

# PathScan® Total Bad Sandwich ELISA Kit

✓ 1 Kit  
(96 assays)

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

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

**Web** ■ www.cellsignal.com

New 03/07

This product is for *in vitro* research use only and is not intended for use in humans or animals.

## Species Cross-Reactivity: H, Mk

**Introduction:** CST's PathScan® Total Bad Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of total Bad protein. A Bad rabbit mAb\* has been coated onto the microwells. After incubation with cell lysates, Bad protein (phospho and nonphospho) is captured by the coated antibody. Following extensive washing, a Bad mouse mAb\* is added to detect the captured Bad protein. Anti-mouse IgG, HRP-linked Antibody #7076\* is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop color. The magnitude of absorbance for this developed color is proportional to the quantity of total bad protein.

\* Antibodies in kit are custom formulations specific to kit.

## Companion Products:

Phospho-Bad (Ser112) Antibody #9291

Phospho-Bad (Ser112) (40A9) Rabbit mAb #5284

Phospho-Bad (Ser112) (7E11) Mouse mAb #9296

Phospho-Bad (Ser136) Antibody #9295

Phospho-Bad (Ser136) (5D8) Monoclonal Antibody #5282

Phospho-Bad (Ser155) Antibody #9297

Bad Antibody #9292

Bad (11E3) Rabbit mAb (IP Preferred) #9268

Cell Lysis Buffer (10X) #9803

Anti-mouse IgG, HRP-linked Antibody #7076

**Specificity/Sensitivity:** CST's PathScan® Total Bad Sandwich ELISA Kit #7162 detects endogenous levels of Bad protein. A significant induction of Bad phosphorylation at Ser112 can be detected in TPA-treated OVCAR8 cells using PathScan® Phospho-Bad (Ser112) Sandwich ELISA Kit #7182. However, the level of total Bad protein (phospho and nonphospho) detected by PathScan® Total Bad Sandwich ELISA Kit #7162 remains unchanged (Figure 1). In Figure 3, Western analysis of protein captured in microwells coated with the Bad antibody shows a major band corresponding to the Bad protein.

Products Included	Volume	Solution Color
Bad Antibody Coated Microwells*	96 tests	
Bad Detection Antibody	11 ml	green
Anti-Mouse IgG HRP-Linked Antibody	11 ml	red
TMB Substrate	11 ml	colorless
STOP Solution	11 ml	colorless
Sealing Tape	2 sheets	
20X Wash Buffer	25 ml	colorless
Sample Diluent	25 ml	blue
10X Cell Lysis Buffer #9803**	15 ml	yellowish

\* 12 8-well modules -Each module is designed to break apart for 8 tests.

\*\*Kit should be stored at 4°C with the exception of 10X Cell Lysis Buffer, which is stored at -20°C (packaged separately).

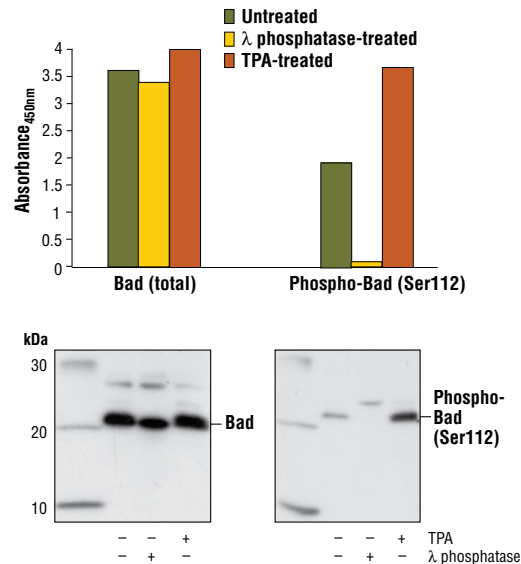


Figure 1. Treatment of OVCAR8 cells with TPA stimulates phosphorylation of Bad at Ser112, detected by PathScan® Phospho-Bad (Ser112) Sandwich ELISA Kit #7182, but does not affect the level of total Bad protein detected by PathScan® Total Bad Sandwich ELISA Kit #7162. λ phosphatase treatment of control cell lysates (4000 U/mL for 60 minutes at 37°C) abolishes the basal phosphorylation of Bad as shown by both Sandwich ELISA and Western analysis. The absorbance readings at 450 nm are shown in the top figure, while the corresponding Western blots using Phospho-Bad (Ser112) Antibody #9296 (right panel) or Bad Antibody #9254 (left panel), are shown in the bottom figure.

**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 (3,4). 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).

**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.

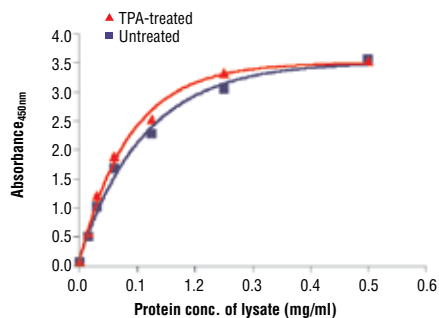


Figure 2. The relationship between the protein concentration of untreated and TPA-treated OVCAR8 cell lysates and the absorbance at 450 nm is shown. Cells were serum starved overnight and then treated with 200 nM TPA for 30 min. at 37°C.

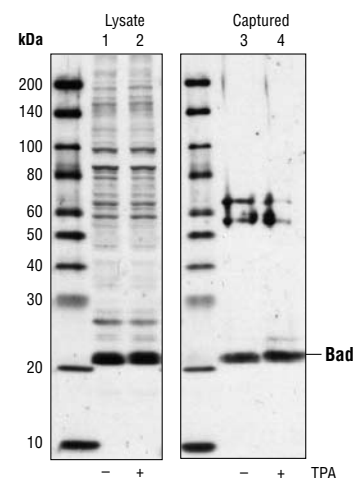
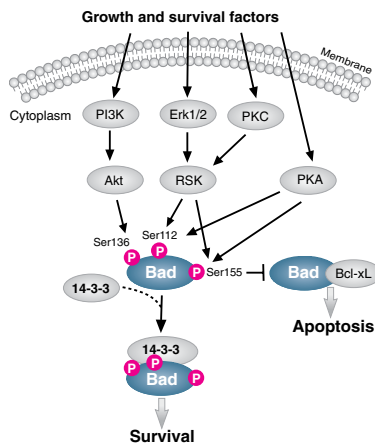


Figure 3. Kit specificity as demonstrated by Western analysis of the ELISA microwell captured protein. Lysates were prepared from OVCAR8 cells and incubated in microwells coated with the Bad capture antibody. Wells were washed, and the captured protein was solubilized in SDS gel loading buffer. Western analysis of OVCAR8 cell starting lysate (lanes 1 & 2) and the captured protein (lanes 3 & 4) was performed using Bad Mouse mAb #9254. The major band detected in the captured material corresponds to the Bad protein (lanes 3 & 4).



## Sandwich ELISA Protocol

### A Reagent Preparation

1. Bring all microwell strips to room temperature before use.
2. Prepare 1X Wash Buffer by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in Milli-Q or equivalently purified water.
3. **1X Cell Lysis Buffer from CST #9803:** 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylene diamine tetraacetate (EDTA), 1 mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 μg/ml leupeptin. This buffer can be stored at 4°C for short-term use (1–2 weeks).

### 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 plus 1 mM phenyl-methylsulfonyl fluoride (PMSF) to each plate (10 cm in diameter) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 minutes at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

### C Test Procedure

1. After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed and stored at 4°C immediately.
2. Add 100 μl of Sample Diluent (supplied in each PathScan® Sandwich ELISA Kit, blue color) to a microcentrifuge tube. Transfer 100 μl of cell lysate into the tube and vortex for a few seconds. Generally, sample applied to the well can be diluted 1:1 when the suggested cell lysis buffer is used for cell extraction. Individual data sheets for each kit provide information regarding an appropriate dilution factor for lysates and kit assay results. However, dilution factors need to be titrated when specific cell lysates are used.

3. Add 100 μl of each diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hours at 37°C. Alternatively, the plate can be incubated overnight at 4°C, which gives the best detection of target protein.
4. Gently remove the tape and wash wells:
  - a. Discard plate contents into a receptacle.
  - b. Wash 4 times with 1X Wash Buffer, 200 μl each time for each well.
  - c. For each wash, strike plates on fresh towels hard enough to remove the residual solution in each well, but do not allow wells to completely dry at any time.
  - d. Clean the underside of all wells with a lint-free tissue.
5. Add 100 μl of Detection Antibody (green color) to each well. Seal with tape and incubate the plate for 1 hour at 37°C.
6. Repeat wash procedure as in Step 4.
7. Add 100 μl of HRP-linked secondary antibody (red color) to each well. Seal with tape and incubate the plate for 30 minutes at 37°C.
8. Repeat wash procedure as in Step 4.
9. Add 100 μl of TMB Substrate to each well. Seal with tape and incubate the plate for 10 minutes at 37°C or 30 minutes at 25°C.
10. Add 100 μl of STOP Solution to each well. Shake gently for a few seconds.

**NOTE:** Initial color of positive reaction is blue, which changes to yellow upon addition of STOP Solution.

11. Read results.
  - a. Visual Determination — Read within 30 minutes after adding STOP Solution.
  - b. Spectrophotometric Determination — Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 minutes after adding STOP Solution.