#12001 Store at 4°C

PathScan® Phospho-Smad2 (Ser465/467)/ Smad3 (Ser423/425) Sandwich ELISA Kit

Cell Signaling TECHNOLOGY®

☑ 1 Kit
(96 assays)

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New 12/12

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

Entrez-Gene ID #4087, 4088
Swiss-Prot Acc. #Q15796, P84022

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 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 SSXS 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).

Products Included	Volume	Solution Color
Smad2/3 Mouse Ab Coated Microwells*	96 tests	
P-Smad2 (Ser465/467)/Smad3 (Ser423/425) Rabbit Detection Ab	11 ml	green
Anti-rabbit 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).

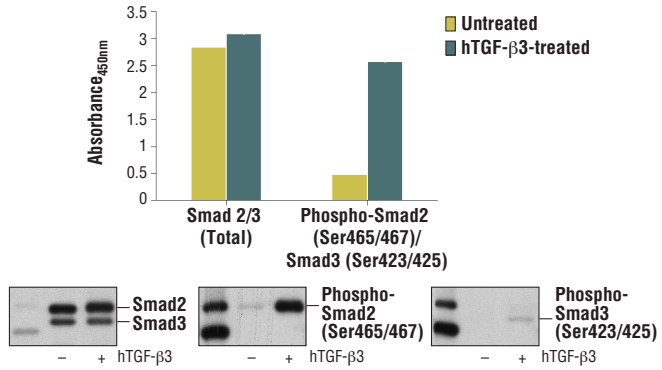
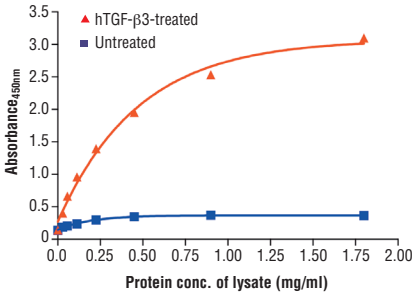


Figure 1. Treatment of HeLa cells with hTGF-β3 #8425 stimulates phosphorylation of Smad2 at Ser465/467 or Smad3 at Ser423/425, as detected by PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA Kit, but does not affect the level of total Smad2 or Smad3 protein detected by PathScan® 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.



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.

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.

#12001

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_3VO_4, 1µg/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 phenylmethylsulfonyl 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.