

Phospho-Bad Antibody Sampler Kit

1 Kit
 (4 x 40 µl)



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This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

| Products Included | Product # | Quantity | Mol. Wt. | Isotype |
|--|-----------|----------|------------|------------|
| Phospho-Bad (Ser112) (40A9) Rabbit mAb | 5284 | 40 µl | 23 kDa | Rabbit IgG |
| Phospho-Bad (Ser136) (185D10) Rabbit mAb | 5286 | 80 µl | 23 kDa | Rabbit IgG |
| Phospho-Bad (Ser155) Antibody | 9297 | 40 µl | 23 kDa | Rabbit IgG |
| Bad (D24A9) Rabbit mAb | 9239 | 40 µl | 23 kDa | Rabbit IgG |
| pCMV-Tag4A-mBad/GrpE | 2888 | 20 µg | 48, 52 kDa | |
| Anti-rabbit IgG, HRP-linked Antibody | 7074 | 100 µl | | Goat |

See www.cellsignal.com for individual component applications, species cross-reactivity, dilutions and additional application protocols.

Description: The Phospho-Bad Antibody Sampler Kit provides an economical means to investigate the role of Bad protein in apoptosis. The kit contains primary and secondary antibodies to perform four Western blots with each antibody.

Background: Bad is a proapoptotic member of the Bcl-2 family that promotes cell death by displacing Bax from binding to Bcl-2 and Bcl-xL (1,2). Survival factors, such as IL-3, 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 promotes binding of Bad to 14-3-3 proteins to prevent an association between Bad with Bcl-2 and Bcl-xL (2). Akt phosphorylates Bad at Ser136 to promote cell survival (3,4). Bad is phosphorylated at Ser112 both *in vivo* and *in vitro* by p90RSK (5,6) and mitochondria-anchored PKA (7). Phosphorylation of Ser155 in the BH3 domain by PKA plays a critical role in blocking the dimerization of Bad and Bcl-xL (8-10).

Specificity/Sensitivity: Bad (D24A9) Rabbit mAb detects endogenous levels of total Bad protein. Phospho-Bad (Ser112) (40A9) Rabbit mAb detects endogenous levels of Bad only when phosphorylated at Ser112. The Ser112 nomenclature is based upon the mouse sequence. The analogous phosphorylation sites are Ser75 in human and Ser113 in rat. Phospho-Bad (Ser136) (185D10) Rabbit mAb detects immunoprecipitated or transfected levels of Bad protein only when phosphorylated at Ser136. The Ser136 nomenclature is based upon the mouse sequence. The analogous phosphorylation sites are Ser99 in human and Ser137 in rat. Phospho-Bad (Ser155) Antibody detects transfected levels of Bad only when phosphorylated at Ser155. The Ser155 nomenclature is based upon the mouse sequence. The analogous phosphorylation sites are Ser118 in human and Ser156 in rat.

Source/Purification: Bad (D24A9) Rabbit mAb (#9239) was prepared by immunizing animals with a synthetic peptide corresponding to residues surrounding Pro102 of human Bad. Phospho-specific antibodies (#5284, 5286, 9297) were prepared by respectively immunizing animals with synthetic phosphopeptides corresponding to residues surrounding Ser112, Ser136, Ser155 of mouse Bad. Polyclonal Phospho-Bad (Ser155) Antibody (#9297) was purified by protein A and peptide affinity chromatography. The construct pCMV-Tag4A-mBad/GrpE expresses a fusion protein of mouse Bad with the *E. coli* heat shock protein GrpE. The fusion protein runs as a 48 to 52 kDa doublet formed by alternative start sites.

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) Peso, L. et al. (1997) *Science* 278, 687-689.
- (5) Bonni, A. et al. (1999) *Science* 286, 1358-1362.
- (6) Tan, Y. et al. (1999) *J. Biol. Chem.* 274, 34859-34867.
- (7) Harada, H. et al. (1999) *Mol. Cell* 3, 413-422.
- (8) Tan, Y. et al. (2000) *J. Biol. Chem.* 275, 25865-25869.
- (9) Lizcano, J. et al. (2000) *Biochem. J.* 349, 547-557.
- (10) Datta, S. et al. (2000) *Mol. Cell* 6, 41-51.

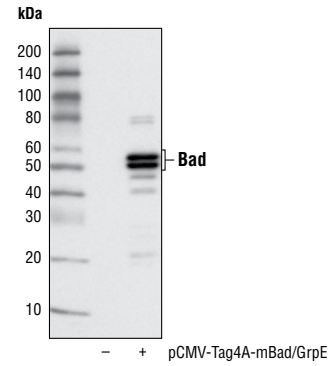
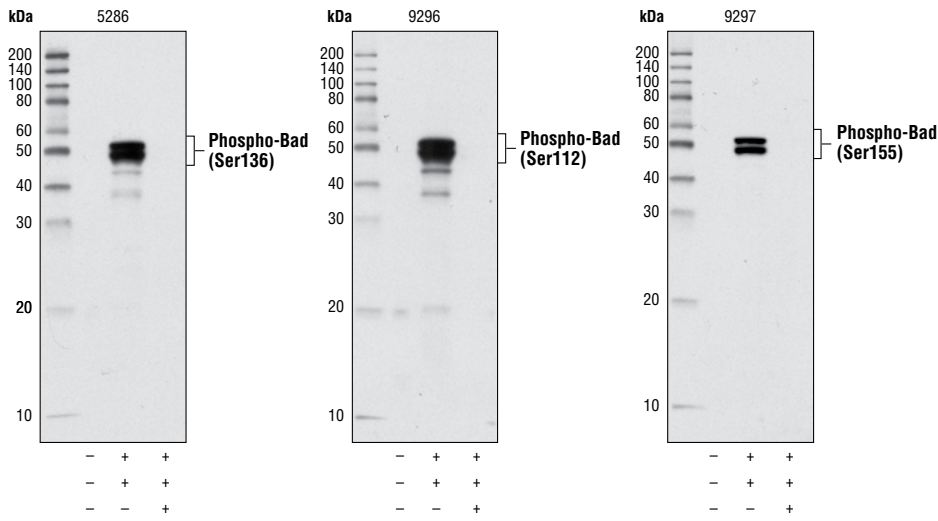
Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at -20°C. *Do not aliquot the antibodies.*

Recommended Antibody Dilutions:

Western blotting 1:1000

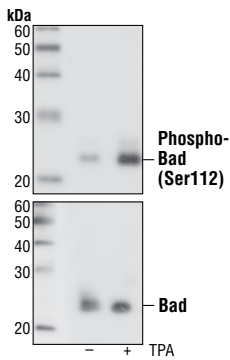
Please visit www.cellsignal.com for a complete listing of recommended companion products.

Selected rabbit monoclonal antibodies are produced under license (granting certain rights including those under U. S. Patent No. 5,675,063 and/or U.S.S.N. 11/476,277) from Epitomics, Inc. U.S.S.N. 11/476,277) from Epitomics, Inc.

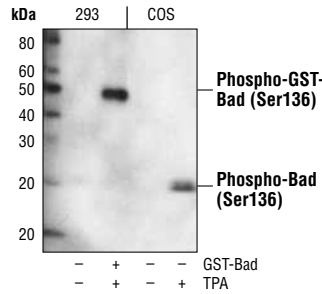


Western blot analysis of HeLa cells, mock transfected or transfected with pCMV-Tag4A-mBad/GrpE, using **Bad (D24A9) Rabbit mAb #9239**.

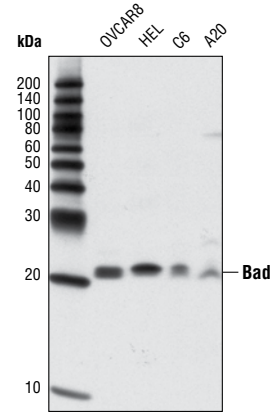
Western blot analysis of HeLa cells, mock transfected or transfected with pCMV-Tag4A-mBad/GrpE, untreated or TPA- and/or λ -phosphatase-treated, using phospho- and total Bad antibodies #5286, #9296, #9297. Note that overexpressed fusion protein runs as a 48 to 52 kDa doublet that is likely formed as a result of alternative start sites.



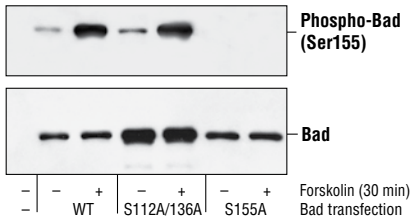
Western blot analysis of extracts from COS cells, untreated or TPA-treated, using **Phospho-Bad (Ser112) (40A9) Rabbit mAb #5284** (upper) or **Bad Antibody #9292** (lower).



Western blot analysis of extracts from control 293, GST-Bad transfected 293 and COS cells, untreated or TPA-treated, using **Phospho-Bad (Ser136) (185D10) Rabbit mAb #5286**.



Western blot analysis of extracts from various cell lines using **Bad (D24A9) Rabbit mAb #9239**.



Western blot analysis of cell extracts from 293 cells transfected with Wild-type Bad, Bad (S112A/S136A), Bad (S155A) and treated with forskolin (30 μ M), using **Phospho-Bad (Ser155) Antibody #9297** (upper) and **Bad Antibody #9292** (lower).

Western Immunoblotting Protocol (Primary Antibody 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.

A Solutions and Reagents

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

1. 1X Phosphate Buffered Saline (PBS)
2. **1X 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
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
4. **10X Tris Buffered Saline (TBS):** 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).
5. Nonfat Dry Milk (weight to volume [w/v])
6. **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%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
8. Bovine Serum Albumin (BSA)
9. **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%).
10. **Phototope[®]-HRP Western Blot Detection System #7071:** Includes biotinylated protein ladder, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO[®] chemiluminescent reagent and peroxide.
11. Prestained Protein Marker, Broad Range (Premixed Format) #7720
12. 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 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 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.
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 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.