

PathScan® Phospho-4E-BP1 (Thr37/Thr46) Sandwich ELISA Kit



✓ 1 Kit
(96 assays)

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

Species Cross-Reactivity: H, M, R, Mk

Description: CST's PathScan® Phospho-4E-BP1 (Thr37/46) Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of 4E-BP1 when phosphorylated at Thr37/46. A Phospho-4E-BP1 (Thr37/46) Rabbit Antibody has been coated onto the microwells. After incubation with cell lysates, phospho-4E-BP1 (Thr37/46) is captured by the coated antibody. Following extensive washing, a 4E-BP1 Mouse Detection Antibody is added to detect the captured phospho-4E-BP1 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 4E-BP1 phosphorylated at Thr37/46.

Antibodies in kit are custom formulations specific to kit.

Specificity/Sensitivity: CST's PathScan® Phospho-4E-BP1 (Thr37/46) Sandwich ELISA Kit detects endogenous levels of phospho-4E-BP1 when phosphorylated at Thr37/46. As shown in Figure 1, using the Phospho-4E-BP1 (Thr37/46) ELISA Kit #7216, a significant induction of 4E-BP1 phosphorylation at Thr37/46 is detected in serum and amino acid starved HEK-293T cells treated with insulin for 30 minutes after replenishing the amino acids. This kit detects proteins from the indicated species, as determined through in-house testing, but may also detect homologous proteins from other species.

Background: Translation repressor protein 4E-BP1 (also known as PHAS-1) inhibits cap-dependent translation by binding to the translation initiation factor eIF4E. Hyperphosphorylation of 4E-BP1 disrupts this interaction and results in activation of cap-dependent translation (1). Both the PI3 kinase/Akt pathway and FRAP/mTOR kinase regulate 4E-BP1 activity (2,3). Multiple 4E-BP1 residues are phosphorylated *in vivo* (4). While phosphorylation by FRAP/mTOR at Thr37 and Thr46 does not prevent the binding of 4E-BP1 to eIF4E, it is thought to prime 4E-BP1 for subsequent phosphorylation at Ser65 and Thr70 (5).

- Background References:**
- (1) Pause, A. et al. (1994) *Nature* 371, 762-7.
 - (2) Brunn, G.J. et al. (1997) *Science* 277, 99-101.
 - (3) Gingras, A.C. et al. (1998) *Genes Dev* 12, 502-13.
 - (4) Fadden, P. et al. (1997) *J Biol Chem* 272, 10240-7.
 - (5) Gingras, A.C. et al. (1999) *Genes Dev* 13, 1422-37.

Product Includes	Item #	Kit Quantity	Color	Storage Temp
4E-BP1 (T37/46) RmAb Coated Microwells*	10345	96 tests		4°C
4E-BP1 Mouse Detection mAb	9453	1 each	Green (Lyophilized)	4°C
Anti-mouse IgG, HRP-linked Antibody (ELISA Formulated)	13304	1 each	Red (Lyophilized)	4°C
Detection Ab Diluent 3	14632	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.

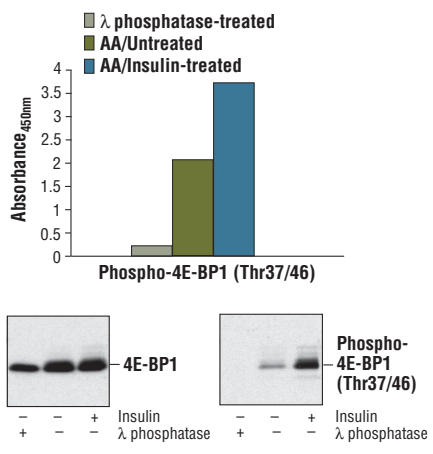


Figure 1: Treatment of HEK-293T cells with amino acids and insulin stimulates phosphorylation of 4E-BP1 at Thr37/46, as detected by PathScan® Phospho-4E-BP1 (Thr37/46) Sandwich ELISA Kit #7216, but does not affect the level of total 4E-BP1 protein detected by Western analysis. HEK-293T cells (70-80% confluent) were starved overnight and deprived of amino acids for 1 hour. The amino acids were replenished for 1 hour. Cells were either untreated or stimulated with 100 nM insulin for 30 minutes at 37°C. λ phosphatase treatment of control cell lysates (4000 U/mL for 60 minutes at 37°C) abolishes the basal phosphorylation of 4E-BP1 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 4E-BP1 Antibody #9452 (left panel) or Phospho-4E-BP1 (Thr37/46) Antibody #9459 (right panel), are shown in the bottom figure.

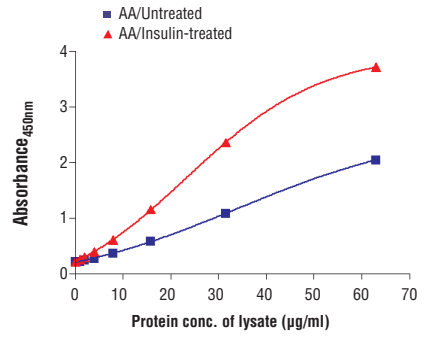


Figure 2: The relationship between the protein concentration of lysate from amino acid (AA)/untreated and AA/insulin-treated HEK-293T and the absorbance at 450 nm is shown.

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

A Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **Microwell strips:** Bring all to room temperature before use.
2. **Detection Antibody:** Supplied lyophilized as a green colored cake or powder. Add 1.0 ml of Detection Antibody Diluent 3 (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 3 in a clean tube and gently mix. Unused working solution may be stored for 4 weeks at 4°C.
3. **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.
4. **Detection Antibody Diluent 3:** Green colored diluent for reconstitution and dilution of the detection antibody (11 ml provided).
5. **HRP Diluent:** Red colored diluent for reconstitution and dilution of the HRP-Linked Antibody (11 ml provided).
6. **Sample Diluent:** Blue colored diluent provided for dilution of cell lysates.
7. **1X Wash Buffer:** Prepare by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in purified water.
8. **Cell Lysis Buffer:** 10X Cell Lysis Buffer #9803: This buffer can be stored at 4°C for short-term use (1–2 weeks). Recommended: Add 1 mM phenylmethyl-sulfonyl fluoride (PMSF) immediately before use.
9. **TMB Substrate** (#7004).
10. **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.

1. Aspirate media when the culture reaches 80–90% confluence. Treat cells by adding fresh media containing regulator for desired time.
2. Remove media and rinse cells once with ice-cold 1X PBS.
3. 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.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 min (14,000 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

1. Remove media by low speed centrifugation (~1200 rpm) when the culture reaches 0.5–1.0 × 10⁶ viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
2. Collect cells by low speed centrifugation (~1200 rpm) and wash once with 5–10 ml ice-cold 1X PBS.
3. Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X Cell Lysis Buffer plus 1 mM PMSF.
4. Sonicate lysates on ice.
5. Microcentrifuge for 10 min (14,000 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

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. 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.
3. 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.
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 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.
6. Repeat wash procedure (Section C, Step 4).
7. 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.
8. Repeat wash procedure (Section C, Step 4).
9. 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.
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 min after adding STOP Solution.
 - b. **Spectrophotometric Determination:** Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 min after adding STOP Solution.