

## Certificate of Analysis

### Anti-Phosphotyrosine, clone 4G10

(mouse monoclonal IgG<sub>2bκ</sub>)

Catalog # 05-321

Lot # 22584

**Immunogen:** Phosphotyramine-KLH.

**Antibody Class:** IgG<sub>2bκ</sub> mouse monoclonal antibody produced *in vitro* by mouse-mouse hybridoma 4G10 (FOX-NY [NS-1 derivative] myeloma x spleen cells). Purified by Protein A-Sepharose chromatography.

**Formulation:** 100mg of mouse monoclonal IgG<sub>2bκ</sub> in 100ml of 0.01M Tris-HCl, pH 8.0, 0.15M NaCl, 0.02% sodium azide. Liquid.

**Storage and Stability:** Stable for 6 months at 4°C from date of shipment. **NOTE: DO NOT FREEZE.** For maximum recovery of the product, centrifuge the original vial prior to removing the cap. If the product has accidentally been frozen and thawed, spin it at 13,000 x g for 10 minutes at 4°C. Save the supernatant for application.

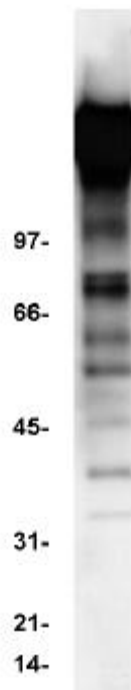
**FOR IN VITRO RESEARCH USE ONLY  
NOT FOR USE IN HUMANS OR ANIMALS**

### Quality Control Testing

**Immunoblot Analysis:** 0.5-2µg/ml of this lot detected tyrosine-phosphorylated proteins in a modified RIPA lysate from EGF-treated human A431 carcinoma cells.<sup>1,2,3.</sup>

**Included Positive Antigen Control:** Catalog #12-302, EGF-stimulated A431 cell lysate is provided as a free positive antigen control for western immunoblotting. Aliquot as desired, refreeze immediately, and store at -20°C. The lysate is stable for 6 months at -20°C. Before use, **add 2.5ml of 2-mercaptoethanol/100ml of lysate and boil for 5 minutes to reduce the preparation.** Load 20µg of reduced lysate per lane for immunoblot analysis.

**Immunoprecipitation:** 2-4µg of this lot can immunoprecipitate quantitatively the phosphotyrosine-containing proteins in the lysate of a confluent culture (10cm dish) of cells expressing an activated tyrosine kinase. To preserve phosphotyrosine, add 0.2mM sodium orthovanadate to the lysis buffer.



#### 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 (1µg/ml). Proteins were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a chemiluminescence detection system.

### References:

1. Cohen, B., *et al.*, Proc. Natl. Acad. Sci. USA. **87**: 4458-4462, 1990.
2. Druker, B.J., *et al.*, New Eng. J. Med. **321**: 1383-1391, 1989.
3. Kanakura, Y., *et al.*, J. Biol. Chem. **266**: 490-495, 1991.

### Immunoprecipitation Protocol

1. Before beginning the immunoprecipitation, dilute the cell lysate to roughly  $1\mu\text{g}/\mu\text{l}$  total cell protein in a microcentrifuge tube with PBS.
2. Add **2-4mg of anti-Phosphotyrosine, clone 4G10** to  $500\mu\text{g}$ -1mg cell lysate.
3. Gently rock the reaction mixture at  $4^{\circ}\text{C}$  overnight.
4. Capture the immunocomplex by adding  $100\mu\text{l}$  ( $50\mu\text{l}$  packed beads) of washed Protein A agarose bead slurry, Catalog # 16-125.
5. Gently rock the reaction mixture at  $4^{\circ}\text{C}$  for 2 hours.
6. Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at  $14,000 \times g$ ), and drain off the supernatant. Wash the beads 3 times with either ice-cold cell lysis buffer or PBS.
7. Resuspend the agarose beads in  $50\mu\text{l}$  2X Laemmli sample buffer.
8. The agarose beads can either be frozen for later use or boiled for 5 minutes. The beads are collected by a microcentrifuge pulse and SDS-PAGE and subsequent immunoblot analysis can be performed on a sample of the supernatant.

### 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;  $1\mu\text{g}/\text{ml}$  aprotinin, leupeptin, pepstatin; 1mM  $\text{Na}_3\text{VO}_4$ ; 1mM NaF) and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Block the blotted nitrocellulose in freshly prepared 3% nonfat dry milk (Catalog # 20-200) in PBS (PBS-MLK) for 20 minutes at  $20$ - $25^{\circ}\text{C}$  with constant agitation.
3. Incubate the nitrocellulose with  **$0.5$ - $2\text{mg}/\text{ml}$  of anti-Phosphotyrosine, clone 4G10**, diluted in freshly prepared PBS-MLK overnight with agitation at  $4^{\circ}\text{C}$ .
4. Wash the nitrocellulose twice with water.
5. Incubate the nitrocellulose in the secondary reagent of choice (a goat anti-mouse HRP conjugated, Catalog # 12-349, 1:2000 dilution, was used) in PBS-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 with a 30 second exposure was used).