

Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100)

✓ 300 µg
(40 Western mini-blot)

rev. 01/02



Cell Signaling
TECHNOLOGY™

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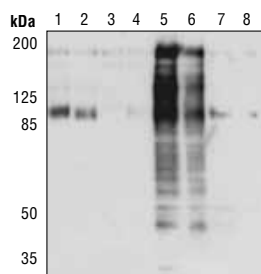
Background: Since the original observations that protein tyrosine kinases (PTKs) play critical roles in oncogenic transformation (1,2), it has become clear that both PTKs and phospho-tyrosine phosphatases control a wide range of critical biological processes (3,4). Antibodies specific for phospho-Tyr (5,6) have been invaluable reagents in these studies. The Phospho-Tyrosine Monoclonal Antibodies developed by Cell Signaling Technology (P-Tyr-100, #9411 and P-Tyr-102, #9416) provide exceptionally sensitive new tools for studying Tyr phosphorylation and monitoring Tyr kinase activity in high throughput drug discovery.

Description: Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) is a high affinity antiphospho-Tyr monoclonal antibody. ELISAs using a wide variety of phospho-peptides indicate (a) that P-Tyr-100 binds phospho-Tyr in a manner largely independent of the surrounding amino acid sequence, and (b) that P-Tyr-100 binds to a larger number of phospho-Tyr-containing peptides than any other antiphospho-Tyr monoclonal antibody, including 4G10 and PY20. 2D Western blot analyses of pervanadate-treated Jurkat lysates indicate that P-Tyr-100 interacts with a broader range of Tyr-phosphorylated proteins than does 4G10.

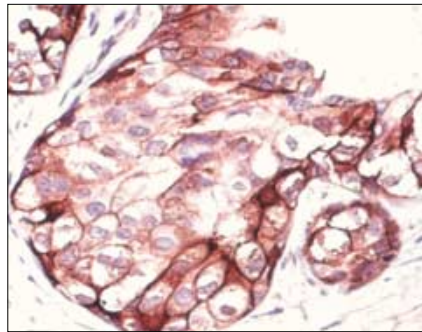
Source: Monoclonal antibody (isotype: IgG1) is derived from mice immunized with phospho-Tyr peptides coupled to KLH.

Purification: Antibody is supplied in HEPES buffer with 50% glycerol and less than 0.02% sodium azide.

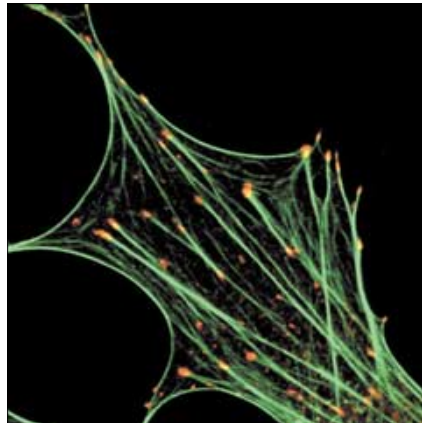
Sensitivity: When used in conjunction with our Phototope®-HRP Western Detection System, Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) detects endogenous levels of many tyrosine-phosphorylated proteins.



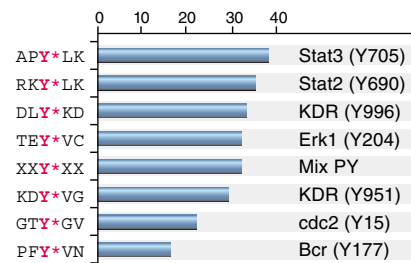
Tyrosine phosphorylation in rat hippocampal dentate gyrus ischemia. Control (lanes 1 and 2); 15 minute transient cerebral ischemia (lanes 3 and 4); reperfusion for 4 hours (lanes 5 and 6); and 24 hours (lanes 7 and 8). Western blot analysis using Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100). (Provided by Dr. Bingren Hu, University of Miami School of Medicine, Florida.)



Immunohistochemical staining of proteins with phosphorylated tyrosine residues in paraffin-embedded human breast carcinoma, using Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100).



Serum-starved Swiss NIH/3T3 cells were stimulated with 10 µM lysophosphatidic acid (LPA) for 10 minutes and then fixed with PFA and stained with phalloidin for F-actin (green) and Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) (red). LPA causes heavy tyrosine phosphorylation of proteins in focal adhesions, present at the tips of actin stress fibers. (Provided by Dr. Harry Mellor, University of Bristol, UK.)



Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) ELISAs: Signal to noise ratio of phospho- versus nonphospho-peptides. (Y denotes phosphorylated tyrosine.)*

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	1:2000
Immunoprecipitation	1:100
Immunohistochemistry (Paraffin)	1:100
ELISA	1:1000

Companion Products:

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

Phospho-Tyrosine Monoclonal Antibody (P-Tyr-102) #9416

Nitro-Tyrosine Polyclonal Antibody #9691

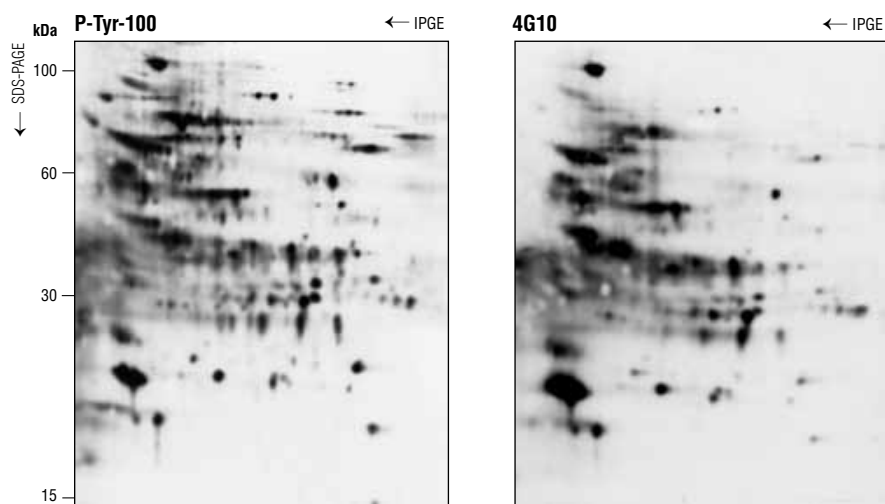
Phototope®-HRP Western Detection System:

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



Comparison of P-Tyr-100 and 4G10 Phospho-Tyrosine Monoclonal Antibodies: Western blot analysis of whole cell lysates of Jurkat cells treated with 1 mM pervanadate for 30 minutes prior to lysis. Proteins were separated by 2D electrophoresis prior to blotting.

Specificity: Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) is highly specific for phospho-Tyr in peptides/proteins, shows no cross-reactivity with the corresponding nonphosphorylated peptides and does not cross-react with peptides containing phospho-Ser or phospho-Thr instead of phospho-Tyr.

Species Cross-reactivity: It is expected that P-Tyr-100 will react with peptides/proteins containing phospho-Tyr from all species.

Applications: Western blotting, immunoprecipitation, immunohistochemistry, ELISA

Selected Application References:

Asao, H. et al. (2001) Cutting edge: the common γ -chain is an indispensable subunit of the IL-21 receptor complex. *J. Immunol.* 167(1), 1–5.

Chang, C.I. et al. (2000) The involvement of tyrosine kinases, Cyclic AMP/protein kinase A, and p38 mitogen-activating protein kinase in IL-13-mediated arginase I induction in macrophages: Its implications in IL-13-inhibited nitric oxide production. *J. Immunol.* 165, 2134–2141.

Farhang-Fallah, J. et al. (2000) Cloning and characterization of PHIP, a novel insulin receptor substrate-1 pleckstrin homology domain interacting protein. *J. Biol. Chem.* 275, 40492–40497.

References:

- (1) Varmus, H. and Bishop, J.M. (1986) *Cancer Surv.* 5, 153–158.
- (2) Hunter, T. and Cooper, J.A. (1985) *Annu. Rev. Biochem.* 54, 897–930.
- (3) Fischer, E.H. (1999) *Adv. Enzyme Regul.* 39, 359–369.
- (4) Hunter, T. (1997) *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 353, 583–605.
- (5) Ward, S.G. et al. (1992) *J. Biol. Chem.* 267, 23862–23869.
- (6) Glenney, J.R. (1988) *J. Immunol. Methods* 109, 277–285.

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 cm² 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 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 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.

Immunoprecipitation Followed by Western Immunoblotting Protocol

Solutions and Reagents

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

Cell Lysis Buffer (1X):

- 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 -Glycerolphosphate
- 1 mM Na_3VO_4
- 1 $\mu\text{g/ml}$ Leupeptin

Note: We recommend adding 1 mM PMSF before use.

Protein A Agarose Beads:

- (Can be stored for 2 weeks at 4°C)
- Add 5 ml of 1X PBS to 1.5 g of Protein A Agarose Beads.
- Shake 2 hours at 4°C; spin down. Wash pellet twice with PBS.
- Resuspend beads in 1 volume of PBS.

3X SDS Sample Buffer:

- 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

Transfer Buffer:

- 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)

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):

- For 1 liter of 10X TBS, use 24.2 g Tris base and 80 g NaCl.
- Adjust pH to 7.6 with HCl (use at 1X).

Primary Antibody Dilution Buffer:

- 1X TBS, 0.05% 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 10 μl Tween-20 (100%).

Wash Buffer TBS/T:

- 1X TBS, 0.1% Tween-20

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

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 1X ice-cold Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm²) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate 4 times for 5 seconds each on ice.
6. Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at -80°C.

Immunoprecipitation

1. Take 200 μl cell lysate and add primary antibody; incubate with gentle rocking overnight at 4°C.
2. Add Protein A 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 5 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.
5. Heat the sample to 95–100°C for 2–5 minutes.
6. Load the sample (15–30 μl) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting (*see Western Immunoblotting Protocol*).

Immunohistochemistry Protocol for Paraffin Sections

Solutions and Reagents

Xylene

Ethanol

Distilled H₂O (dH₂O)

Hematoxylin

10X PBS (Phosphate Buffered Saline):

0.58 M sodium phosphate dibasic (Na₂HPO₄), 0.17 M sodium phosphate monobasic (NaH₂PO₄), 0.68 M NaCl. To prepare 1 liter of 10X PBS: Combine 82.33 g Na₂HPO₄•4H₂O, 23.45 g NaH₂PO₄•H₂O 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 dH₂O. Adjust pH to 6.0.

1% Hydrogen Peroxide:

To prepare, add 10 ml 30% H₂O₂ to 290 ml dH₂O.

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 dH₂O; 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 dH₂O 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 dH₂O three times for 5 minutes each.
6. Incubate sections in 1% hydrogen peroxide for 10 minutes.
7. Wash sections in dH₂O 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 dH₂O.
19. If desired, counterstain sections in hematoxylin for 10 seconds.
20. Wash sections in dH₂O 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.

ELISA Protocol

Solutions and Reagents

Carbonate Buffer:

15 mM Na₂CO₃, 35 mM NaHCO₃, 0.2g/L NaN₃ (pH 9.6),
Use 1 μM synthetic peptide in Carbonate Buffer

10X PBS (phosphate buffered saline):

0.58 M sodium phosphate dibasic (Na₂HPO₄), 0.17 M sodium phosphate monobasic (NaH₂PO₄), 0.68 M NaCl. To prepare 1 liter of 10X PBS: 82.33 g Na₂HPO₄•4H₂O, 23.45 g NaH₂PO₄•H₂O 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-Rabbit IgG (AP-linked) Antibody (#7051-1)

Anti-Mouse IgG (AP-linked) Antibody (#7056)

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 MgCl₂ 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 in PBST (1:1000). 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.