

Anti-Phosphotyrosine Western Blotting Kit (ECL Detection System)

Catalog # 17-153
Lot # 17095

Kit Components

Anti-Phosphotyrosine, Catalog # 05-321, Lot # 17047. One vial containing **100mg** of mouse monoclonal IgG_{2bκ} 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 # 16714. Two vials, each vial containing **100mg** 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 # 14605. 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 # 16309
One vial containing **20g** non-fat skim milk.

ECL Western Blot Detection Reagent A, Catalog # 17-153a, Lot # 17111. One vial containing **20ml**. Store at 2-8°C.

ECL Western Blot Detection Reagent B, Catalog # 17-153b, Lot # 17112. One vial containing **20ml**. Store at 2-8°C.

ECL Autorad Orientation Markers, Catalog # 20-135, Lot # 17113. 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.

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.

FOR LABORATORY RESEARCH USE ONLY
NOT RECOMMENDED OR INTENDED FOR DIAGNOSIS OF DISEASE IN HUMANS OR ANIMALS
DO NOT USE IN HUMANS OR ANIMALS

Technical Information for Kit Components

Anti-Phosphotyrosine (monoclonal IgG_{2bκ})

Immunogen: Phosphotyramine-KLH.

Antibody Class: IgG_{2bκ} 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.

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 Na₃VO₄, 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.

Storage and Stability: Stable for 1 year at -20°C. 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.

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.