Rabbit (polyclonal) Anti-c-Ret [pS^{696}] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number/Size:	44-274 (10 blot size)
Lot Number:	See product label
Volume/Concentration:	See product label
Form of Antibody:	Rabbit polyclonal immunoglobulin in Dulbecco's phosphate buffered saline (without Mg^{2+} and Ca^{2+}), pH 7.3 (+/- 0.1), 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-Ret. The final product is generated by affinity chromatography using c-Ret-derived peptide that is phosphorylated at serine 696.
Immunogen:	The antiserum was produced against a chemically synthesized phosphopeptide derived from a region of human c-Ret that contains serine 696.
Target Summary:	c-Ret is a 170 kDa tyrosine kinase receptor for glial cell line-derived neurotrophic factors (GDNFs: neurturin, persephin, and artemin) and plays an important role in the development of the enteric nervous system and the kidney. In humans, inactivating mutations of c-Ret results in Hirschsprung's disease, and constitutively active c-Ret mutations result in endocrine neoplasia and familial medullary thyroid carcinoma. Activation of the c-Ret can initiate a cascade of events including the activation of the Ras → Raf → ERK1&2 pro-growth signaling pathway. c-Ret is regulated by phosphorylation on multiple tyrosine and serine sites. Phosphorylation of c-Ret on serine 696 by protein kinase A (PKA) promotes lamellipodia formation of neuronal cells induced by GDNF, a critical step in neuritegenesis.
Reactivity:	Human c-Ret. Mouse (83% homologous) and rat (92%) c-Ret have not been tested, but are expected to react.
Applications:	The antibody has been used for Western blotting applications.
Suggested Working Dilutions:	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. The optimal antibody concentration should be determined empirically for each specific application.
Storage:	Store at −80°C. Upon initial thawing, apportion into working aliquots and store at −80°C. Avoid repeated freeze-thaw cycles to prevent denaturing the antibody.
Expiration Date:	Expires one year from date of receipt when stored as instructed.
Positive Controls Used:	GDNF-stimulated Neuro-2A cells or TT medullary thyroid carcinoma cells.

This product is for research use only. Not for use in diagnostic procedures.

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Related Products:

Antibodies:

Dok-2 [pY^{142}] (mouse), Cat. # 44-264	PAK1/2/3 [pS^{141}], Cat. # 44-940G
c-Jun [pS^{73}], Cat. # 44-292G	Pro-Growth Sample Pack, Cat. # 44-587G
Vav1 [pY^{160}], Cat. # 44-482	Tyrosine kinase inhibitor PP1, Cat. # PHZ1213

References:

Fukuda, T., et al. (2002) Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase. J. Biol. Chem. 277(21):19114-19121.

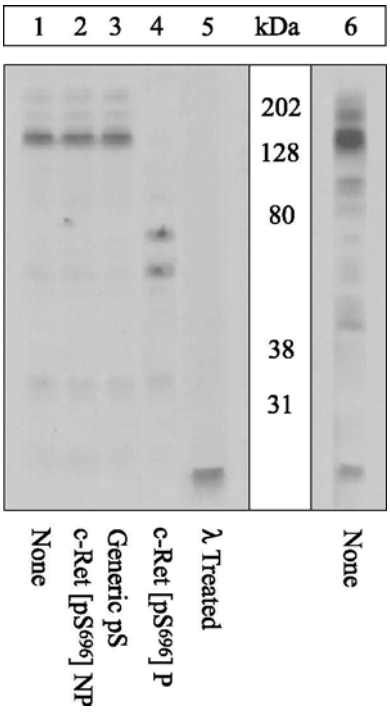
Carlomagno, F., et al. (2002) The kinase inhibitor PP1 blocks tumorigenesis induced by RET oncogenes. Cancer Res. 62(4):1077-1082.

Cohen, M.S., et al. (2002) Inhibition of medullary thyroid carcinoma cell proliferation and RET phosphorylation by tyrosine kinase inhibitors. Surgery 132(6):960-967.

You, L., et al. (2001) Glial cell-derived neurotrophic factor (GDNF)-induced migration and signal transduction in corneal epithelial cells. Invest. Ophthalmol. Vis. Sci. 42(11):2496-2504.

Jing, S., et al. (1996) GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. Cell 85(7):1113-1124.

Edery, P., et al. (1994) Mutations of the RET proto-oncogene in Hirschsprung's disease. Nature 367(6461):378–380.



Peptide Competition and Phosphatase Treatment

Lysates prepared from Neuro-2A cells stimulated with GDNF (1-5) or TT cells (6) were resolved by SDS-PAGE on a 10% polyacrylamide gel and transferred to PVDF. Membranes were either left untreated (1-4, 6) or treated with lambda (λ) phosphatase (5), blocked with a 3% BSA-TBST buffer overnight at 4°C and incubated with 0.50 µg/mL c-Ret [pS^{696}] antibody for two hours at room temperature in a 3% BSA-TBST buffer, following prior incubation with: no peptide (1, 5, 6), the non-phosphopeptide corresponding to the immunogen (2), a generic phosphoserine containing peptide (3), or, the phosphopeptide immunogen (4). After washing, membranes were 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 peptide corresponding to c-Ret [pS^{696}] blocks the antibody signal and that phosphatase stripping eliminates the signal, thereby 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 catalog number 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 PVDF membrane slightly larger than the gel. Soak the membrane in methanol for 1 minute, then rinse with ddH_2O 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 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 (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 $Na_4P_2O_7$
2 mM Na_3VO_4
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
3 gm BSA
0.1 mL Tween 20

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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:

- The molecular mass of an IgG molecule is 150,000 daltons.
- Each mole of antibody binds two moles of peptide.
- 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.

Procedure:

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:
   - tube 1: water only no peptide control
   - tube 2: phosphopeptide
   - tube 3: non-phosphopeptide
9. Prepare 2x peptide stock solutions (2.66 µM) or water control by pipetting the following:
   - tube 1: water control stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL water.
   - tube 2: phosphopeptide 2x stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL reconstituted (100 µM) phosphopeptide.
   - 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:
    - ♦ Incubate these strips for 2 hours at room temperature, followed by several washes to remove unbound antibody.
    - ♦ Transfer each strip to a new solution containing a labeled secondary antibody (example goat anti-rabbit IgG-alkaline phosphatase conjugate).
    - ♦ 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.

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