#8999 Store at -20°C

PathScan® Signaling Nodes Multiplex IF Kit

Cell Signaling TECHNOLOGY®

☑ 1 Kit
(100 immunocytochemical stainings)

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

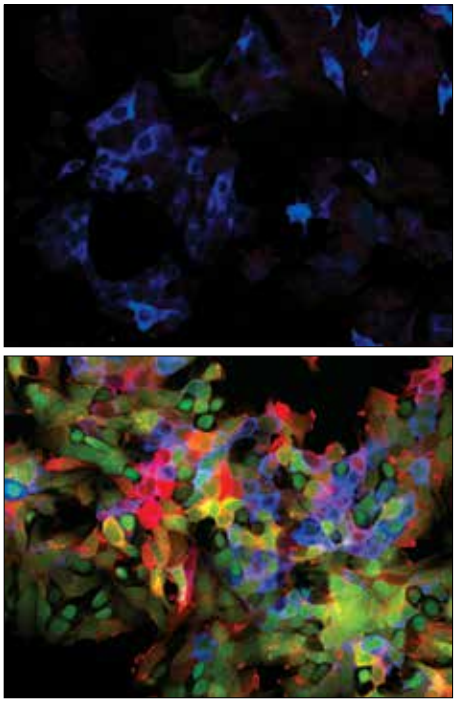
Products Included	Product #	Volume	Applicaton	Dilution	Species Cross-Reactivity
Primary Cocktail	8998	100 µl	IF-IC	1:100	H, M, R, Mk
Detection Cocktail	8997	100 µl	IF-IC	1:100	N/A

Kit Analytes	Detection Dye	$Ex_{(max)}$ (nm)	$Em_{(max)}$ (nm)
Phospho-Akt (Ser473)	Alexa Fluor® 555	555	565
Phospho-p44/42 Erk1/2 (Thr202/Tyr204)	Alexa Fluor® 488	495	519
Phospho-S6 Ribosomal Protein (Ser235/236)	Alexa Fluor® 647	650	665

Description: Traditional biochemical and lysate-based assays (e.g., western blot, immunoprecipitation, ELISA) have been integral in the analysis of individual signaling events, however they are limited in their ability to monitor the phosphorylation and subcellular localization of multiple proteins on a per cell basis. PathScan® Signaling Nodes Multiplex IF Kit offers a novel method to simultaneously monitor signaling through key pathway nodes using manual immunofluorescence microscopy, or automated imaging and laser scanning high content platforms. These kits contains a cocktail of three high quality primary antibodies targeted against phospho-Akt (Ser473), phospho-p44/42 (Thr202/Tyr204), and phospho-S6 (Ser235/236) and a detection cocktail utilizing the Alexa Fluor® series of fluorescent dyes. Antibody formulation and dye pairings have been pre-optimized and each kit contains enough reagents for 100 assays (based on a working volume of 100 µL/test).

Specificity/Sensitivity: Phospho-Akt (Ser473) antibody detects endogenous levels of Akt only when phosphorylated at Ser473. Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody detects endogenous levels of p44 and p42 MAP kinase (Erk1 and Erk2) when dually phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2), and singly phosphorylated at Thr202. This antibody does not cross-react with the corresponding phosphorylated residues of either JNK/SAPK or p38 MAP kinases. Phospho-S6 ribosomal protein (Ser235/236) antibody detects endogenous levels of ribosomal protein S6 only when phosphorylated at Ser235 and Ser236.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with synthetic phosphopeptides corresponding to residues surrounding Ser473 of human Akt, Thr202/Tyr204 of human p44 MAP kinase, and Ser235/Ser236 of human ribosomal protein S6.

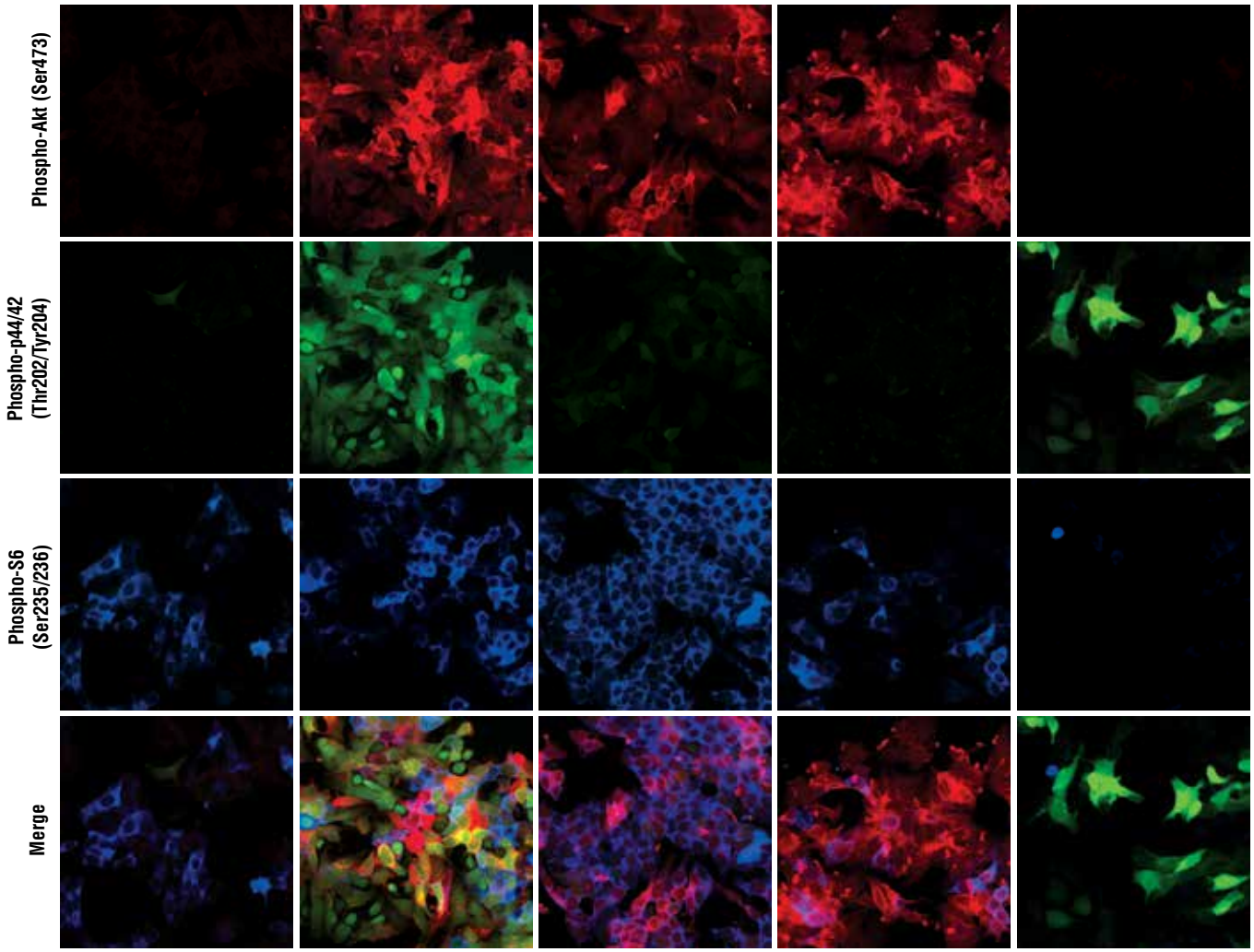


Immunofluorescent analysis of MCF7 cells, serum-starved (left) or insulin-treated (right), using PathScan® Signaling Nodes Multiplex IF Kit. Red = Phospho-Akt (Ser473), green = Phospho-p44/42 (Thr202/Tyr204), and blue pseudocolor = Phospho-S6 (Ser235/236).

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 either cocktail.*

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



Immunofluorescent analysis of insulin-treated MCF7 cells (human breast adenocarcinoma), following pretreatment with kinase specific inhibitors LY294002 (PI3 Kinase Inhibitor) #9901 or U0126 (MEK1/2 Inhibitor) #9903 for the indicated times.

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 a downstream effector of phosphoinositide-3 kinase (PI3K), and is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 (4), as well as by phosphorylation within the carboxy terminus at Ser473 by the mTOR-rictor complex (TORC2) (5). This pathway is down-regulated following dephosphorylation of phosphatidyl-inositol 3,4,5 triphosphate by PTEN, as well as by deactivation of PI3K with targeted small molecule inhibitors such as wortmannin and LY294002 (2,3,6,7).

p70 S6 kinase, a mitogen activated Ser/Thr protein kinase downstream of PI3K and the mTOR-raptor complex (mTORC1), phosphorylates the S6 protein of the 40S ribosomal subunit leading to an increase in translation of mRNA transcripts that contain an oligopyrimidine tract in their 5' untranslated region (8). These particular mRNA transcripts (5'TOP) encode proteins involved in cell cycle progression, as well as ribosomal proteins and elongation factors necessary for translation (8,9). Important S6 ribosomal protein phosphorylation sites include several residues (Ser235, Ser236, Ser240 and Ser244) located within a small, carboxy-terminal region of the S6 protein (10,11).

Both p44 and p42 mitogen-activated protein (MAP) kinases (Erk1 and Erk2, respectively) play a critical role in the regulation of cell growth and differentiation (12-15). MAP kinases are activated by a wide variety of extracellular signals including growth and neurotrophic factors, cytokines, hormones, and neurotransmitters. Activation of MAP kinases occur through phosphorylation of Thr202/Tyr204 on human Erk1 and Thr185/Tyr187 on human Erk2 at the sequence T*EY* by a pair of upstream MAP kinase kinases (MEK1/2) (16,17). Erk proteins are negatively regulated by a family of dual specificity (Thr/Tyr) MAPK phosphatases, known as DUSPs or MKPs (18), along with MEK inhibitors such as U0126 and PD98059. Erk dependent phosphorylation of TSC2 at Ser663 leads to the functional inactivation of the TSC1/TSC2 inhibitory complex, and subsequent downstream activation of S6 ribosomal protein through the mTORC1/p70 S6K signaling cascade (19).

Background References:

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(4) Alessi, D.R. et al. (1996) *EMBO J* 15, 6541-51.

(5) Sarbassov, D.D. et al. (2005) *Science* 307, 1098-101.

(6) Myers, M.P. et al. (1998) *Proc Natl Acad Sci U S A* 95, 13513-8.

(7) Vlahos, C.J. et al. (1994) *J Biol Chem* 269, 5241-8.

(8) Peterson, R.T. and Schreiber, S.L. (1998) *Curr Biol* 8, R248-50.

(9) Jefferies, H.B. et al. (1997) *EMBO J* 16, 3693-704.

(10) Ferrari, S. et al. (1991) *J Biol Chem* 266, 22770-5.

(11) Flotow, H. and Thomas, G. (1992) *J Biol Chem* 267, 3074-8.

(12) Marshall, C.J. (1995) *Cell* 80, 179-85.

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(14) Hill, C.S. and Treisman, R. (1995) *Cell* 80, 199-211.

(15) Cowley, S. et al. (1994) *Cell* 77, 841-52.

(16) Sturgill, T.W. et al. (1988) *Nature* 334, 715-8.

(17) Payne, D.M. et al. (1991) *EMBO J* 10, 885-92.

(18) Owens, D.M. and Keyse, S.M. (2007) *Oncogene* 26, 3203-13.

(19) Ma, L. et al. (2005) *Cell* 121, 179-93.

PathScan® Multiplex IF Kit Protocol

***IMPORTANT:** Please refer to the **APPLICATIONS** section on the front page of the datasheet to determine if this kit has been validated and approved for use on cultured cell lines (**IF-IC**), paraffin-embedded samples (**IF-P**), or frozen tissue sections (**IF-F**)

A Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **10X Phosphate Buffered Saline (PBS):** To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 7.4.
2. **Formaldehyde,** 16%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4°C in dark, dilute in warm PBS for use.
3. **Blocking Buffer (1X PBS/5% normal goat serum/0.3% Triton X-100):** To prepare 25 ml, add 2.5 ml 10X PBS, 1.25 ml normal goat serum and 21.25 ml dH_2O and mix well. While stirring, add 75 µl Triton X-100.
4. **Antibody Dilution Buffer (1X PBS/1% BSA/0.3% Triton X-100):** To prepare 25 ml, add 2.5 ml 10X PBS to 22.5 ml dH2O, mix. Add 0.25 g BSA and mix well. While stirring, add 75 µl Triton X-100.

Reagents specific to IF-P application:

1. Xylene.
2. Ethanol, anhydrous denatured, histological grade, 100% and 95%.
3. **Antigen Unmasking:**
   a. **Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.
   b. **EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 8.0.

B Specimen Preparation

I. Cultured Cell Lines (IF-IC)

NOTE: Cells should be grown, treated, fixed, and stained directly in multiwell plates, chamber slides, or on coverslips.

1. Aspirate culture medium, and then cover cells to a depth of 2–3 mm with 4% formaldehyde diluted in 1X PBS warmed to 37°C.
   NOTE: Formaldehyde is toxic, use only in fume hood.
2. Allow cells to fix for 15 minutes at room temperature.
3. Aspirate fixative, rinse three times in PBS for 5 minutes each.
4. Proceed with immunostaining (Section C).

II. Paraffin Sections (IF-P)

NOTE: Do not allow slides to dry at any time during this procedure.

Deparaffinization/Rehydration:

1. Incubate sections in three washes of xylene for 5 minutes each.
2. Incubate sections in two washes of 100% ethanol for 10 minutes each.
3. Incubate sections in two washes of 95% ethanol for 10 minutes each.
4. Rinse sections twice in dH_2O for 5 minutes each.

NOTE: Consult product datasheet for specific recommendation for the unmasking solution.

Antigen Unmasking:

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0 then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.
2. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.

III. Frozen/Cryostat Sections (IF-F)

NOTE: Fresh frozen/unfixed sections should be fixed immediately in 4% formaldehyde as follows to preserve signaling epitopes.

1. Cover sections with 4% formaldehyde diluted in 1X PBS warmed to 37°C.
   NOTE: Formaldehyde is toxic, use only in fume hood.
2. Allow sections to fix for 15 minutes at room temperature.
3. Rinse slides three times in PBS for 5 minutes each.

C Immunostaining

NOTE: All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid, light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

1. Block specimen in Blocking Buffer for 60 minutes.
2. While blocking, prepare primary cocktail by diluting as indicated on datasheet in Antibody Dilution Buffer.
3. Aspirate blocking solution, apply diluted primary cocktail.
4. Incubate overnight at 4°C.
5. Rinse three times in PBS for 5 minutes each.
6. Prepare detection cocktail by diluting as indicated on datasheet in Antibody Dilution Buffer.
7. Incubate 1-2 hours at room temperature in the dark.
8. Rinse three times in PBS for 5 minutes each.
9. Coverslip slides with Prolong® Gold Antifade Reagent.
10. For best results examine specimens immediately using appropriate excitation wavelengths. For long-term storage, store slides at 4°C protected from light.

#8999 MSDS

Material Safety Data Sheet (MSDS) for PathScan® Signaling Nodes Multiplex IF Kit

I. Identification:

Product name: PathScan® Signaling Nodes Multiplex IF Kit
Product Catalog: 8999
CAS#: None
Manufacturer Supplier: Cell Signaling Technology
3 Trask Lane
Danvers, MA 01923 USA
1-978-867-2300 TEL
1-978-867-2400 FAX
1-978-578-6737 Emergency TEL

II. Composition/Information on Ingredients:

This Product is For Research Use Only.
The components of this kit are composed of antibodies in aqueous buffer solution.

Hazardous Ingredient:	Percent (%w/v)	CAS#	EC#
Glycerol	53%	56-81-5	200-289-5
Sodium azide	<0.02%	26628-22-8	247-852-1

III. Hazard Identification:

This product is a kit containing antibodies in aqueous solution.
Emergency Overview of Hazardous ingredient substance : Glycerol (CAS# 56-81-5)
According to OSHA, 29 CFR 1910.1200(d): Irritant. Avoid contact and inhalation.
Target organ: Kidneys.
According to (EC) No1272/2008: Eye Irritation (Category 2)
Not a hazardous substance or mixture according to EC-directives 67/548/EEC or 1999/45/EC.
Caution: This substance has not been thoroughly tested.

IV. First Aid Measures:

Inhalation: Remove to fresh air. If breathing is difficult, get medical attention.
Ingestion: If swallowed, rinse mouth with water provided person is conscious. Get medical attention.
Skin exposure: : In case of contact, wash skin with soap and water.
Eye exposure: In case of contact with eyes, immediately flush eyes with water for at least 15 minutes. Get medical attention.

V. Fire Fighting Measures:

Flash Point: Data not available.
Autoignition Temperature: Data not available.
Fire extinguishing media: water spray, dry chemical, foam, or carbon dioxide.
Firefighting: wear protective clothing and self-contained breathing apparatus to prevent contact with skin and eyes.

VI. Accidental Release Measures:

Absorb liquid with an absorbent material. Transfer contaminated absorbent to a chemical waste container for disposal.

VII. Handling And Storage:

Avoid inhalation and contact with eyes and skin. Avoid prolonged or repeated exposure.
Store at 4°C in tightly closed container.

VIII. Exposure Controls/Personal:

Engineering Controls: Maintain adequate ventilation, eye wash and quick-drench facilities in work area.
Personal Protective Equipment: Lab coat, chemical resistant gloves and chemical safety glasses.
Occupational Exposure Limits: Data not available.

IX. Physical And Chemical Properties:

Physical State:	liquid
Appearance:	colorless
Odor:	odorless
pH:	data not available
Boiling Point:	data not available
Melting Point:	data not available
Volatile Organic Compounds (VOC):	data not available
Solubility (water):	soluble

X. Stability and Reactivity:

Stability: : Stable under recommended conditions.
Conditions to avoid: No data available
Hazardous Decomposition: May form carbon dioxides under fire conditions.
Materials to aviod: Strong oxidizing reagents.

XI. Toxicological Information:

Acute toxicity: No data available.
Skin corrosion/irritation: No data available.
Eye damage/eye irritation: No data available.
Mutagenicity: No data available.
Carcinogenicity: No data available.
IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible, or confirmed human carcinogen by IARC.
Reproductive toxicity: No data available.
Specific target organ toxicity: No data available.

Potential Health Effects:
Inhalation: May be harmful if inhaled.
Ingestion: May be harmful if swallowed.
Skin: May be harmful if absorbed through skin. May cause skin irritation.
Eyes: May cause skin irritation.

XII. Ecological Information:

Toxicity: No data available.
Persistance and degradability: No data available.
Bioaccumulative potential: No data available.
Mobility in soil: No data available.
PBT and vPvP assessment: No data available.
Other adverse effects: No data available.

XIII. Disposal Considerations:

Dispose of in accordance with federal, state and local environment regulations.

XIV. Transport Information:

D.O.T.: This product is considered to be non-hazardous for transport.
IATA: This product is considered to be non-hazardous for transport.
IMDG: This product is considered to be non-hazardous for transport.

XV. Regulatory Information:

This safety datasheet complies with the requirement of regulations 29 CFR 1910.1200(d) and (EC) No.1907/2006.

XVI. Other Information:

This product is not intended for use in humans. To the best of our knowledge, this document is accurate. It is intended to serve as a guide for safe use of this product in a laboratory setting by experienced personnel. The burden of safe use of this material rests entirely with the user. The above information is believed to be accurate but is not necessarily all-inclusive and shall be used only as a guide. Cell Signaling Technology, Inc., shall not be held liable for any damage resulting from the handling of or from contact with the above product.