#7100 Store at -20°C

PathScan® Multiplex Western Cocktail I:

Phospho-p90RSK, Phospho-Akt, Phospho-p44/42 MAPK and Phospho-S6

Ribosomal Protein Detection Cocktail I

☑ 1 Kit
(5 western blots)

rev. 07/11/11

Cell Signaling TECHNOLOGY®

Orders ■ 877-616-CELL (2355)
orders@cellsignal.com
Support ■ 877-678-TECH (8324)
info@cellsignal.com
Web ■ www.cellsignal.com

Applications	Species Cross-Reactivity*	Source
W Endogenous	H, M, R	Rabbit

Kit Components

No.	Name	Quantity	Source
5301	PathScan® Multiplex Western Cocktail I	250 µl	Rabbit
	Treated and Untreated Control Cell Extracts	50 µl/each	
7074	Anti-rabbit IgG, HRP-linked Antibody	100 µl	Goat
7075	Anti-biotin, HRP-linked Antibody	100 µl	Goat
7003	20X LumiGLO® Reagent and 20X Peroxide	5 ml/each	
7727	Biotinylated Protein Ladder Detection Pack	100 µl	

Description: The Pathscan® Multiplex Western Cocktail I Detection Kit offers a unique method to assay the activation of multiple pathways on one membrane without stripping and reprobing. This method saves the user valuable time, while increasing accuracy and minimizing reagent waste. The system allows the user to simultaneously detect levels of phospho-p90RSK, phospho-Akt, phospho-p44/42 MAPK (Erk1/2) and phospho-S6 ribosomal protein. The kit also includes Rab11 antibody to control protein loading. In addition, each Pathscan® Multiplex Western Cocktail Detection Kit contains treated and untreated cell lysates and the Phototope®-HRP Western Detection System. The kit includes enough primary and secondary antibodies to perform five Western blot experiments.

Background: Akt, also referred to as PKB or Rac, plays a critical role in controlling the balance between survival and apoptosis (1-3). This protein kinase is activated by insulin and various growth and survival factors, and functions in a wortmannin-sensitive pathway involving PI3 kinase (2,3). Akt is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 (4) and also by phosphorylation within the carboxy-terminus at Ser473.

Both p44 and p42 MAP kinases (Erk1 and Erk2) play a critical role in the regulation of cell growth and differentiation (5-8). MAP kinases are activated by a wide variety of extracellular signals including growth and neurotrophic factors, cytokines, hormones and neurotransmitters. Activation of MAP kinases occurs through phosphorylation of threonine and tyrosine (202 and 204 of human MAP kinase [Erk1] or 183 and 185 of rat Erk2) at the sequence T*EY* by a single upstream MAP kinase kinase (MEK) (9,10). One of the downstream targets of p44/42 MAPK is p90RSK.

To effectively promote growth and cell division in a sustained manner, growth factors and mitogens must upregulate translation (11,12). Growth factors and mitogens induce the activation of p70 S6 kinase, which in turn phosphorylates the S6 ribosomal protein. Phosphorylation of S6 correlates with an increase in translation, particularly of mRNAs with an oligopyrimidine tract in their 5+ untranslated regions (12).

Specificity/Sensitivity: Each phospho-antibody in this kit recognizes only the phosphorylated form of its specific target. The Rab11 antibody detects total levels of target protein to control for protein loading. All the antibodies detect endogenous levels of target proteins.

Source/Purification: Antibodies are produced by immunizing animals with synthetic peptides. Polyclonal antibodies are purified by protein A and peptide affinity chromatography.

Antibody Cocktail Components and Molecular Weights

No.	Antibody	Molecular Weight
9335	Phospho-p90RSK (Ser380) (9D9) Rabbit mAb	90 kDa
4060	Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb	60 kDa
4370	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) Rabbit mAb	42, 44 kDa
4858	Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) Rabbit mAb	32 kDa
5589	Rab11 (D4F5) XP® Rabbit mAb	25 kDa

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C. *Do not aliquot the antibody.*

***Species cross-reactivity is determined by Western blot, using the individual antibody cocktail components.**

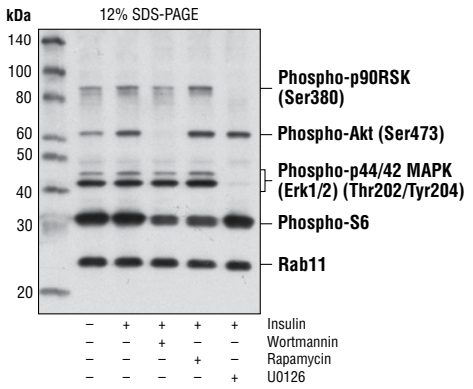
Recommended Antibody Dilutions:

Western blotting	1:200

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

Background References:

(1) Franke, T.F. (1997) *Cell* 88, 435–437.
(2) Burgering, B.T. and Coffer, P.J. (1995) *Nature* 376, 599–602.
(3) Franke, T.F. et al. (1995) *Cell* 81, 727–736.
(4) Alessi, D.R. et al. (1996) *EMBO J.* 15, 6541–6551.
(5) Marshall, C.J. (1995) *Cell* 80, 179–185.
(6) Hunter, T. (1995) *Cell* 80, 225–236.
(7) Hill, C.S. and Treisman, R. (1995) *Cell* 80, 199–211.
(8) Cowley, S. et al. (1994) *Cell* 77, 841–852.
(9) Sturgill, T.W. et al. (1988) *Nature* 334, 715–718.
(10) Payne, D.M. et al. (1991) *EMBO J.* 10, 885–892.
(11) Dufner, A. and Thomas, G. (1999) *Exp. Cell. Res.* 253, 100–109.
(12) Peterson, R.T. and Schreiber, S.L. (1998) *Curr. Biol.* 8, R248–R250.



Western blot analysis of extracts from CHO cells, untreated or insulin-treated following pretreatment with wortmannin (PI3 kinase inhibitor), rapamycin (mTOR inhibitor) and/or U0126 (MEK inhibitor) as indicated, using PathScan® Multiplex Western Cocktail I to detect phosphorylation of p90RSK, Akt, p44/42 MAPK and S6 ribosomal protein.

IMPORTANT: 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.

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 **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

#7100

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.

1. 1X Phosphate Buffered Saline (PBS)
2. **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
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
4. **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).
5. Nonfat Dry Milk (weight to volume [w/v])
6. **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%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
8. Bovine Serum Albumin (BSA)
9. **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%).
10. **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.
11. Prestained Protein Marker, Broad Range (Premixed Format) #7720
12. Biotinylated Protein Ladder Detection Pack #7727
13. **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.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. 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.
4. Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
5. Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. 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.

8. Electrotransfer to nitrocellulose or PVDF membrane.

C Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
3. Wash three times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.
6. 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.
7. Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

1. 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.

2. 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.