

mTOR Substrates Antibody Sampler Kit

✓ 1 Kit
(5 x 40 µl)

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rev. 06/08/11

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-mTOR (Ser2448) (D9C2) XP® Rabbit mAb	5536	40 µl	289 kDa	Rabbit IgG
mTOR (7C10) Rabbit mAb	2983	40 µl	289 kDa	Rabbit IgG
Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb	9234	40 µl	70, 85 kDa	Rabbit IgG
Phospho-p70 S6 Kinase (Ser371) Antibody	9208	40 µl	70, 85 kDa	Rabbit IgG
Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb	2855	40 µl	15-20 kDa	Rabbit IgG
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl		Goat

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

Description: The mTOR Substrates Antibody Sampler Kit provides an economical means to evaluate the signaling of mTOR to downstream substrates including p70 S6 Kinase and 4E-BP1. The kit contains enough primary and secondary antibodies to perform four Western blot experiments per primary antibody.

Background: The mammalian target of rapamycin (mTOR, FRAP, RAFT) is a Ser/Thr protein kinase (1-3) that functions as an ATP and amino acid sensor to balance nutrient availability and cell growth (4,5). When sufficient nutrients are available, mTOR responds to a phosphatidic acid-mediated signal to transmit a positive signal to p70 S6 kinase and participate in the inactivation of the eIF4E inhibitor, 4E-BP1 (6). These events result in the translation of specific mRNA subpopulations. mTOR is phosphorylated at Ser2448 via the PI3 kinase/Akt signaling pathway and autophosphorylated at Ser2481 (7,8). mTOR plays a key role in cell growth and homeostasis and may be abnormally regulated in tumors. For these reasons, mTOR is currently under investigation as a potential target for anti-cancer therapy (9).

The regulatory associated protein of mTOR (Raptor) interacts with mTOR to mediate mTOR signaling to downstream targets (10,11). Raptor binds to mTOR substrates, such as 4E-BP1 and p70 S6 kinase, through their TOR signaling (TOS) motifs and is required for mTOR-mediated substrate phosphorylation (12,13). Binding of the FKBP12-rapamycin complex to mTOR inhibits mTOR-raptor interaction, which suggests a mechanism for the inhibition of mTOR signaling by rapamycin (14). This mTOR-raptor interaction and its regulation by nutrients and/or rapamycin are dependent on a protein called GβL (15). GβL is part of the rapamycin-insensitive complex between mTOR and rictor (rapamycin-insensitive companion of mTOR) and may mediate rictor-mTOR signaling to PKCα and other downstream targets (16). The rictor-mTOR complex has been identified as the previously elusive PDK2 responsible for the phosphorylation of Akt/PKB at Ser473, which is required for PDK1 phosphorylation of Akt/PKB at Thr308 and full activation of Akt/PKB (17).

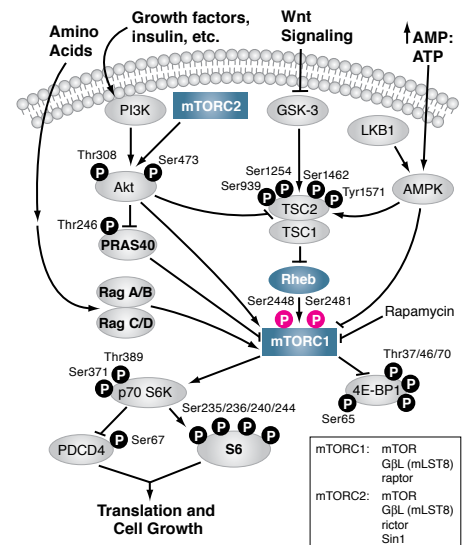
Specificity/Sensitivity: Each antibody in the mTOR Substrates Antibody Sampler Kit detects endogenous levels of its target protein. While activation state antibodies typically detect only target proteins phosphorylated at indicated residues, some cross-reaction can occur with related proteins phosphorylated at analogous sites.

Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Ser371 of human p70 S6 kinase. Polyclonal antibodies are purified by protein A and peptide affinity chromatography. Phospho-specific rabbit monoclonal antibodies are produced by immunizing animals with synthetic phosphopeptides corresponding to residues surrounding Thr389 of human p70 S6 kinase, Thr37 and Thr46 of mouse 4E-BP1 and the Ser2448 site of human mTOR. The mTOR (7C10) Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Ser2481 of human mTOR.

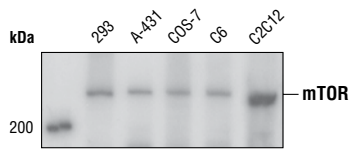
Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol and less than 0.02% sodium azide. Store at -20°C. Do not aliquot the antibodies.

Recommended Antibody Dilutions:
Western blotting 1:1000

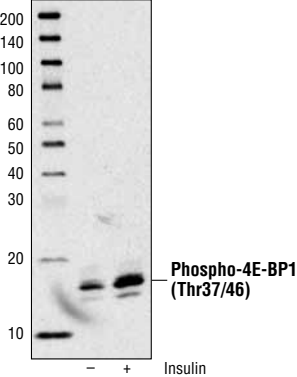
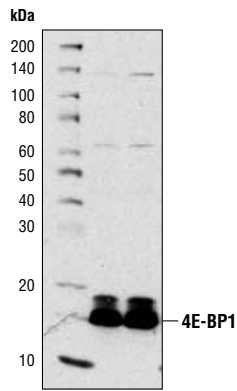
Please visit www.cellsignaling.com for a complete listing of recommended companion products.



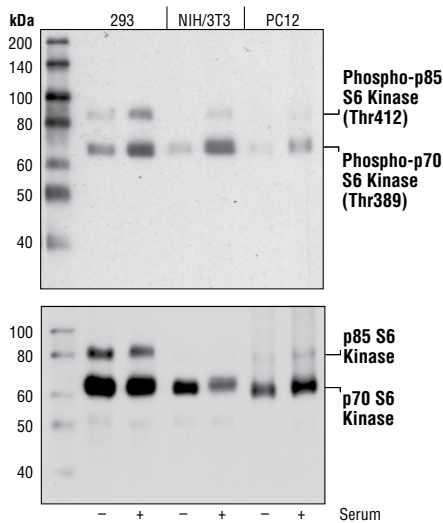
Selected rabbit monoclonal antibodies are produced under license (granting certain rights including those under U. S. Patent No. 5,675,063 and/or U.S.S.N. 11/476,277) from Epitomics, Inc. U.S.S.N. 11/476,277) from Epitomics, Inc.



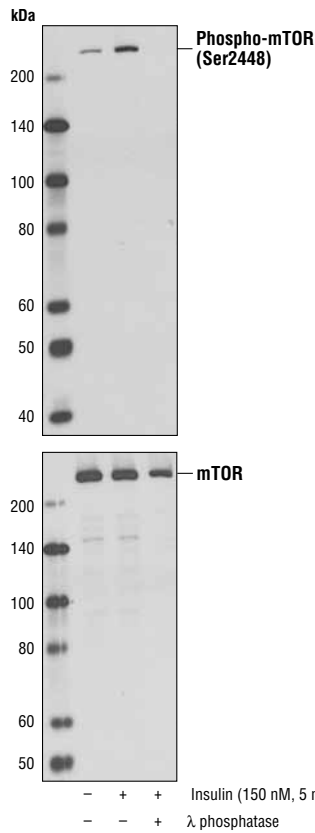
Western blot analysis of extracts from various cell types using **mTOR (7C10) Rabbit mAb #2983**.



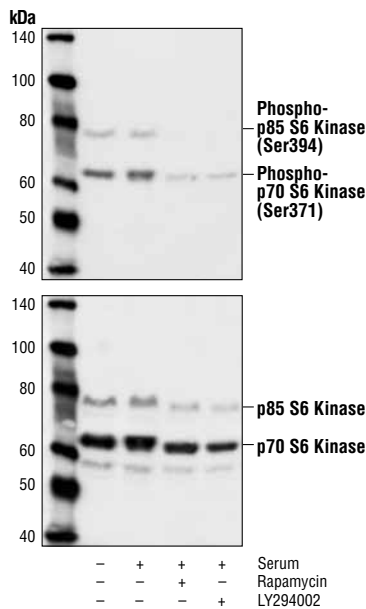
Western blot analysis of extracts from 293T cells using **4E-BP1 Antibody #9452 (upper)** and **Phospho-4E-BP1 (Thr37/46) Antibody #2855 (lower)**. The cells were starved for 24 hours in serum-free medium and underwent a 1 hour amino acid deprivation. Amino acids were replenished for 1 hour. Cells were then either untreated (-) or treated with 100 nM insulin (+) for 30 minutes.



Western blot analysis of extracts from serum starved or serum treated (20%) 293, NIH/3T3, and PC12 cells, using **Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb #9234 (upper)** or **p70 S6 Kinase (49D7) Rabbit mAb #2708 (lower)**.



Western blot analysis of extracts from serum-starved NIH/3T3 cells, untreated or insulin-treated (150 nM, 5 minutes), alone or in combination with λ -phosphatase, using **Phospho-mTOR (Ser2448) (D9C2) XP[®] Rabbit mAb #5536 (upper)** or **mTOR (7C10) Rabbit mAb #2983**.



Western blot analysis of lysates from 293 cells grown in low serum, then treated with 20% serum for 30 minutes alone or after 1 hour preincubation with rapamycin (10 nM) #9904 or LY294002 (50 uM) #9901, using **Phospho-p85 S6 Kinase (Ser394) (upper)** or **p85 S6 Kinase (lower)**.

Background References:

- (1) Sabers, C.J. et al. (1995) *J Biol Chem* 270, 815-22.
- (2) Brown, E.J. et al. (1994) *Nature* 369, 756-8.
- (3) Sabatini, D.M. et al. (1994) *Cell* 78, 35-43.
- (4) Gingras, A.C. et al. (2001) *Genes Dev* 15, 807-26.
- (5) Dennis, P.B. et al. (2001) *Science* 294, 1102-5.
- (6) Fang, Y. et al. (2001) *Science* 294, 1942-5.
- (7) Navé, B.T. et al. (1999) *Biochem J* 344 Pt 2, 427-31.
- (8) Peterson, R.T. et al. (2000) *J Biol Chem* 275, 7416-23.
- (9) Huang, S. and Houghton, P.J. (2003) *Curr Opin Pharmacol* 3, 371-7.
- (10) Hara, K. et al. (2002) *Cell* 110, 177-89.
- (11) Kim, D.H. et al. (2002) *Cell* 110, 163-75.
- (12) Beugnet, A. et al. (2003) *J Biol Chem* 278, 40717-22.
- (13) Nojima, H. et al. (2003) *J Biol Chem* 278, 15461-4.
- (14) Oshiro, N. et al. (2004) *Genes Cells* 9, 359-66.
- (15) Kim, D.H. et al. (2003) *Mol Cell* 11, 895-904.
- (16) Sarbassov, D.D. et al. (2004) *Curr Biol* 14, 1296-302.
- (17) Sarbassov, D.D. et al. (2005) *Science* 307, 1098-101.

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.

- 1X Phosphate Buffered Saline (PBS)
- 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
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 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).
- Nonfat Dry Milk (weight to volume [w/v])
- 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%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
- Bovine Serum Albumin (BSA)
- 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%).
- 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.
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- 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.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- 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.
- Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- Microcentrifuge for 5 minutes.
- 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.

- 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.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- 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.
- Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

- 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.

- 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.