

# PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) 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

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For Research Use Only. Not For Use In Diagnostic Procedures.

## Species Cross-Reactivity: H, M, Mi

**Description:** The PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that recognizes endogenous levels of phospho-Smad2 (Ser465/467) and Smad3 (Ser423/425) proteins. A Smad2/3 Mouse Antibody has been coated on the microwells. After incubation with cell lysates, Smad2/3 proteins (phospho and nonphospho) are captured by the coated antibody. Following extensive washing, a Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Detection Antibody is added to detect captured phospho-Smad2 (Ser465/467) and phospho-Smad3 (Ser423/425) proteins. Anti-rabbit IgG, HRP-linked Antibody is then used to recognize the bound detection antibody. HRP substrate TMB is added to develop color. The magnitude of the absorbance for this developed color is proportional to the quantity of phospho-Smad2 (Ser465/467) and phospho-Smad3 (Ser423/425) proteins.

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

**Specificity/Sensitivity:** PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA Kit detects endogenous levels of phospho-Smad2 (Ser465/467) and Smad3 (Ser423/425) proteins in human cells, as shown in Figure 1. The kit sensitivity is shown in Figure 2. This kit detects proteins from the indicated species, as determined through in-house testing, but may also detect homologous proteins from other species.

**Background:** Members of the Smad family of signal transduction molecules are components of a critical intracellular pathway that transmit TGF-β signals from the cell surface

Product Includes	Item #	Kit Quantity	Color	Storage Temp
Smad2/3 Mouse mAb Coated Microwells*	61684	96 tests		4°C
P-Smad2 (Ser465/467)/Smad3 (Ser423/425) Rabbit Detection Ab	33925	1 each	Green (Lyophilized)	4°C
Anti-rabbit IgG, HRP-linked Antibody (ELISA Formulated)	13272	1 each	Red (Lyophilized)	4°C
Detection Antibody Diluent	13339	11 ml	Green	4°C
HRP Diluent	13515	11 ml	Red	4°C
TMB Substrate	7004	11 ml		4°C
STOP Solution	7002	11 ml		4°C
Sealing Tape	54503	2 sheets		4°C
ELISA Wash Buffer (20X)	9801	25 ml		4°C
ELISA Sample Diluent	11083	25 ml	Blue	4°C
Cell Lysis Buffer (10X)	9803	15 ml		-20°C

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

into the nucleus. Three distinct classes of Smads have been defined: the receptor-regulated Smads (R-Smads), which include Smad1, 2, 3, 5, and 8; the common-mediator Smad (co-Smad), Smad4; and the antagonistic or inhibitory Smads (I-Smads), Smad6 and 7 (1-5). Activated type I receptors associate with specific R-Smads and phosphorylate them on a conserved carboxy terminal SXSX motif. The phosphorylated R-Smad dissociates from the receptor and forms a heteromeric complex with the co-Smad (Smad4), allowing translocation of the complex to the nucleus. Once in the nucleus, Smads can target a variety of DNA binding proteins to regulate transcriptional responses (6-8).

## Background References:

- (1) Heldin, C.H. et al. (1997) *Nature* 390, 465-471.
- (2) Attisano, L. and Wrana, J.L. (1998) *Curr. Opin. Cell Biol.* 10, 188-194.
- (3) Derynck, R. et al. (1998) *Cell* 95, 737-740.
- (4) Massague, J. (1998) *Annu. Rev. Biochem.* 67, 753-791.
- (5) Whitman, M. (1998) *Genes Dev.* 12, 2445-2462.
- (6) Wu, G. et al. (2000) *Science* 287, 92-97.
- (7) Attisano, L. and Wrana, J.L. (2002) *Science* 296, 1646-1647.
- (8) Moustakas, A. et al. (2001) *J. Cell Sci.* 114, 4359-4369.

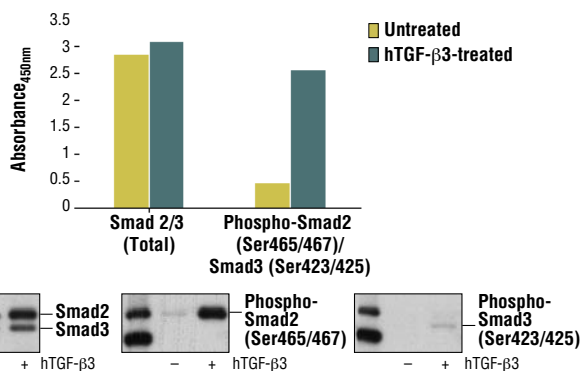


Figure 1. Treatment of HeLa cells with hTGF-β3 #8425 stimulates phosphorylation of Smad2 at Ser465/467 or Smad3 at Ser423/425, as detected by PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA Kit, but does not affect the level of total Smad2 or Smad3 protein detected by PathScan® Total Smad2/3 Sandwich ELISA Kit #12000. The absorbance readings at 450 nm are shown in the top figure, while the corresponding western blots using Smad2/3 (D7G7) XP® Rabbit mAb #8685 (left panel), Phospho-Smad2 (Ser465/467) (138D4) Rabbit mAb #3108 (center panel), or a phospho-Smad3 (Ser423/425) Rabbit mAb (right panel) are shown in the bottom figure.

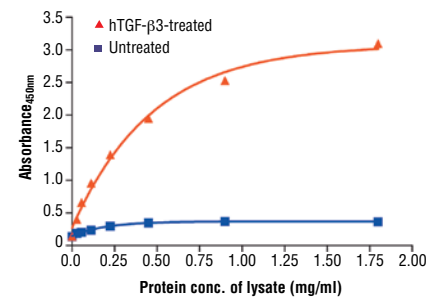


Figure 2. The relationship between the protein concentration of lysates from untreated and TGF-β3-treated HeLa cells and the absorbance at 450 nm as detected by the PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA Kit is shown. Starved HeLa cells (85% confluence) were treated with 10 ng/ml of hTGF-β3 #8425 for 30 min at 37°C.

U.S. Patent No. 5,675,063

**Applications Key:** W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide

**Species Cross-Reactivity Key:** H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine

Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

## PathScan® Sandwich ELISA Protocol (for kits with Lyophilized Antibodies)

### A Solutions and Reagents

**NOTE:** Prepare solutions with purified water.

- Microwell strips:** Bring all to room temperature before use.
- Detection Antibody:** Supplied lyophilized as a green colored cake or powder. Add 1.0 ml of Detection Antibody Diluent (green solution) to yield a concentrated stock solution. Incubate at room temperature for 5 min with occasional gentle mixing to fully reconstitute. To make the final working solution, add the full 1.0 ml volume of reconstituted Detection Antibody to 10.0 ml of Detection Antibody Diluent in a clean tube and gently mix. Unused working solution may be stored for 4 weeks at 4°C.
- HRP-Linked Antibody\*:** Supplied lyophilized as a red colored cake or powder. Add 1.0 ml of HRP Diluent (red solution) to yield a concentrated stock solution. Incubate at room temperature for 5 min with occasional gentle mixing to fully reconstitute. To make the final working solution, add the full 1.0 ml volume of reconstituted HRP-Linked Antibody to 10.0 ml of HRP Diluent in a clean tube and gently mix. Unused working solution may be stored for 4 weeks at 4°C.
- Detection Antibody Diluent:** Green colored diluent for reconstitution and dilution of the detection antibody (11 ml provided).
- HRP Diluent:** Red colored diluent for reconstitution and dilution of the HRP-Linked Antibody (11 ml provided).
- Sample Diluent:** Blue colored diluent provided for dilution of cell lysates.
- 1X Wash Buffer:** Prepare by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in purified water.
- Cell Lysis Buffer:** 10X Cell Lysis Buffer #9803 or 1X Cell Lysis Buffer #7018: This buffer can be stored at 4°C for short-term use (1–2 weeks). Recommended: Add 1 mM phenylmethylsulfonyl fluoride (PMSF) immediately before use.
- TMB Substrate** (#7004).
- STOP Solution** (#7002).

\*Note: Some PathScan® ELISA Kits may include HRP-Linked Streptavidin in place of HRP-Linked Antibody.

### B Preparing Cell Lysates

**For adherent cells.**

- Aspirate media when the culture reaches 80–90% confluence. Treat cells by adding fresh media containing regulator for desired time.
- Remove media and rinse cells once with ice-cold 1X PBS.
- Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 min.
- Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
- Sonicate lysates on ice.
- Microcentrifuge for 10 min (x14,000 rpm) 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.

**For suspension cells**

- Remove media by low speed centrifugation (~1200 rpm) when the culture reaches 0.5–1.0 x 10<sup>6</sup> viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
- Collect cells by low speed centrifugation (~1200 rpm) and wash once with 5–10 ml ice-cold 1X PBS.
- Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X Cell Lysis Buffer plus 1 mM PMSF.
- Sonicate lysates on ice.
- Microcentrifuge for 10 min (x14,000 rpm) 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

- 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.
- Cell lysates can be undiluted or diluted with Sample Diluent (supplied in each PathScan® Sandwich ELISA Kit, blue color). Individual datasheets for each kit provide a sensitivity curve that serves as a reference for selection of an appropriate starting lysate concentration. The sensitivity curve shows typical kit assay results across a range of lysate concentration points.
- Add 100 µl of each undiluted or diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hr at 37°C. Alternatively, the plate can be incubated overnight at 4°C.
- Gently remove the tape and wash wells:
  - Discard plate contents into a receptacle.
  - Wash 4 times with 1X Wash Buffer, 200 µl each time for each well.
  - 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.
  - Clean the underside of all wells with a lint-free tissue.
- Add 100 µl of reconstituted Detection Antibody (green color) to each well (refer to Section A, Step 2). Seal with tape and incubate the plate at 37°C for 1 hr.
- Repeat wash procedure (Section C, Step 4).
- Add 100 µl of reconstituted HRP-Linked secondary antibody (red color) to each well (refer to Section A, Step 3). Seal with tape and incubate the plate for 30 min at 37°C.
- Repeat wash procedure (Section C, Step 4).
- Add 100 µl of TMB Substrate to each well. Seal with tape and incubate the plate for 10 min at 37°C or 30 min at 25°C.
- 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.

- Read results.
  - Visual Determination:** Read within 30 min after adding STOP Solution.
  - Spectrophotometric Determination:** Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 min after adding STOP Solution.