

## Anti-Phosphotyrosine Immunoblotting Kit (ECL Detection System)

Catalog # 17-153

Lot # 18368

### Kit Components

**Anti-Phosphotyrosine**, Catalog # 05-321, Lot # 18265. One vial containing **100ng** of mouse monoclonal IgG<sub>2bκ</sub> in **100ml** of 0.01M Tris-HCl, 0.15M NaCl, pH 8.0 containing 0.02% sodium azide. Protein was determined by a Bradford microtiter protein assay. See page two for more information.

**Phosphorylated A431 Protein Preparation (Positive control)**, Catalog # 12-302, Lot # 18020. Two vials, each vial containing **100ng** in **100ml** of RIPA/non-reducing sample buffer. Concentration: 1μg/μl. See page two for more information.

**Goat Anti-Mouse IgG (H+L) HRP Conjugate**, Catalog # 12-326, Lot # 18373. One vial containing **100μl** of goat IgG, conjugated with horseradish peroxidase, in 0.02M potassium phosphate, 0.15M NaCl, pH 7.2, with 10mg/ml BSA, 50% glycerol and 0.1% thimerosal. See page two for more information.

**Note: This product is light sensitive.**

**Blocking Reagent**, Catalog # 17-105b, Lot # 18097

One vial containing **20g** non-fat skim milk.

**ECL Western Blot Detection Reagent A**, Catalog # 17-153a, Lot # 18366. One vial containing **20ml**. Store at 2-8°C.

**ECL Western Blot Detection Reagent B**, Catalog # 17-153b, Lot # 18367. One vial containing **20ml**. Store at 2-8°C.

**ECL Autorad Orientation Markers**, Catalog # 20-135, Lot # 18372. One packet containing 5 markers. Use one-half marker per nitrocellulose blot.

**Note:** Markers are light sensitive but must be activated before use by exposing to light for 1-5 minutes.

**FOR IN VITRO RESEARCH USE ONLY. NOT RECOMMENDED OR INTENDED FOR  
DIAGNOSIS OF DISEASE IN HUMANS OR ANIMALS  
DO NOT USE IN HUMANS OR ANIMALS**

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### Kit Description

**Quantity:** 10 blots per kit.

**Quality Control:** The reagents in this kit have been matched to optimize the range and sensitivity of detection using EGF-stimulated human A431 cells as a source of phosphotyrosine containing proteins.

**Storage and Stability:** Stable for 6 months at 2-8°C from date of shipment. **Note:** Upon receipt, aliquot and store Catalog # 12-302, Phosphorylated A-431 Protein Preparation and Catalog # 12-326, Goat Anti-Mouse IgG (H+L), at -20°C for optimal performance. Store Catalog # 05-321 at 4°C for optimal performance.

**Use:** Read the enclosed protocol before use.

## Technical Information for Kit Components

### Anti-Phosphotyrosine (monoclonal IgG<sub>2bκ</sub>)

**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.

**Physical Form:** 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.

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#### 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.

### EGF-Stimulated A431 Protein Prep. (in non-reducing sample buffer)

**Product Description:** Cellular protein preparation from A431 cells containing phosphorylated proteins, including the EGF receptor. A431 cells were cultured for 20 minutes in the presence of 50ng/ml EGF. Cells were lysed in modified RIPA buffer (50mM Tris-HCl, pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM PMSF, 1µg/ml aprotinin, 1µg/ml leupeptin, 1µg/ml pepstatin, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM NaF) and diluted with non-reducing sample buffer (31mM Tris-HCl, pH 6.8, 5% glycerol, 1% SDS, 0.002% bromphenol blue).

**Physical Form:** Frozen solution.

**Storage and Stability:** Stable for 6 months at -20°C from date of shipment. For maximum recovery of product, centrifuge the original vial after thawing and prior to removing the cap. Aliquot to avoid repeated freezing and thawing.

**Note:** 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 immunoblot analysis. This preparation may be used as a positive control for some of Upstate Biotechnology's antibodies.

### Anti-Mouse IgG, HRP-conjugated (goat polyclonal IgG)

**Immunogen:** Highly purified whole mouse IgG (heavy and light chains).

**Physical Form:** Frozen liquid.

maximum recovery of the product, centrifuge the original vial after thawing and prior to removing the cap.

**Storage and Stability:** Stable for 1 year at -20°C. Aliquot to avoid repeated freezing and thawing. For

## Western Immunoblotting Analysis Protocol

**Safety Precautions:** Safety information for ECL Reagents A and B is provided on the Material Safety Data Sheets (MSDS) enclosed with the kit components. Read carefully prior to use.

### Reagent Preparation:

1. **Blocking Buffer:** Dissolve 0.9g of "Blocking Reagent," Catalog # 17-105b in 30ml of phosphate buffered saline (PBS), pH 7.4.
2. **Primary Antibody Solution:** Add 10-20µg of Anti-Phosphotyrosine Antibody, Catalog # 05-321, to 10ml of **freshly** prepared Blocking Buffer. Mix well and store at 2-8°C.  
**Note: This solution can be reused ONCE within one week.**
3. **Secondary Antibody Solution:** Add 10µl of Goat Anti-Mouse IgG (H+L) HRP-Conjugate, Catalog # 12-326, to 10ml of Blocking Buffer. Mix well and store in the dark at 2-8°C, or prepare immediately before use.

### Procedure:

#### **Prior to Immunodetection:**

1. Prepare the samples and "Positive Control" (Catalog # 12-302) for electrophoresis and immunoblotting. Load the SDS-PAGE gel with the samples to be tested and 10µl of the Positive Control.  
**Note: A positive control should be included with every analysis.**
2. Perform electrophoresis and blot to nitrocellulose filter paper.
3. Wash the nitrocellulose filter two times with fresh changes of PBS (phosphate buffered saline) or distilled water.

#### **Immunodetection:**

1. Block the blotted nitrocellulose filter by immersing in 10ml of **freshly** prepared Blocking Buffer for 30 minutes at room temperature.
2. Incubate the nitrocellulose filter in the "Primary Antibody Solution" overnight at 2-8°C. **The solution can be saved for one additional use!!**
3. Wash the nitrocellulose filter two times with fresh changes of PBS or distilled water.
4. Incubate the nitrocellulose filter in the "Secondary Antibody Solution" for one and one-half hours at room temperature.
5. Wash the nitrocellulose filter two times with fresh changes of PBS or distilled water.
6. Wash the nitrocellulose filter in PBS-0.05% Tween 20 for 5 minutes with constant rocking.
7. Rinse the nitrocellulose filter four-five times with fresh changes of PBS or distilled water.
8. Lay the nitrocellulose on a transparency sheet, Blot excess water from nitrocellulose membrane with paper towel being careful not to allow blot to dry out.
9. Combine 2ml ECL Reagent A and 2ml ECL Reagent B. Mix thoroughly.

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**Immunodetection:**

10. Cover the nitrocellulose, using a pipet, with a uniform layer of the ECL Reagent mixture.
11. Incubate for precisely 1 minute at room temperature.
12. Drain and wick the excess ECL reagent mixture with a paper towel, making sure that the nitrocellulose does not dry out.
13. Place an ECL/Autorad orientation marker (Catalog # 20-135) next to the nitrocellulose.
14. Cover the nitrocellulose with a second transparency sheet being careful to remove all bubbles on the nitrocellulose. **Note:** Expose as soon as possible, may be stored in the dark for up to 30 minutes.
15. Place the transparency covered nitrocellulose in a film cassette, cover entirely with a piece of film and close the cassette securely.
16. Start with a 40 second exposure, remove the film from the cassette and develop. Re-exposure for longer or shorter periods may be necessary depending on intensity of staining.
17. Develop the exposed film for 2 minutes in developer, rinse for 30 seconds in water and fix for 4 minutes in fixer.