295.
Morrison, D.K., et al. (1993) Identification of the major phosphorylation sites of the Raf-1 kinase. *J. Biol. Chem.* 268(23):17309-17316.

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 concentration of 0.1-1.0 µg/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 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 $\times / = 50$.

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 3 mL of a 2x (1 $\mu\text{g}/\text{mL}$) 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.
3. Apportion the unused PSSA into working aliquots and store 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 μL nanopure water
 - tube 2: 2 mL diluted PSSA solution plus 10 μL phosphopeptide
 - tube 3: 2 mL diluted PSSA solution plus 10 μL 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

Total cell lysates (1) or immunoprecipitates (2-7) prepared from Hek293 cells overexpressing c-Raf and stimulated with EGF were resolved by SDS-PAGE on a 10% polyacrylamide gel and transferred to PVDF. Membranes were either untreated (1-6), or treated with lambda (λ) phosphatase (7), blocked with a 5% BSA-TBST buffer overnight at 4°C, then incubated with 0.50 μ g/mL c-Raf [pS259] antibody for two hours at room temperature in a 3% BSA-TBST buffer, following prior incubation with: no peptide (1, 2, 6, 7), the non-phosphopeptide corresponding to the immunogen (3), a generic phosphoserine-containing peptide (4), or, the phosphopeptide immunogen (5). After washing, membranes were incubated with goat F(ab')₂ anti-rabbit IgG alkaline phosphatase and signals were detected using the Tropix WesternStar™ method. The data show that only the peptide corresponding to c-Raf [pS259] blocks the antibody signal, thereby demonstrating the specificity of the antibody. The data also show that phosphatase stripping eliminates the signal, verifying that the antibody is phospho-specific.

