PhosphoPlus® Bad (Ser112/136) Antibody Kit

✓ (10 Western mini-blots)

Cell Signaling TECHNOLOGY®

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

Support ■ 877-678-TECH (8324)
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rev. 12/01/05

Kit Components:

■ **Phospho-Bad (Ser112) Antibody**
rabbit polyclonal IgG, affinity purified
#9291 100 µl

■ **Phospho-Bad (Ser136) Antibody**
rabbit polyclonal IgG, affinity purified
#9295 200 µl

■ **Bad Antibody**
rabbit polyclonal IgG, affinity purified
#9292 100 µl

■ **Bad Control Proteins**
#9293 4 Western mini blots

■ **Bad Eukaryotic Expression Plasmid (pEBG-mBad)**
#9294 20 µg

■ **Phototope®-HRP Western Detection System**

Anti-rabbit IgG, HRP-linked Antibody
#7074 50 µl

Anti-biotin, HRP-linked Antibody
#7075 100 µl

20X Lumiglo® Reagent and Peroxide
#7003 5 ml each

Biotinylated Protein Ladder Detection Pack
#7727 100 µl

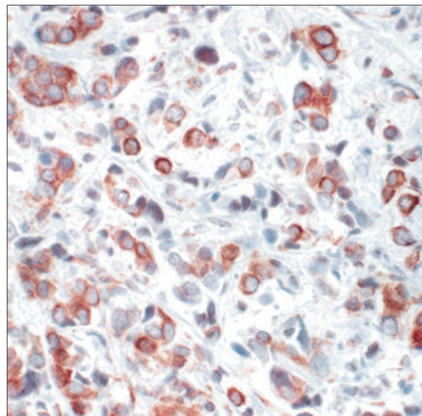
Description: The PhosphoPlus® Bad (Ser112/136) Antibody Kit provides reagents and protocols for the rapid analysis of the phosphorylation status of Bad at serines 112 and 136.

Bad Control Proteins: Nonphosphorylated and phosphorylated Bad peptides fused to a carrier protein, to serve as positive and negative controls. **These proteins migrate at 76 kDa.**

Bad Eukaryotic Expression Plasmid (pEBG-mBad): Mouse bad is expressed as a glutathione-S-transferase fusion protein under the control of the strong constitutive EF-1α promotor for the purpose of transient transfection. **The resulting fusion protein has a molecular weight of approximately 49 kDa.**

Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide corresponding to residues surrounding Ser112 or Ser136 of mouse Bad (Phospho-Bad (Ser112) Antibody and Phospho-Bad (Ser136) Antibody), or with a synthetic peptide corresponding to residues surrounding Ser112 of mouse Bad (Bad Antibody). Antibodies are purified by protein A and peptide affinity chromatography.

Specificity/Sensitivity: Phospho-Bad (Ser112) Antibody detects **endogenous** levels of Bad only when phosphorylated at Ser112. It does not detect Bad phosphorylated at other sites or related proteins. Phospho-Bad (Ser136) Antibody detects **overexpressed** levels of Bad only when phosphorylated at Ser136. It does not detect Bad phosphorylated at other sites or related proteins. Bad Antibody detects **endogenous** levels of total Bad protein. It does not cross-react with related proteins.



Immunohistochemical analysis of paraffin-embedded human breast carcinoma, showing cytoplasmic localization, using Bad Antibody.

Western blot analysis of extracts from 293 cells transfected with wild-type Bad, Bad (Ser112A), Bad (S136A) or Bad (S112A/S136A), treated with TPA or forskolin, using Phospho-Bad (Ser112) Antibody #9291 (top), Phospho-Bad (Ser136) Antibody #9295 (middle) or Bad Antibody #9292 (bottom).

Storage: Antibodies are 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 antibodies.*

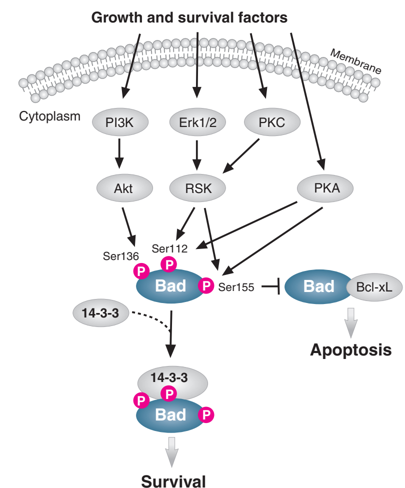
Bad Control Proteins are supplied in SDS Sample Buffer: 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red. Store at –20°C.

Bad Eukaryotic Expression Plasmid (pEBG-mBad) is supplied in 10 mM Tris (pH8.0) 1 mM EDTA. Store at –20°C.

Companion Products:

Phospho-Bad (Ser112) (7E11) Monoclonal Antibody #9296

Phospho-Bad (Ser155) Antibody #9297



Bad Signaling Pathway

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Background: Bad is a proapoptotic member of the Bcl-2 family that can displace Bax from binding to Bcl-2 and Bcl-xL, resulting in cell death (1,2). Survival factors such as IL-3 can inhibit the apoptotic activity of Bad by activating intracellular signaling pathways that result in the phosphorylation of Bad at Ser112 and Ser136 (2). Phosphorylation at these sites results in the binding of Bad to 14-3-3 proteins and the inhibition of Bad binding to Bcl-2 and Bcl-xL (2). Akt has been shown to promote cell survival via its ability to phosphorylate Bad at Ser136 (3,5). Ser112 has been shown to be the substrate *in vivo* and *in vitro* of p90RSK (6,7) and mitochondria-anchored PKA (8). Phosphorylation of Ser155 by PKA plays a critical role in blocking the dimerization of Bad to Bcl-xL because of its position in the BH3 domain (9-11).

Selected Application References:

Bertolotto, C. et al. (2000) Protein kinase C theta and epsilon promote T-cell survival by a Rsk-dependent phosphorylation and inactivation of BAD. *J. Biol. Chem.* 275, 37246–37250. Applications: W.

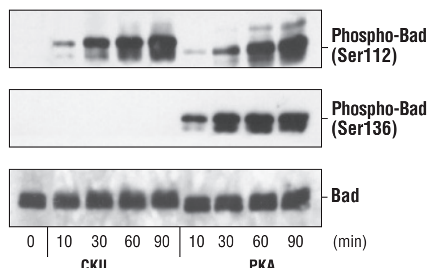
Jamieson, C.A. and Yamamoto, K.R. (2000) Crosstalk pathway for inhibition of glucocorticoid-induced apoptosis by T cell receptor signaling. *Proc. Natl. Acad. Sci. USA* 97, 7319–7324. Applications: W.

Laine, J. et al. (2000) The protooncogene TCL1 is an Akt kinase coactivator. *Mol. Cell* 6, 395–407. Applications: W.

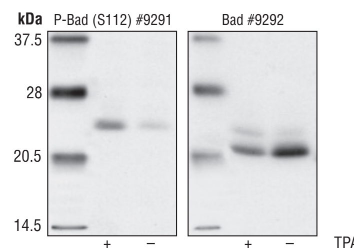
Tan, Y. et al. (1999) p90(RSK) blocks Bad-mediated cell death via a protein kinase C-dependent pathway. *J. Biol. Chem.* 274, 34859–34867. Applications: W.

Background References:

(1) Yang, E. et al. (1995) *Cell* 80, 285–291.
(2) Zha, J. et al. (1996) *Cell* 87, 619–628.
(3) Datta, S.R. et al. (1997) *Cell* 91, 231–241.
(4) Mizushima, S. and Nagata, S. (1990) *Nucleic Acids Res.* 18, 5322.
(5) Peso, L. et al. (1997) *Science* 278, 687–689.
(6) Bonni, A. et al. (1999) *Science* 286, 1358–1362.
(7) Tan, Y. et al. (1999) *J. Biol. Chem.* 274, 34859–34867.
(8) Harada, H. et al. (1999) *Mol. Cell* 3, 413–422.
(9) Tan, Y. et al. (2000) *J. Biol. Chem.* 275, 25865–25869.
(10) Lizcano, J. (2000) *Biochem. J.* 349, 547–557.
(11) Datta, S. et al. (2000) *Mol. Cell* 6, 41–51.



Western blot analysis of GST-Bad phosphorylated by CKII and PKA in vitro, using Phospho-Bad (Ser112) Antibody #9291 (top), Phospho-Bad (Ser136) Antibody #9295 (middle) or Bad Antibody #9292 (bottom).

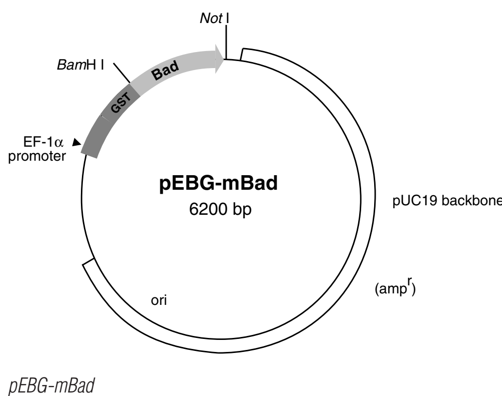


Western blot analysis of extracts from COS cells, untreated or TPA-treated, using Bad Antibody #9292 (left) or Phospho-Bad (Ser112) Antibody #9291 (right).

No. Antibody	W	IP	IHC (Pa)	IC (IF)	Species Cross-reactivity	Mol. Weight	Source
#9291 Phospho-Bad (Ser112) Ab	1:1000	1:200	-	1:50	H, M, R, Mk	23	Rabbit
#9295 Phospho-Bad (Ser136) Ab	1:500	-	-	-	H, M, R	23*, 49*	Rabbit
#9292 Bad Ab	1:1000	1:500	1:50	1:50	H, M, R, Mk	23	Rabbit

***23 kDa = endogenous if possible and 49 kDa = GST-Bad if transfected**

W=Western Blotting IP=Immunoprecipitation IHC=Immunohistochemistry IC=Immunocytochemistry



pEBG-mBad

Applications Key: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **IC**—Immunocytochemistry **F**—Flow cytometry **E**—ELISA **D**—DELFIA®

Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **X**—*Xenopus* **Z**—zebra fish **All**—all species expected
Species enclosed in parentheses are predicted to react based on 100% sequence homology.

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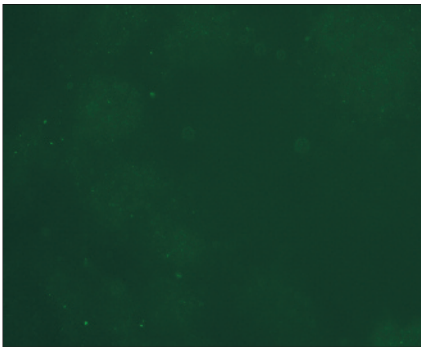
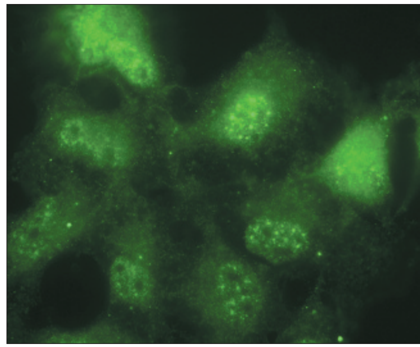
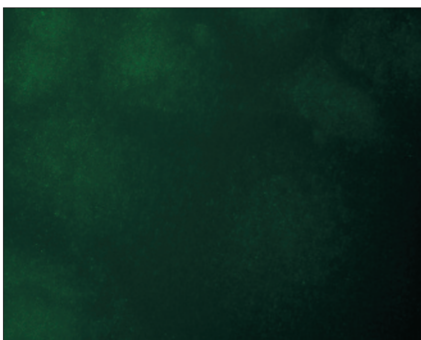
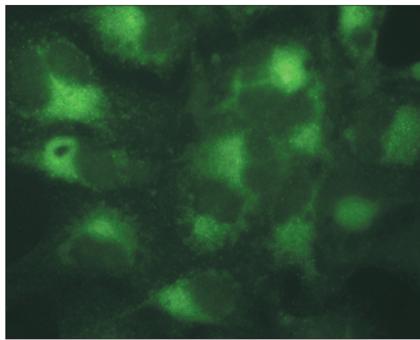




Immunocytochemical staining of TPA-treated COS cells, using Phospho-Bad (Ser112) Antibody #9291 with (left) or without (right) peptide blocking.





Immunocytochemical staining of untreated COS cells, using Bad Antibody #9292 with (left) or without (right) peptide blocking.

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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% 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:
Biotinylated protein marker, secondary anti-rabbit 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: CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein markers (#7727, 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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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 µg/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

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 cm^2) 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 2 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)*.

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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, 23.45 g $NaH_2PO_4 \bullet H_2O$ 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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Immunocytochemistry Protocol

This procedure works well with 50% confluent cells in a 12-well plate.

Solutions and Reagents

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

FBS Media:
0.5% fetal bovine serum (FBS)

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, use 82.33 g Na_2HPO_4, 23.45 g $NaH_2PO_4 \bullet H_2O$ and 40 g NaCl. Adjust pH to 7.4.

3% paraformaldehyde in PBS:
Prepare fresh before using; heat at 65°C to dissolve then keep on ice.

TBS:
50 mM Tris-HCl (pH 7.4), 150 mM NaCl

TBS/Triton (TBS/T):
50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100

Blocking Buffer:
5% Normal Horse Serum in TBST

BSA (bovine serum albumin)

0.6% Hydrogen Peroxide:
200 µl 30% H_2O_2 in 10 ml TBS

ABC Reagent (VectaStain ABC Kit, Vector Laboratories, Inc., Burlingame, CA)
Prepare 30 minutes before using.

DAB Reagent:
Add 6.7 µl 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. Culture cells in medium containing 0.5% FBS for 2 days. CST recommends plating cells directly in 0.5% FBS media to reduce basal level of phosphorylation.
2. Aspirate media. Add fresh media without FBS. Culture for 2 hours.
3. Aspirate media. Treat cells by adding fresh media without FBS containing growth factor for desired time.
4. Aspirate media from cultures; wash cells with PBS; aspirate.
5. Fix cells in 3% paraformaldehyde for 30 minutes at 4°C (rocking).
6. Wash three times for 5 minutes each with 1 ml of TBS/T.
7. Wash once with TBS for 2 minutes.
8. Incubate in 100% MeOH at –20°C for 10 minutes.
9. Wash three times for 5 minutes each with 1 ml of TBS/T.
10. Incubate in blocking buffer (5% normal horse serum in TBS/T) for 60 minutes at room temperature.
11. Incubate with primary antibody overnight at 4°C in 5% BSA TBS/T at 1:400.
12. Wash once for 15 minutes with TBS/T.
13. Wash once for 15 minutes with 0.1% BSA in TBS/T.
14. Incubate with biotinylated secondary antibody (diluted appropriately [1:500 for anti-mouse biotinylated secondary antibody from Vector ABC Kit] in TBS/T/3% BSA) for 1 hour at room temperature.
15. Wash three times for 5 minutes each with 1 ml of TBS/T.
16. Incubate for exactly 30 minutes in 0.6% H_2O_2 (200 µl 30% H_2O_2 in 10 ml TBS).
17. Wash three times for 5 minutes each with 1 ml of TBS/T.
18. Incubate 1 hour with 0.5–1 ml ABC reagent at room temperature (2 drops solution A into 5 ml PBS, mix, then add 2 drops solution B, mix).
19. Wash 3 times for 5 minutes each with 1 ml of TBS.
20. Add 1 ml DAB reagent. Monitor reaction progress under microscope. Reaction should proceed for 10 minutes.
21. Terminate reaction by adding 1 ml of water.
22. Aspirate and wash once with 1 ml of water at 4°C.

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