Store at –20°C

#9391

Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101)

- Small 100 µl (50 Western mini-blots)
- Large 300 µl (150 Western mini-blots)

rev. 01/02

Cell Signaling TECHNOLOGY™

Orders ■ 877-616-CELL (2355) orders@cellsignal.com

Support ■ 877-678-TECH (8324) info@cellsignal.com

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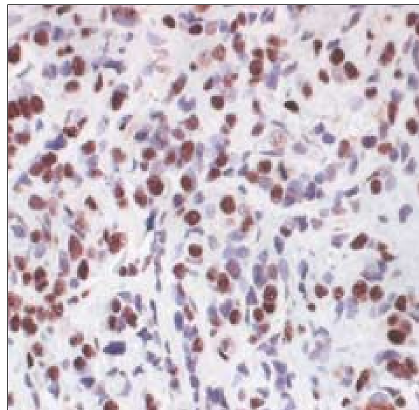
Background: Proline-directed protein kinases, including MAPKs, CDKs and GSKs, can phosphorylate serine/threonine residues followed by prolines (1). Proline-directed phosphorylation plays a central role in many critical biological processes including transcriptional regulation (2), checkpoint control (3), apoptosis (4), carcinogenesis (5) and neurological degeneration (6), yet few of the physiological substrates of these kinases are known. Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) provides a powerful tool for investigating the cellular role of proline-directed phosphorylation, as well as monitoring and characterizing the *in vitro* activity of proline-directed protein kinases (MAPKs, CDKs) and phosphatases.

Description: Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) binds the phospho-Thr-Pro motif in a manner largely independent of the surrounding amino acid sequence in ELISA. 2D Western analyses indicate that Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) specifically detects a broad range of phosphorylated proteins.

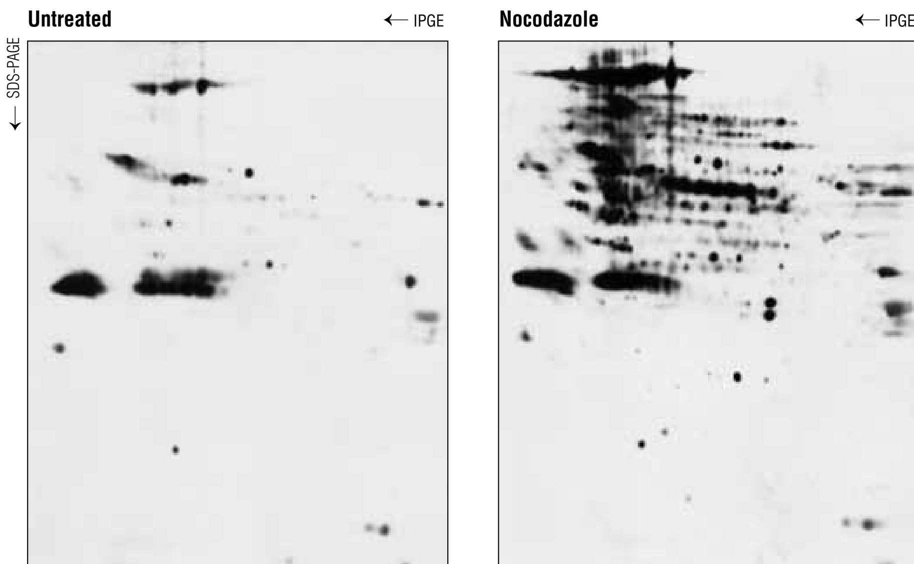
Source: The monoclonal antibody is produced by immunizing mice with a synthetic phospho-threonine-proline peptide (KLH coupled). Phospho-(Thr) MAPK/CDK Substrate Monoclonal Antibody is a mouse IgM clone but can be recognized by anti-mouse IgG (whole molecule) secondary antibody.

Purification: Antibodies are purified by protein A chromatography.

Sensitivity: When used in conjunction with our Phototope®-HRP Western Detection System, Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) detects endogenous levels of many proteins containing a phospho-Thr-Pro motif.



Immunohistochemical staining of proteins containing phospho-threonine-proline motifs in paraffin-embedded human breast carcinoma, using Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101).



Western blot analysis, using Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101), of whole cell lysates of Jurkat cells untreated and treated with 1 µg/ml nocadazole for 12 hours prior to lysis. Proteins were separated by 2D electrophoresis prior to blotting.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C. *Do not aliquot the antibody.*

Recommended Antibody Dilutions:

Western blotting	1:5000
Immunohistochemistry (Paraffin)	1:50
ELISA	1:1000

Companion Products:

Phospho-(Ser) PKC Substrate Antibody #2261

Phospho-(Thr) PDK1 Substrate Antibody #2291

Phospho-Threonine-X-Arginine Antibody #2351

Phospho-(Ser) 14-3-3 Binding Motif Antibody #9601

Phospho-(Ser/Thr) Akt Substrate Antibody #9611

Phospho-(Ser/Thr) PKA Substrate Antibody #9621

Phospho-(Ser/Thr) Phe Antibody #9631

Phospho-Threonine Antibody (P-Thr-Polyclonal) #9381

Phototope®-HRP Western Detection System:
Anti-mouse IgG, HRP-linked Antibody #7076

Prestained Protein Marker, Broad Range (Premixed Format) #7720

Biotinylated Protein Marker Detection Pack #7726

LumiGLO® Reagent and Peroxide #7003

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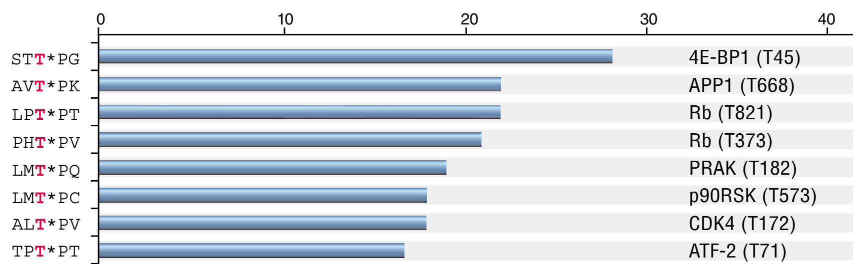
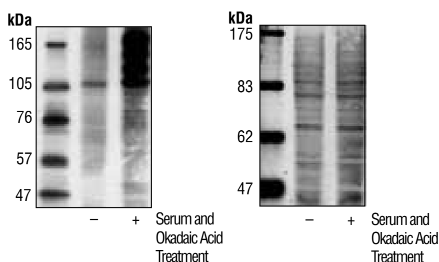


Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) ELISAs: Signal to noise ratio of phospho- versus non-phospho peptides. (T denotes phosphorylated threonine.)*



Western blot analysis of extracts from serum and Okadaic acid treated COS cells, using Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) (left) and total protein staining (right).

Specificity: Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) is highly specific for peptides containing phospho-Thr-Pro. It reacts neither with non-phosphorylated peptides with the same sequence nor with threonine-phosphorylated peptides lacking downstream prolines. Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) does not react with phospho-Tyr but does cross-react with 1 of 30 phospho-Ser-Pro peptides that have been tested.

Species Cross-reactivity: It is expected that this antibody will react with peptides/proteins containing phospho-Thr-Pro from all species.

Applications: Western blotting, immunohistochemistry, ELISA

Selected Application References:

Conover, C.A. et al. (2000) Insulin-like Growth Factor (IGF) binding protein-3 potentiation of IGF action is mediated through the phosphtidylinositol-3-kinase pathway and is associated with alteration in Protein Kinase B/AKT sensitivity. *Endocrinology* 141(9), 3098–3103.

Hall, C. et al. (2001) HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. *Mol. Cell. Biol.* 21, 1854–1865.

Marinissen, M.J.; Chiariello, M.; Gutkind, J.S. (2001) Regulation of gene expression by the small GTPase Rho through the ERK6 (p38γ) MAP Kinase pathway. *Genes & Development* 15, 535–553.

Rashid, T. et al. (2001) Phosphorylation of Pak1 by the p35/Cdk5 kinase affects neuronal morphology. *J. Biol. Chem.* 276, 49043–49052.

Wada, T. et al. (2001) Impaired synergistic activation of stress-activated protein kinase SAPK/JNK in mouse embryonic stem cells lacking SEK1/MKK4. *J. Biol. Chem.* 276(33), 30892–30897.

References:

(1) Pearson, R.B. and Kemp, B.E. (1991) *Meth. Enzymol.* 200, 62–81.
(2) Seger, R. and Krebs, E.G. (1995) *FASEB J.* 9, 726–735.
(3) Nurse, P. (2000) *Cell* 100, 71–78.
(4) Cross, T.G. et al. (2000) *Exp. Cell Res.* 256, 34–41.
(5) Yang, C.C. et al. (1998) *J. Prot. Chem.* 17, 329–335.
(6) Reynolds, C.H. et al. (2000) *J. Neurochem.* 74, 1587–1595.

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Western Immunoblotting Protocol

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

Solutions and Reagents

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

Transfer Buffer:
 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)

SDS Sample Buffer (1X):
 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

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%).

10X TBS (Tris-buffered saline):
 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).

Primary Antibody Dilution Buffer:
 1X TBS, 0.1% Tween-20 with 5% blocking agent; 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:
 Biotinylated protein marker, secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO™ chemiluminescent reagent, peroxide.

Wash Buffer TBS/T:
 1X TBS, 0.1% Tween-20

Blotting Membrane

This protocol has been optimized for nitrocellulose membranes, which we recommend. PVDF membranes may also be used.

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^2 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 to shear DNA and 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: We recommend loading prestained molecular weight markers (#7720) to verify electrotransfer and biotinylated protein markers (#7726, 10 µl/lane) to determine molecular weights.

8. Electrotransfer to nitrocellulose membrane.

Membrane Blocking and Antibody Incubations

Note: Volumes are for 10 cm x 10 cm (100 cm^2) 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 3 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 3 times for 5 minutes each with 15 ml of TBS/T.
6. Incubate membrane with 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.
7. Wash 3 times for 5 minutes each with 15 ml of TBS/T.

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 ten-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.

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Immunohistochemistry Protocol for Paraffin Sections

Solutions and Reagents

Xylene

Ethanol

Distilled H_2O (dH_2O)

Hematotoxylin

10X PBS (Phosphate Buffered Saline):
0.58 M sodium phosphate dibasic (Na_2HPO_4), 0.17 M sodium phosphate monobasic (NaH_2PO_4), 0.68 M NaCl. To prepare 1 liter of 10X PBS: Combine 82.33 g Na_2HPO_4•$4H_2O$, 23.45 g NaH_2PO_4•H_2O and 40 g NaCl. Adjust pH to 7.4.

10 mM Sodium Citrate Buffer:
To prepare 1 liter, add 2.94 g sodium citrate to 1 liter dH_2O. Adjust pH to 6.0.

1% Hydrogen Peroxide:
To prepare, add 10 ml 30% H_2O_2 to 290 ml dH_2O.

Blocking Solution:
5% horse serum or goat serum in PBS

ABC Reagent (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA):
Prepare according to manufacturer's instructions 30 minutes before use.

DAB Reagent:
Add 6.7 µg of 30% hydrogen peroxide to 10 ml dH_2O; add this mixture to 10 ml of 1 mg/ml DAB (diaminobenzidine tetrahydrochloride) in PBS, filter.

Protocol

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 dH_2O for 5 minutes each.
3. Wash sections in PBS for 5 minutes.
4. For antigen unmasking, heat sections in 10 mM sodium citrate buffer (pH 6.0) for 1 minute at full power followed by 9 minutes at medium power. (Keep slides fully immersed in buffer and maintain temperature at or just below boiling.) Cool slides for 20 minutes after antigen unmasking.
5. Wash sections in dH_2O three times for 5 minutes each.
6. Incubate sections in 1% hydrogen peroxide for 10 minutes.
7. Wash sections in dH_2O three times for 5 minutes each.
8. Wash section in PBS for 5 minutes.
9. Block each section with 100–400 µl blocking solution for 1 hour at room temperature.
10. Remove blocking solution and add 100–400 µl diluted primary antibody to each section. (Dilute antibody in blocking solution.) Incubate overnight at 4°C.
11. Remove antibody solution and wash sections in PBS three times for 5 minutes each.
12. Add 100–400 µl secondary antibody, diluted in blocking solution, to each section. Incubate 30 minutes at room temperature.
13. If using ABC biotin/avidin method, make ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
14. Remove secondary antibody solution and wash sections three times with PBS for 5 minutes each.
15. Add 100–400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.
16. Remove ABC reagent and wash sections three times in PBS for 5 minutes each.
17. Add 100–400 µl DAB reagent to each section and monitor staining closely.
18. As soon as the section turns brown, immerse slides in dH_2O.
19. If desired, counterstain sections in hematotoxylin for 10 seconds.
20. Wash sections in dH_2O two times for 5 minutes each.
21. 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.
22. Mount coverslips.

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ELISA Protocol

Solutions and Reagents

Carbonate Buffer:
 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2 g/L NaN_3 (pH 9.6). Use 1 µM synthetic peptide in Carbonate Buffer.

10X PBS (phosphate buffered saline):
 0.58 M sodium phosphate dibasic (Na_2HPO_4), 0.17 M sodium phosphate monobasic (NaH_2PO_4), 0.68 M NaCl. To prepare 1 liter of 10X PBS: 82.33 g $Na_2HPO_4•4H_2O$, 23.45 g $NaH_2PO_4•H_2O$ and 40 g NaCl. Adjust pH to 7.4.

PBST:
 1X PBS containing 0.05% Tween-20

Blocking Buffer:
 10 mg/ml BSA in PBST

Antibody Dilution Buffer:
 1.0 mg/ml BSA in PBST

Anti-mouse IgG, AP-linked Antibody (#7052-1)

Anti-rabbit IgG, AP-linked Antibody (#7051-1)

Enzyme Substrate:
 1 mg/ml p-Nitrophenyl Phosphate (NPP) (Sigma #N-2765) in 1 M diethanolamine buffer (pH 9.8)

1 M Diethanolamine Buffer:
 97 ml diethanolamine (Sigma #D-8885), 100 mg $MgCl_2$ and 0.2 g sodium azide, add 800 ml deionized water, adjust pH to 9.8 with 10 M HCl, add deionized water to 1 L.

Coating Plates

1. 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 2–6 hours at 37°C. If the peptide does not absorb, try other buffers in the pH 4 to 8 range.
2. Discard the uncoated synthetic peptide; wash the coated wells at least three times with PBST.
3. Block remaining active sites by incubating the plate with 200 µl/well of Blocking Buffer for 1 hour at 37°C.
4. Wash the wells at least three times with PBST.

ELISA

1. Prepare appropriate dilution of primary antibody with Antibody Dilution Buffer. Add 100 µl to wells and incubate 2 hours at 37°C or overnight at 4°C.
2. Wash the wells at least three times with PBST.
3. Dilute secondary antibody PBST. Add 100 µl to wells and incubate 1 hour at 37°C.
4. Wash the well at least three times with PBST.
5. Add 100 µl enzyme substrate and incubate 1–3 hours at 37°C.
6. Read the absorbance at 405 nm with a microtiter plate reader.

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