#7180 Store at -20°C

PathScan® PDGFR Activity Assay:
Phospho-PDGFR, Phospho-SHP2, Phospho-Akt, and Phospho-p44/42 MAPK (Erk1/2) Multiplex Western Detection Kit

Cell Signaling TECHNOLOGY®

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☑ 1 Kit
(5 western blots)

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

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

Kit Components

No.	Name	Quantity	Source
5304	PathScan® Multiplex Western Detection Cocktail II	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 Detection Kit offers a unique method to assay the phosphorylation of multiple proteins 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 the phosphorylation of PDGF Receptor β, SHP2, Akt, and p44/42 MAPK proteins in response to PDGF. The kit also includes an eIF4E antibody to control protein loading. In addition, each Pathscan® Multiplex Western 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 mini-blot experiments.

Background: Platelet-derived growth factor (PDGF) is a dimeric molecule that exists as homodimers or heterodimers of related polypeptide chains (A and B). Two types of PDGF receptors have been identified. The PDGF α-receptor binds all three isoforms with high affinity, whereas the β-receptor binds only PDGF-BB with high affinity, PDGF-AB with low affinity and does not appear to bind PDGF-AA (1). PDGF exerts its stimulatory effects on cells by binding to these two related protein tyrosine kinase receptors. Ligand binding induces receptor dimerization and autophosphorylation, allowing binding and activation of cytoplasmic SH2-domain-containing signal transduction molecules. Thereby, a number of different signaling pathways are initiated, leading to cell growth, actin reorganization, migration and differentiation (2-4). In clinical studies, PDGF expression has been shown in a number of different solid tumors, from glioblastomas to prostate carcinomas. In these various tumor types, the biologic role of PDGF signaling can vary from autocrine stimulation of cancer cell growth to more subtle paracrine interactions involving adjacent stroma and even angiogenesis. Targeting PDGF signaling becomes an effective way for tumor treatment (5).

Specificity/Sensitivity: Each phospho-antibody in this kit recognizes only the phosphorylated form of its specific target. The eIF4E control antibody detects total levels of eIF4E independent of phosphorylation state to determine protein loading. All of the antibodies detect endogenous levels of the target proteins.

Source/Purification: Antibodies are produced by immunizing rabbits with synthetic peptides, and are purified by combinations of Protein A and peptide affinity chromatography.

Antibody Cocktail Components and Molecular Weights

No.	Antibody	Molecular Weight
4549	Phospho-PDGF Receptor β (Tyr751) (C63G6) Rabbit mAb	190 kDa
3751	Phospho-SHP-2 (Tyr542) Antibody	72 kDa
9271	Phospho-Akt (Ser473) Antibody	60 kDa
4370	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) Rabbit mAb	42, 44 kDa
2067	eIF4E (C46H6) 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.*

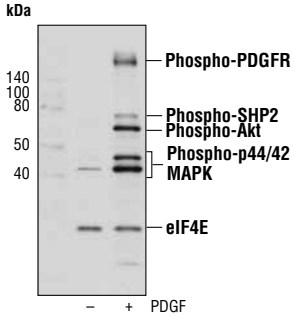
***Species cross-reactivity is determined by Western blot, using the individual antibody cocktail components.**

Recommended Antibody Dilutions:
Western Blotting 1:200

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

Background References:

(1) Westermark, B. et al. (1990) *Ciba Found. Symp.* 150, 6–22.
(2) Heldin, C.H. (1997) *FEBS Lett.* 410, 17–21.
(3) Bornfeldt, K.E. et al. (1995) *Ann. N. Y. Acad. Sci.* 766, 416–430.
(4) Renhowe, P.A. (2002) *Curr. Opin. Drug Discov. Devel.* 5, 214–224.
(5) George, D. (2001) *Semin. Oncol.* 28, 27–33.



Western blot analysis of extracts from serum-starved NIH/3T3 cells untreated or PDGF-treated (#8912, 50 ng/ml for 20 minutes), using PathScan PDGF Receptor Activity Assay cocktail to detect phosphorylation of PDGFR, SHP2, Akt and p44/42 MAPK.

Rabbit monoclonal antibody is produced under license (granting certain rights including those under U. S. Patent No. 5,675,063) from Epitomics, Inc.

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.

#7180

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.