

PathScan® Multiplex Western Cocktail II:

Phospho-p90RSK, Phospho-p53, Phospho-p38 MAPK and Phospho-S6 Ribosomal Protein Detection Cocktail II

✓ 250 µl
(5 Western mini-blots)

Orders ■ 877-616-CELL (2355)
orders@cellsignaling.com

Support ■ 877-678-TECH (8324)
info@cellsignaling.com

Web ■ www.cellsignaling.com

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This product is for *in vitro* research use only and is not intended for use in humans or animals.

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

Description: The PathScan® Multiplex Western Cocktail II 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-p53, phospho-p38 MAPK and phospho-S6 ribosomal protein. The cocktail also includes eIF4E antibody to control protein loading.

Specificity/Sensitivity: Each phospho-antibody in this cocktail recognizes endogenous levels of only the phosphorylated form of its specific target. The eIF4E antibody detects endogenous levels of its target protein independent of phosphorylation and is provided to control for protein loading.

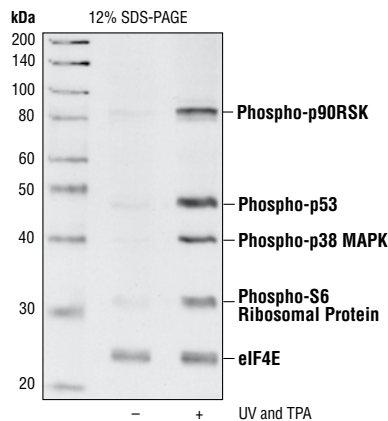
Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with synthetic peptides. Antibodies are purified by protein A and peptide affinity chromatography.

Background: The 90 kDa ribosomal S6 kinases (RSK1-3) are a family of serine/threonine kinases broadly expressed in response to many growth factors, polypeptide hormones and neurotransmitters (1). p90RSK is activated by Erk1 and Erk2 *in vitro* and *in vivo* via phosphorylation (2). Several sites, such as Ser380, Thr359 and Ser363, are important for its activation (3).

The p53 tumor suppressor protein plays a major role in cellular response to DNA damage and other genomic aberrations. Activation of p53 can lead to either cell cycle arrest and DNA repair or apoptosis (4). p53 is phosphorylated at multiple sites *in vivo* and by several different protein kinases *in vitro* (5,6). DNA damage induces phosphorylation of p53 at Ser15 and Ser20 and leads to reduced interaction of p53 with its negative regulator, oncoprotein MDM2 (7).

p38 MAP kinase controls cellular responses to cytokines and stress (8–11). Like the SAPK/JNK pathway, p38 MAP kinase is activated by a variety of cellular stresses including osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS), UV light and growth factors (8–12). MKK3, MKK6 and SEK activate p38 MAP kinase by phosphorylation at Thr180 and Tyr182.

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 (13). This group of mRNAs (5'TOP) encodes proteins involved in cell cycle progression and proteins that are part of the translational machinery, such as ribosomal proteins and elongation factors (13,14).



Western blot analysis of extracts from Mv1Lu mink lung epithelial cells untreated or treated with UV and TPA, using PathScan® Multiplex Western cocktail II to detect phosphorylation of p90RSK, p53, p38 MAPK and S6 ribosomal protein.

Species Cross-Reactivity and Molecular Weight

No.	Antibody	Species Cross-Reactivity	MW (kDa)
9341	Phospho-p90RSK (Ser380) Antibody	H, M, R, Hm	90
9284	Phospho-p53 (Ser15) Antibody	H, M, R, Mk	53
9211	Phospho-p38 MAP Kinase (Thr180/Tyr182) Antibody	H, M, R	43
2211	Phospho-S6 Ribosomal Protein (Ser235/236) Antibody	H, M, R, (C, X)	32
9742	eIF4E Antibody	H, M, R	25

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry IC—Immunocytochemistry IF—Immunofluorescence

Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken X—Xenopus

Species enclosed in parentheses are predicted to react based on 100% sequence homology.

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.

Recommended Antibody Dilutions:
Western blotting 1:200

Companion Products:

Phospho-S6 Ribosomal Protein (Ser235/236) Antibody #2211

Phospho-p38 MAP Kinase (Thr180/Tyr182) Antibody #9211

Phospho-p53 (Ser15) Antibody #9284

Phospho-p90RSK (Ser380) Antibody #9341

Anti-rabbit IgG, HRP-linked Antibody #7074

Phototope®-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody #7071

Biotinylated Protein Ladder Detection Pack #7727

20X LumiGLO® Reagent and 20X Peroxide #7003

PathScan® Multiplex Western Cocktail II: Phospho-p90RSK, Phospho-p53, Phospho-p38 MAPK and Phospho-S6 Ribosomal Protein Detection Kit #7110

Background References:

- (1) Frodin, M. and Gammeltoft, S. (1999) *Mol. Cell. Endocrinol.* 151, 65–77.
- (2) Lazar, D.F. et al. (1995) *J. Biol. Chem.* 270, 20801–20807.
- (3) Dalby, K.N. et al. (1998) *J. Biol. Chem.* 273, 1496–1505.
- (4) Levine, A.J. (1997) *Cell* 88, 323–331.
- (5) Meek, D.W. (1994) *Semin. Cancer Biol.* 5, 203–210.
- (6) Milczarek, G.J. et al. (1997) *Life Sci.* 60, 1–11.
- (7) Shieh, S.Y. et al. (1997) *Cell* 91, 325–334.
- (8) Han, J. et al. (1994) *Science* 265, 808–811.
- (9) Lee, J.C. et al. (1994) *Nature* 372, 739–746.
- (10) Rouse, J. et al. (1994) *Cell* 78, 1027–1037.
- (11) Freshney, N.W. et al. (1994) *Cell* 78, 1039–1049.
- (12) Raingeaud, J. et al. (1995) *J. Biol. Chem.* 270, 7420–7426.
- (13) Peterson, R.T. and Schreiber, S.L. (1998) *Curr. Biol.* 8, R248–R250.
- (14) Jefferies, H.B. et al. (1997) *EMBO J.* 16, 3693–3704.

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.

F—Flow cytometry E—ELISA D—DELFIATM

Z—zebra fish B—bovine All—all species expected

Western Immunoblotting Protocol (Primary Ab 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.