

Applications	Reactivity	Sensitivity	Isotype
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W IP IHC-P E-P All Endogenous Rabbit IgG

Applications Key: W=Western Blotting IP=Immunoprecipitation IHC-P=Immunohistochemistry (Paraffin) E-P=ELISA (Peptide)

Reactivity Key: All=All species expected

Species cross-reactivity is determined by western blot.

Protocols

2325:

ELISA-Peptide Assay Protocol

A. Solutions and Reagents

- Carbonate Buffer:** 15 mM Na₂CO₃, 35 mM NaHCO₃, 0.2 g/L NaN₃ (pH 9.6). Use 1 μM synthetic peptide in carbonate buffer.
- 10X Phosphate Buffered Saline (PBS):** To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na₂HPO₄) and 2.4 g potassium phosphate, monobasic (KH₂PO₄) to 1 L dH₂O. Adjust pH to 7.4.
- Wash Buffer:** 1X PBS containing 0.05% Tween-20 (PBST)
- Blocking Buffer:** 10 mg/ml bovine serum albumin (BSA) in PBST
- Antibody Dilution Buffer:** 3% BSA in PBST
- DELFLIA[®] Europium-labeled Anti-mouse IgG for mouse primary antibodies or Anti-rabbit IgG ([PerkinElmer Life Sciences #AD0124](#)) for rabbit primary antibodies.
- DELFLIA[®] Enhancement Solution ([PerkinElmer Life Sciences #1244-105](#))
(DELFLIA[®] is a registered trademark of PerkinElmer, Inc.)

B. Protocol

- Coat the wells of a 96-well microtiter plate with 100 μl of 1 μM synthetic peptide in carbonate buffer by incubating overnight at 4 °C or for 2 to 6 hours at 37 °C. If the peptide does not bind or absorb, try other buffers in the pH 4–8 range.
- Wash plate three times 200 μl/well with wash buffer.
- Block plate with 200 μl/well blocking buffer for 1 hour at 37°C. Wash plate three times with wash buffer. (May leave dry plate at 4 °C for 1–2 months if desired.)
- Prepare appropriate dilution of primary antibody with antibody dilution buffer. Add 100 μl to wells and incubate at 37 °C for 1 hour.
- Wash three times with wash buffer.
- Add 67 ng/well DELFLIA Europium-labeled Anti-mouse IgG, diluted in 100 μl/well antibody dilution buffer. Incubate at 37 °C for 30 minutes.
- Wash five times with wash buffer.

8. Add 100 µl enhancement solution and incubate at 37 °C for 15 minutes. Read plate at 615 nm with an appropriate time-resolved plate reader.

Immunohistochemistry Protocol (Paraffin)

***IMPORTANT:** See product data sheet for the appropriate antibody diluent and antigen unmasking procedure. **IHC**

Protocol: Unmasking buffer/antibody diluent.

A. Solutions and Reagents

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH₂O)
4. Hematoxylin (optional)
5. **Wash Buffer:**

1X TBS/0.1% Tween-20 (1X TBST): To prepare 1 L add 100 ml 10X TBS to 900 ml dH₂O. Add 1 ml Tween-20 and mix.

10X Tris Buffered Saline (TBS): To prepare 1 L add 24.2 g Trizma[®] base (C₄H₁₁NO₃) and 80 g sodium chloride (NaCl) to 1 L dH₂O. Adjust pH to 7.6 with concentrated HCl.
6. ***Antibody Diluent:**
 - a. **SignalStain[®] Antibody Diluent #8112**
 - b. **TBST/5% normal goat serum (#5425):** To 5 ml 1X TBST add 250 µl normal goat serum.
 - c. **PBST/5% normal goat serum (#5425):** To 5 ml 1X PBST add 250 µl normal goat serum.

1X PBS/0.1% Tween-20 (1X PBST): To prepare 1 L add 100 ml 10X PBS to 900 ml dH₂O. Add 1 ml Tween-20 and mix.

10X Phosphate Buffered Saline (PBS): To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na₂HPO₄) and 2.4 g potassium phosphate, monobasic (KH₂PO₄) to 1 L dH₂O. Adjust pH to 7.4.
7. ***Antigen Unmasking:**
 - a. **Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trisodium salt dihydrate (C₆H₅Na₃O₇•2H₂O) to 1 L dH₂O. Adjust pH to 6.0.
 - b. **EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 1 L dH₂O. Adjust pH to 8.0.
 - c. **TE:** 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1L add 1.21 g Trizma[®] base (C₄H₁₁NO₃) and 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 950 ml dH₂O. Adjust pH to 9.0, then adjust final volume to 1000 ml with dH₂O.
 - d. **Pepsin:** 1 mg/ml in Tris-HCl pH 2.0.
8. **3% Hydrogen Peroxide:** To prepare, add 10 ml 30% H₂O₂ to 90 ml dH₂O.
9. **Blocking Solution:** TBST/5% normal goat serum (#5425): to 5 ml 1X TBST add 250 µl normal goat serum.
10. Biotinylated secondary antibody.
11. **ABC Reagent:** (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer's instructions 30 minutes before use.
12. **DAB Reagent or suitable substrate:** Prepare according to manufacturer's recommendations.

B. Deparaffinization/Rehydration

NOTE: Do not allow slides to dry at any time during this procedure.

1. **Deparaffinize/hydrate sections:**
 - a. Incubate sections in three washes of xylene for 5 minutes each.
 - b. Incubate sections in two washes of 100% ethanol for 10 minutes each.
 - c. Incubate sections in two washes of 95% ethanol for 10 minutes each.
2. Wash sections twice in dH₂O for 5 minutes each.

C. *Antigen Unmasking

NOTE: Consult product data sheet for specific recommendation for the unmasking solution.

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0 then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.
2. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.
3. **For TE:** Bring slides to a boil in 10 mM TE/1 mM EDTA, pH 9.0 then maintain at a sub-boiling temperature for 18 minutes. Cool on the bench for 30 minutes.
4. **For Pepsin:** Digest for 10 minutes at 37 °C.

D. Staining

1. Wash sections in dH₂O three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes.
3. Wash sections in dH₂O twice for 5 minutes each.

NOTE: Consult product data sheet for recommended antibody diluent.
4. Wash sections in wash buffer for 5 minutes.
5. Block each section with 100-400 µl blocking solution for 1 hour at room temperature.
6. Remove blocking solution and add 100-400 µl primary antibody diluted in recommended antibody diluent to each section. Incubate overnight at 4 °C.
7. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
8. Add 100-400 µl biotinylated secondary antibody, diluted in TBST per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.
9. If using ABC avidin/biotin method, prepare ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
10. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
11. Add 100-400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.
12. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
13. Add 100-400 µl DAB or suitable substrate to each section and monitor staining closely.
14. As soon as the sections develop, immerse slides in dH₂O.
15. If desired, counterstain sections in hematoxylin per manufacturer's instructions.

16. Wash sections in dH₂O two times for 5 minutes each.
17. Dehydrate sections:
 - a. Incubate sections in 95% ethanol two times for 10 seconds each.
 - b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
 - c. Repeat in xylene, incubating sections two times for 10 seconds each.
18. Mount coverslips.

Immunoprecipitation Protocol / (For Analysis By Western Immunoblotting)

For **shorter assay times** please try our [Immunoprecipitation Protocol Utilizing Magnetic Separation / \(For Analysis By Western Immunoblotting\)](#).

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. 1X Phosphate Buffered Saline (PBS)
2. **1X Cell Lysis Buffer:** ([#9803](#)) 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml Leupeptin
NOTE: Add 1 mM PMSF immediately prior to use.
3. **Protein A or G Agarose Beads:** (Protein A [#9863](#)) Please prepare according to manufacturer's instructions. Use Protein A for rabbit IgG pull down and Protein G for mouse IgG pull down.
4. **3X SDS Sample Buffer:** ([#7722](#)) 187.5 mM Tris-HCl (pH 6.8 at 25 °C), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue

B. Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X cell lysis buffer to each plate (10 cm) and incubate the plates on ice for 5 minutes.
4. Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate samples on ice three times for 5 seconds each.
6. Microcentrifuge for 10 minutes at 14,000 X g, 4 °C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at -80 °C.

C. Immunoprecipitation

Optional: It may be necessary to perform a lysate pre-clearing step to reduce non-specific binding to the Protein A/G agarose beads (See section below).

1. Take 200 μl cell lysate and add primary antibody. Incubate with gentle rocking overnight at 4 °C.
2. Add either protein A or G agarose beads (20 μl of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4 °C.
3. Microcentrifuge for 30 seconds at 4 °C. Wash pellet five times with 500 μl of 1X cell lysis buffer. Keep on ice during washes.
4. Resuspend the pellet with 20 μl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.

- Heat the sample to 95–100 °C for 2–5 minutes and microcentrifuge for 1 minute at 14,000 X g.
- Load the sample (15–30 µl) on SDS-PAGE gel (12–15%).
- Analyze sample by Western blotting (see Western Immunoblotting Protocol: [Western BSA](#), [Western Milk](#)).

Cell Lysate Pre-Clearing (Optional)

- Take 200 µl cell lysate and add to either Protein A or G agarose beads (20 µl of 50% bead slurry).
- Incubate at 4 °C for 30 – 60 minutes.
- Spin for 10 minutes at 4 °C. Transfer the supernatant to a fresh tube.
- Proceed to step 1 of Immunoprecipitation.

NOTE: For proteins with molecular weights of 50 kDa, we recommend using [Mouse Anti-Rabbit IgG \(Light-Chain Specific\) \(L57A3\) mAb #3677](#) or [Mouse Anti-Rabbit IgG \(Conformation Specific\) \(L27A9\) mAb #3678](#) as a secondary antibody to minimize masking produced by denatured heavy chains. For proteins with molecular weights of 25 kDa, [Mouse Anti-Rabbit IgG \(Conformation Specific\) \(L27A9\) mAb #3678](#) is recommended.

Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4 °C with gentle shaking, overnight.

Products available from Cell Signaling Technology are linked by their respective catalog numbers.

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

- 1X Phosphate Buffered Saline (PBS).**
- 1X SDS Sample Buffer:** ([#7722](#), [#7723](#)) 62.5 mM Tris-HCl (pH 6.8 at 25 °C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
- 10X Tris Buffered Saline (TBS):** ([#9997](#)) To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
- Nonfat Dry Milk:** ([#9999](#)) (weight to volume [w/v]).
- Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T).
- Bovine Serum Albumin (BSA):** ([#9998](#)).
- Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
- Phototope®-HRP Western Blot Detection System:** ([#7071 anti-rabbit](#)) or ([#7072 anti-mouse](#)) Includes biotinylated protein ladder, secondary ([#7074 anti-rabbit](#)) or ([#7076 anti-mouse](#)) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
- Prestained Protein Marker, Broad Range (Premixed Format):** ([#7720](#)).
- Biotinylated Protein Ladder Detection Pack:** ([#7727](#)).

13. **Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B. Protein Blotting

A general protocol for sample preparation is described below.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate).
Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 seconds for complete cell lysis and to shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100 °C for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** CST recommends loading prestained molecular weight markers ([#7720](#), 10 µl/lane) to verify electrotransfer and biotinylated protein ladder ([#7727](#), 10 µl/lane) to determine molecular weights.
8. Electrotransfer to nitrocellulose or PVDF membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
3. Wash three times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4 °C.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.

I. For Unconjugated Primary Antibodies

1. Incubate membrane with **appropriate** HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

II. For HRP Conjugated Primary Antibodies

Skip to Detection of Proteins (Step D).

III. For Biotinylated Primary Antibodies

1. Incubate membrane with HRP-Streptavidin (at the appropriate dilution) in milk for one hour with gentle agitation at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO[®] (0.5 ml 20X LumiGLO[®], 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature. **NOTE:** LumiGLO[®] substrate can be further diluted if signal response is too fast.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO[®] incubation and declines over the following 2 hours.

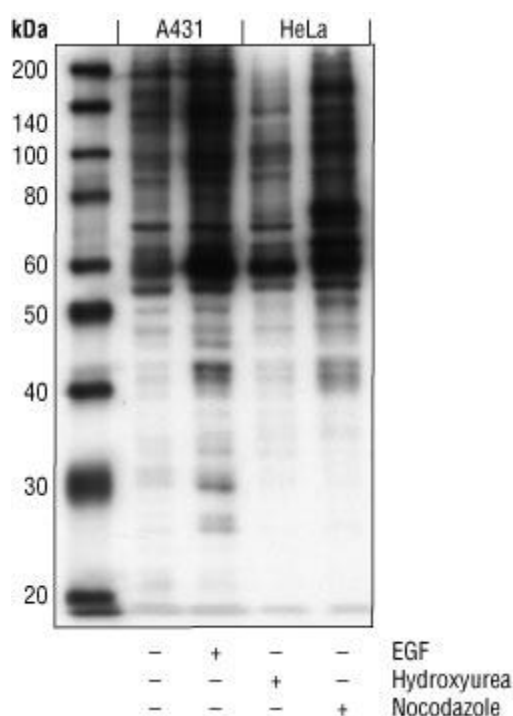
Specificity / Sensitivity

Phospho-MAPK/CDK Substrates (PXS*P or S*PXR/K) (34B2) Rabbit mAb detects phospho-serine in a PXS*P or S*PXR/K motif, as well as a PXS*PXR/K motif. The antibody is phospho-specific, and does not react with phospho-threonine- or phospho-tyrosine-containing peptides/proteins. (U.S. Patent No's.: 6,441,140; 6,982,318; 7,259,022; 7,344,714; U.S.S.N. 11,484,485; and all foreign equivalents.)

Source / Purification

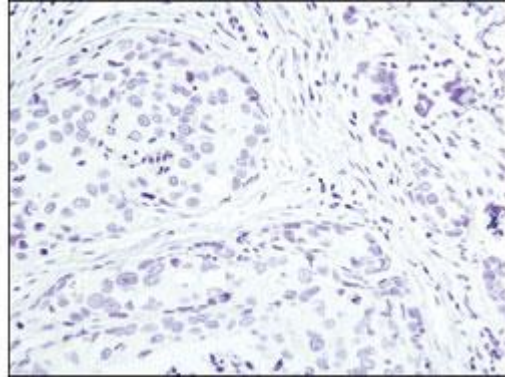
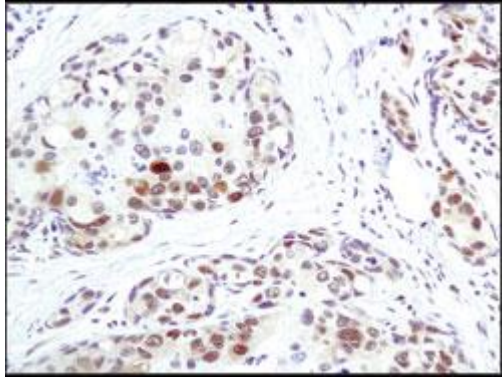
Monoclonal antibody is produced by immunizing animals with synthetic phospho-MAPK/CDK substrate peptides .

Western Blotting



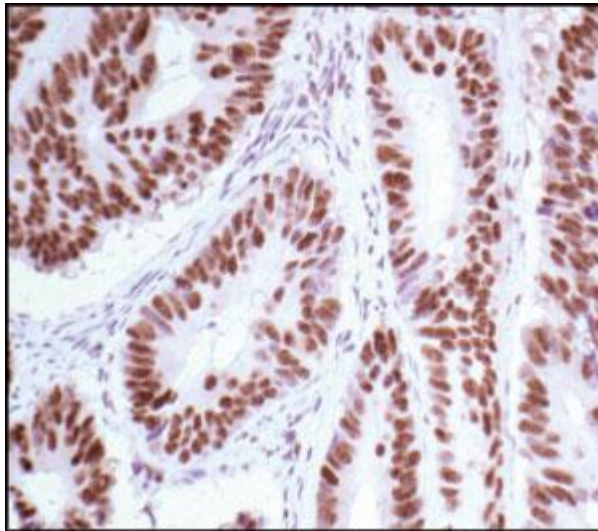
Western blot analysis of extracts from serum-starved A431 cells, untreated or treated in culture with EGF, and HeLa cells, untreated, hydroxyurea-treated (G1/S) or Nocodazole-treated (G2/M), using Phospho-MAPK/CDK Substrate (PXS*P or S*PXR/K) (34B2) Rabbit mAb.

IHC-P (paraffin)



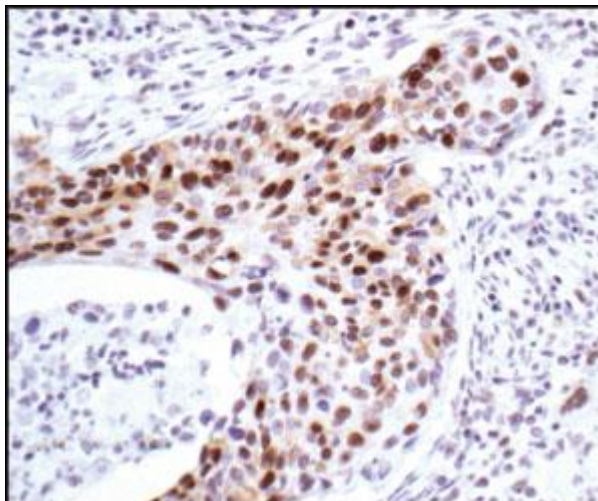
Immunohistochemical analysis of paraffin-embedded human breast carcinoma, control (left) or lambda phosphatase-treated (right), using Phospho-MAPK/CDK Substrates (PXS*P or S*PXR/K) (34B2) Rabbit mAb.

IHC-P (paraffin)



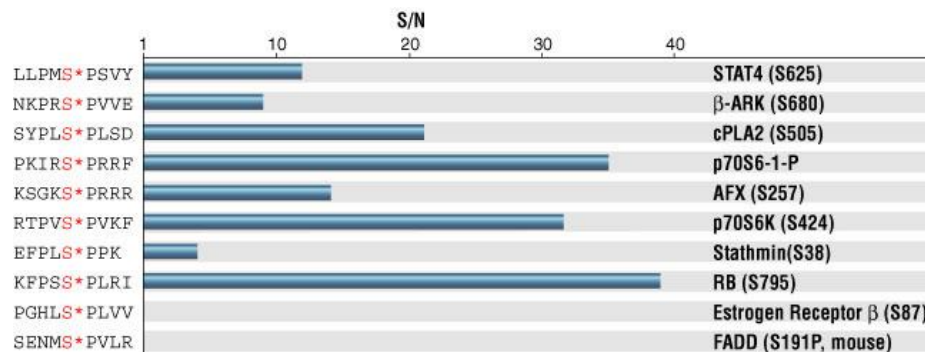
Immunohistochemical analysis of paraffin-embedded human colon carcinoma, using Phospho-MAPK/CDK Substrate (PXS*P or S*PXR/K) (34B2) Rabbit mAb.

IHC-P (paraffin)



Immunohistochemical analysis of paraffin-embedded human lung carcinoma, using Phospho-MAPK/CDK Substrate (PXS*P or S*PXR/K) (34B2) Rabbit mAb.

ELISA-Peptide



Phospho-MAPK/CDK Substrate (PXS*P or S*PXR/K) (34B2) Rabbit mAb ELISA Assay: Signal-to-noise ratio of phospho- versus nonphospho-peptides. (S* denotes phosphorylated serine.)

Background

The MAPK and CDK families of serine/threonine protein kinases play important roles in proliferation and cell cycle control. These kinases phosphorylate threonine or serine followed by a proline residue (1-3). MAPK phosphorylates substrates with the consensus sequence PX(S/T)P, and CDKs phosphorylate substrates containing the consensus sequence (S/T)PXR/K. Cell Signaling Technology has developed antibodies that bind to phospho-threonine followed by proline, motifs PXS*/T*P and/or S*PXR/K, for use in the study and discovery of new MAPK and CDK substrates (4,5).

1. Cross, T.G. et al. (2000) *Exp Cell Res* 256, 34-41.
2. Reynolds, C.H. et al. (2000) *J Neurochem* 74, 1587-95.
3. Seger, R. and Krebs, E.G. (1995) *FASEB J* 9, 726-35.
4. Holmes, J.K. and Solomon, M.J. (1996) *J Biol Chem* 271, 25240-6.
5. Songyang, Z. et al. (1996) *Mol Cell Biol* 16, 6486-93.