



cell signaling solutions

## Certificate of Analysis

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### Anti-Phosphotyrosine

(rabbit immunoaffinity purified IgG)

Catalog # 06-427

Lot # 28136

**Immunogens:** In order to produce broad spectrum polyclonal phosphotyrosine antibodies, rabbits were immunized with three phosphorylated immunogens: (1) phosphotyrosine covalently linked to KLH; (2) the c-Src carboxyl terminal regulatory phosphopeptide (T-S-T-E-P-Q-pY-Q-P-G-E-N-L; Catalog # 12-218) covalently linked to KLH, and; (3) a phosphopeptide associated with high tyrosine kinase activity in human lymphocytes (R-R-L-I-E-D-A-E-pY-A-A-R-G; Catalog # 12-217) covalently linked to KLH. Both of the phosphopeptide haptens serve as strong substrates for tyrosine phosphatases and are part of the two colorimetric tyrosine phosphatase kits provided by Upstate, Inc. (Catalog # 17-125, 17-126).

**Species Cross-reactivity:** Human, mouse and rat. Other species cross-reactivity is unknown.

**Specificity and Purification:** The immunoreactivity of the antibody is totally inhibited by the use of 100mM phenyl phosphate, a phosphotyrosine analog. The phosphotyrosine antibody is purified by immunoaffinity chromatography using either a dual phospho-peptide gel or a BSA-phosphotyrosine gel. All of the phosphotyrosine immunoreactivity present in the antisera is immunoabsorbed whether the antibody is purified by either gel indicating that the antibody is not sequence-specific but specific for phosphotyrosine residues.

**Formulation:** 200µg of immunoaffinity purified rabbit IgG in 900µl of 0.2M Tris-glycine, pH 7.2, 0.15M NaCl, 5mg/ml of BSA containing 0.05% sodium azide. Frozen solution.

**Storage and Stability:** Stable for 1 year at -20°C from date of shipment. Aliquot to avoid repeated freezing and thawing. For maximum recovery of the product, centrifuge the original vial after thawing and prior to removing the cap.

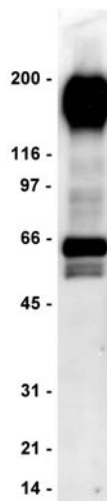
**FOR RESEARCH USE ONLY  
NOT FOR USE IN HUMANS**

### Quality Control Testing

**Immunoblot Analysis:** 0.5-2µg/ml of this lot detected proteins containing phosphotyrosine residues in RIPA lysates from EGF-stimulated human A431 carcinoma cells.

**Included Positive Antigen Control:** Catalog # 12-302, EGF-stimulated A431 cell lysate. **Add 2.5µl of 2-mercaptoethanol/100µl of lysate and boil for 5 minutes to reduce the preparation.** Load 20µg of reduced lysate per lane for minigels.

**Immunoprecipitation:** 4µg of a previous lot immunoprecipitated proteins containing phosphotyrosine residues from a human A431 RIPA lysate.



#### Immunoblot Analysis

Representative blot from a previous lot. EGF-stimulated A431 cell lysate was resolved by electrophoresis, transferred to nitrocellulose and probed with anti-Phosphotyrosine (0.5µg/ml). Proteins were visualized using a goat anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system.

#### References:

1. The cSrc carboxyl terminal regulatory phosphopeptide (T-S-T-E-P-Q-pY-Q-P-G-E-N-L) which binds to the internal SH2 domain of c-Src.  
Song, Z., *et al.*, Cell **72**:767, 1993.  
Luttrel, D.K., *et al.*, Proc. Natl. Acad. Sci. USA **91**: 83, 1994.
2. The phosphopeptide associated with high tyrosine kinase activity in human lymphocytes (R-R-L-I-E-D-A-E-pY-A-A-R-G).  
Trevillyan, J.M., *et al.*, Biochim. Biophys. Acta **845**: 1, 1985.

#### Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a cell lysate sample (cell lysis buffer: 50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EDTA; 1mM PMSF; 1 $\mu$ g/ml aprotinin, leupeptin, pepstatin; 1mM Na<sub>3</sub>VO<sub>4</sub>; 1mM NaF) and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Block the blotted nitrocellulose in freshly prepared PBS containing 3% nonfat dry milk (Catalog # 20-200) and 0.05% Tween-20 (PBST-MLK) for 20 minutes at room temperature with constant agitation.
3. Incubate the nitrocellulose with **0.5-2 $\mu$ g/ml of anti-Phosphotyrosine**, diluted in freshly prepared PBST-MLK, overnight with agitation at 4°C.
4. Wash the nitrocellulose twice with water.
5. Incubate the nitrocellulose in the secondary reagent of choice (a **goat anti-rabbit** HRP conjugated IgG, Catalog # 12-348, 1:5000 dilution, was used) in PBST-MLK for 1.5 hours at room temperature with agitation.
6. Wash the nitrocellulose with water twice.
7. Wash the nitrocellulose in PBS-0.05% Tween 20 for 3-5 minutes.
8. Rinse the nitrocellulose in 4-5 changes of water.
9. Use detection method of choice (enhanced chemiluminescence was used).

#### Immunoprecipitation Protocol

1. Add **4 $\mu$ g of anti-Phosphotyrosine** and 60 $\mu$ l (30 $\mu$ l packed beads) of washed Protein A agarose bead slurry (Catalog # 16-125) to 500 $\mu$ l of PBS in a microcentrifuge tube.
2. Gently rock the reaction mixture at 4°C for 1 hour.
3. Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at 14,000 x g), and drain off the supernatant. Wash the beads 3 times with either ice-cold cell lysis buffer or PBS.
4. Dilute the cell lysate to roughly 1 $\mu$ g/ $\mu$ l total cell protein with PBS.
5. Add 500 $\mu$ g-1mg cell lysate to the reaction mixture.
6. Gently rock the reaction mixture at 4°C for 1 hour.
7. Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at 14,000 x g), and drain off the supernatant. Wash the beads 3 times with either ice-cold cell lysis buffer or PBS.
8. Resuspend the agarose beads in 60 $\mu$ l 2X Laemmli sample buffer.
9. Store the beads frozen for future analysis or boil the beads for 5 minutes.
10. Collect the beads after boiling using a microcentrifuge pulse.
11. Perform SDS-PAGE and immunoblot analysis on a sample of the supernatant fraction.