



Rabbit (polyclonal) Anti-c-Raf [pS⁶²¹] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number/Size:	44504G (10 mini-blot size)
Lot Number:	See product label
Volume:	100 μ L
Form of Antibody:	Rabbit polyclonal immunoglobulin in Dulbecco's phosphate buffered saline (without Mg ²⁺ and Ca ²⁺), pH 7.3 (+/- 0.1), 50% glycerol with 1.0 mg/mL BSA (IgG, protease free) as a carrier.
Preservative:	0.05% sodium azide (Caution: sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.)
Purification:	Purified from rabbit serum by 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 c-Raf. The final product is generated by affinity chromatography using a c-Raf-derived peptide that is phosphorylated at serine 621.
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.
Target Summary:	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.
Reactivity:	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% homologous) have not been tested, but may cross-react in cells expressing high levels of these proteins.
Applications:	The antibody has been used in Western blotting via immunoprecipitation. Other applications may work but have not been tested.
Suggested Working Dilutions:	For Western blotting via immunoprecipitation, we recommend using the antibody at a 1:1000 starting dilution. The exact concentration is not determined for each lot; however, the typical range is 0.1-1.0 mg/mL. The optimal antibody concentration should be determined empirically for each specific application.
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.
Expiration Date:	Expires one year from date of receipt when stored as instructed.
Positive Controls Used:	Immunoprecipitates of Hek293 cells transfected with human c-Raf and stimulated with EGF.

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Related Products:**Antibodies:**

c-Raf, Cat. # 44520G
 c-Raf [pS²⁵⁹], Cat. # 44502
 c-Raf [pSpY^{338/340}], Cat. # 44505G
 c-Raf [pYpY^{340/341}], Cat. # 44506G
 EGFR [pY⁸⁴⁵], Cat. # 44784G

Akt/PKB [pT³⁰⁸], Cat. 44602G
 Akt/PKB [pS⁴⁷³], Cat. # 44623G
 MEK1&2 [pS²²²], Cat. # 44452
 ERK1&2 [pTpY^{185/187}], Cat. # 44680G
 JNK1&2 [pTpY^{183/185}], Cat. # 44682G
 p38 [pTpY^{180/182}], Cat. # 44684G

ELISAs:

Akt [pS⁴⁷³], Cat. # KHO0111
 Akt [pT³⁰⁸], Cat. # KHO0201
 MEK1 [pSpS^{218/222}], Cat. # KHO0321

ERK1&2 [pTpY^{185/187}], Cat. # KHO0091
 JNK1&2 [pTpY^{183/185}], Cat. # KHO0131
 p38 MAPK [pTpY^{180/182}], Cat. # KHO0071

References:

Schulze, A., et al. (2004) The transcriptional response to Raf activation is almost completely dependent on Mitogen-activated Protein Kinase Kinase activity and shows a major autocrine component. *Mol. Biol. Cell.* 15(7):3450-3463.

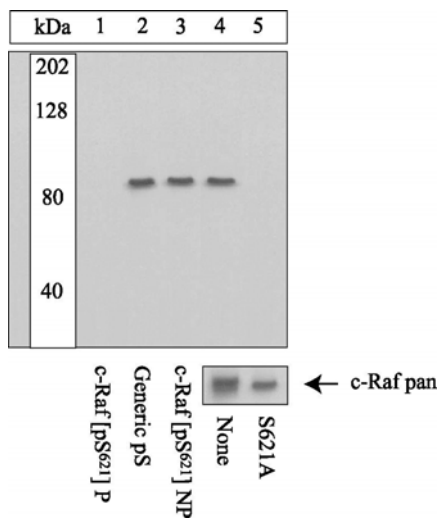
Dhillon, A.S., et al. (2002) Cyclic AMP-dependent protein kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. *Mol. Cell. Biol.* 22(10):3237-3246.

Morel, J.C., et al. (2002) Signal transduction pathways involved in rheumatoid arthritis synovial fibroblast interleukin-18-induced vascular cell adhesion molecule-1 expression. *J. Biol. Chem.* 277(38):34679-34691 (cites the use of cat. # 44506G, 44622G (replaced with 44623G) and 44660G).

Yan, F. and D.B. Polk (2001) Kinase suppressor of ras is necessary for tumor necrosis factor alpha activation of extracellular signal-regulated kinase/mitogen-activated protein kinase in intestinal epithelial cells. *Cancer Res.* 61(3):963-969 (cites the use of cat. # 44506G).

Gu, M., et al. (2000) 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.* 275:11389-11396 (cites the use of cat. # 44504G).

Thorson, J.A., et al. (1998) 14-3-3 Proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. *Mol. Cell. Biol.* 18:5229-5238 (cites the use of cat. 44504G).

**Antibody-Peptide Competition and Mutant Analysis**

Immunoprecipitates of Hek293 cells overexpressing wild-type c-Raf and stimulated with 50 ng/mL EGF for 3 minutes (1-4) or c-Raf mutant S621A (5) were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was blocked with a 5% BSA-TBST buffer overnight at 4°C, then incubated with the c-Raf [pS⁶²¹] antibody for two hours at room temperature in a 3% BSA-TBST buffer, following prior incubation with: the phosphopeptide immunogen (1), a generic phosphoserine-containing peptide (2), the non-phosphopeptide corresponding to the phosphopeptide immunogen (3), or no peptide (4, 5). The inset shows the relative amount of total c-Raf protein in the wild-type vs. the S621A mutant extracts via c-Raf pan antibody. After washing, the membrane was incubated with goat F(ab')₂ anti-rabbit IgG alkaline phosphatase (cat.# ALI4405) and signals were detected using the Tropix WesternStar™ method.

The data show that only the phosphopeptide corresponding to c-Raf [pS⁶²¹] blocks the antibody signal and that the antibody does not recognize the S621A mutant, demonstrating the specificity of the antibody.

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Western Blotting Procedure

1. Lyse approximately 10^7 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 (Invitrogen cat. # FNN0011). 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 nitrocellulose membrane slightly larger than the gel. Alternatively, PVDF activated for 1 minute with methanol and washed with ddH₂O 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) overnight at 4°C or for one hour at room temperature.
12. Incubate the blocked blot with primary antibody at a 1:1000 starting dilution Tris buffered saline supplemented with 3% Ig-free BSA and 0.1% Tween 20 overnight at 4°C or for two 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 (catalog number ALI4405) or goat F(ab')₂ anti-rabbit IgG horseradish peroxidase conjugate (catalog number ALI4404) 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 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 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 Ig-free BSA
0.1 mL Tween 20

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Peptide Competition Experiment

Invitrogen's Phosphorylation Site Specific Antibodies (PSSAs) have been developed to enable the specific and sensitive detection of phosphorylation of particular amino acid residues in target proteins, while circumventing the need for protein purification, phosphopeptide mapping or handling radioactivity. The specificity of a 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.

Invitrogen has developed a line of control peptides specifically for use in peptide competition experiments with our PSSAs. These peptides, available as separate Invitrogen catalog items, are provided in pairs which contain the sequences of the phosphopeptide immunogen and the corresponding non-phosphopeptide.

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. If an optimal antibody dilution has not been determined in your system, please refer to the Suggested Working Dilution on the antibody Product Analysis Sheet for guidance on an appropriate starting dilution. 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 at a dilution of 1:1000 and the peptides are used at a concentration of 333 nM. The total volume of the phosphopeptide and non-phosphopeptide-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 μ L 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 -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 (supplied at 0.1 mg/vial) to a concentration of 66.7 μ M with nanopure water. For a peptide with a molecular mass of 1500 (stated on the peptide Product Analysis Sheet), reconstitution with 1 mL water yields a solution with a concentration of 66.7 μ M.
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 μ L nanopure water
 - tube 2: 2 mL diluted PSSA solution plus 10 μ L phosphopeptide
 - tube 3: 2 mL diluted PSSA solution plus 10 μ L 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 (Cat. # ALI4405) or goat F(ab')₂ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404)].
- 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 pre-incubation 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.

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