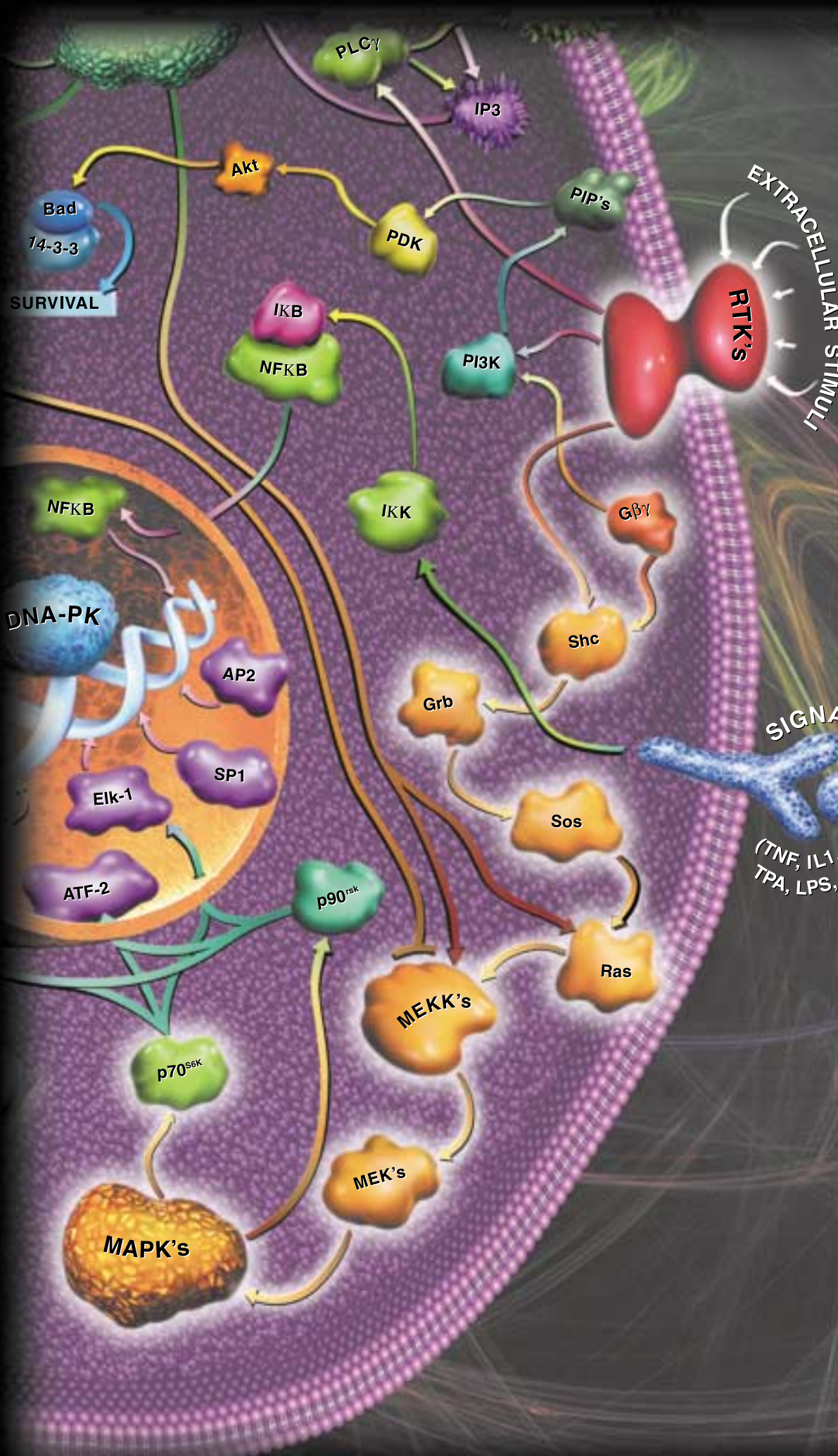


1

CHAPTER

MITOGEN-ACTIVATED PROTEIN KINASE

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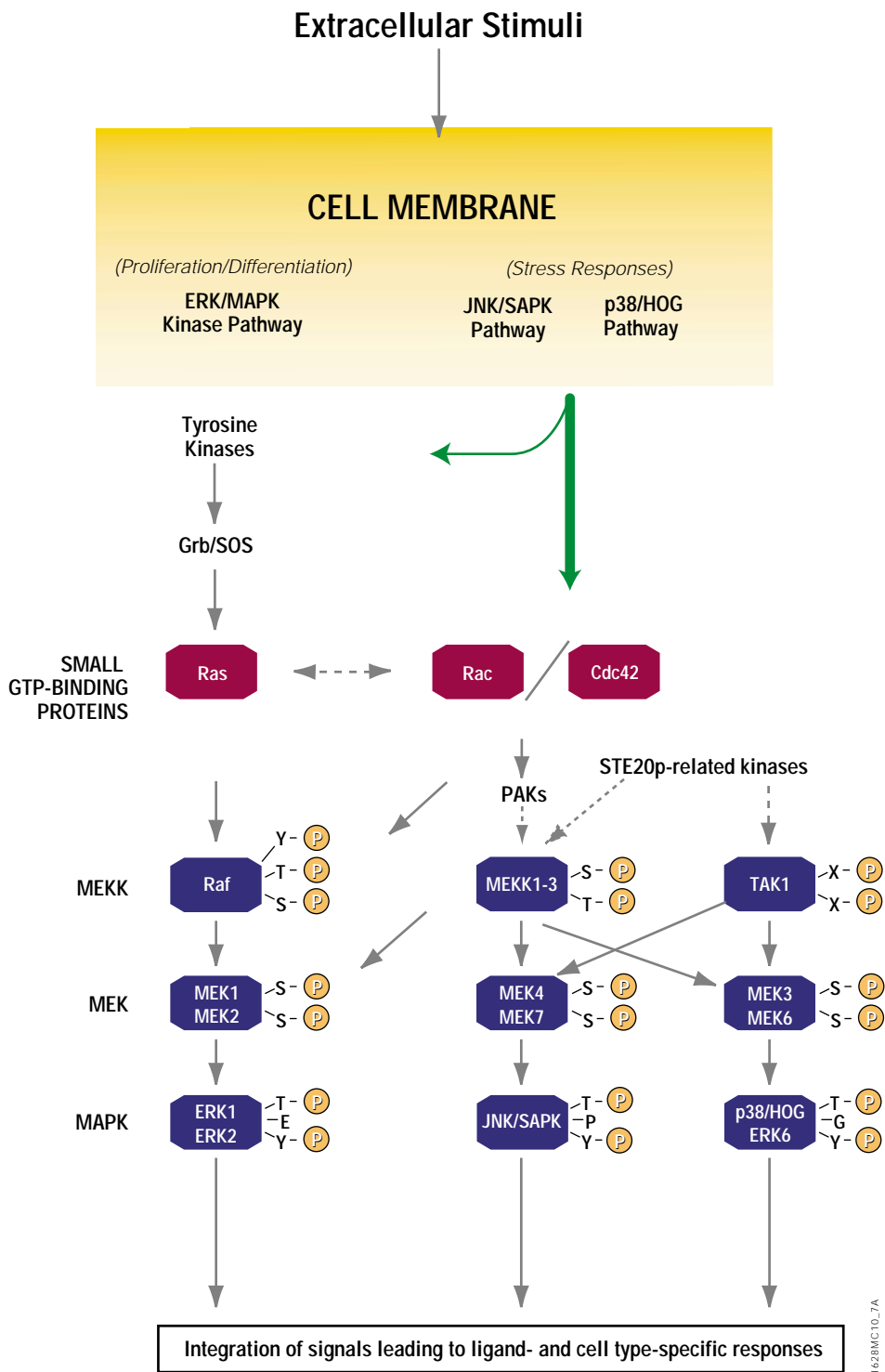


Figure 1.1. Activation of different MAPK signaling cascades by different extracellular stimuli. The ERK, JNK, and p38 cascades all contain the same series of three kinases. A MEK kinase (MEKK) phosphorylates and activates a MAP Kinase Kinase (MEK), then MEK phosphorylates and activates a MAP kinase (MAPK).

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MAPK Signaling

Signal transduction networks permit cells to receive external stimuli and respond to the signals in an appropriate manner. The Mitogen-Activated Protein Kinase (MAPK) signaling pathways play an important role in signal transduction in eukaryotic cells, where they modulate many cellular events including: mitogen-induced cell cycle progression through the G1 phase, regulation of embryonic development, cell movement and apoptosis, as well as cell and neuronal differentiation (1,2). These evolutionarily conserved pathways are organized in three-kinase modules consisting of a MAP kinase, an activator of MAP kinase (MAP Kinase Kinase or MEK) and a MAP Kinase Kinase Kinase (MEK Kinase, MEKK, or MAPK Kinase Kinase). There are at least three distinct MAP kinase signal transduction pathways in mammalian cells, each named after the particular MAPK associated with it. These include the extracellular signal-regulated kinases, ERK1/2 (also known as MAPKs), the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and the p38 kinases (Figure 1.1). Since the budding yeast *Saccharomyces cerevisiae* is known to have more than three distinct MAPK pathways, it is logical to expect that there might be additional MAPK pathways in mammalian cells (2).

MAP kinases are proline-directed serine/threonine kinases that are activated by dual phosphorylation in response to diverse extracellular stimuli. The dual phosphorylation occurs in the activation domain of MAPKs on the threonine and tyrosine residues in the sequence pTxpY (3-6). The dual phosphorylation is facilitated by dual-specificity MAP kinase kinases (MAPKKs or MEKs), which in turn are activated by serine/threonine phosphorylation by MAP kinase kinase kinases (MEKK). Mammalian ERK1 and ERK2, and their upstream activators MEK1 and MEK2, are stimulated by growth and differentiating factors (e.g., epidermal growth factor, platelet-derived growth factor and nerve growth factor [NGF]) through receptor tyrosine kinases (RTKs), heterotrimeric G protein-coupled receptors, or cytokine receptors (reviewed in 7). The yeast homolog of ERK1/2, Fus3p, is involved in pheromone response resulting in cell-cycle arrest and the induction of mating-specific gene expression (8). The mammalian JNKs and p38 kinases are implicated in responses to cellular stress, inflammation and apoptosis. They are activated by lipopolysaccharides, IL-1, TNF- α , ionizing or ultraviolet radiation, the translation inhibitors cycloheximide and anisomycin, tumor promoters, heat shock or hyperosmotic stress. In MDA435 cells, induction of the breast cancer-susceptible gene triggers apoptosis through the JNK pathway (9).

Signal transduction cascades involving ERK/MAPK superfamily enzymes are also regulated by the activities of a variety of protein phosphatases. Several dual-specificity protein phosphatases have been identified that can differentially dephosphorylate the MAPK, JNK or p38 enzymes. For example, the phosphatase MKP2 acts on both MAPK and JNK enzymes, while M3/M6 acts on JNK and p38. In contrast, PAC1 acts primarily on MAPKs while MKP1, also known as CL-100, can act on all three enzymes (10,11). In addition, individual Ser/Thr (e.g., PP2A) or Tyr (e.g., PTP1) phosphatases also appear to regulate the activity of the ERK/MAPK enzymes by dephosphorylating either core residue (7,12,13). Thus, the cell can tightly regulate the activity of the ERK/MAPK enzymes by judicious use of different combinations of MEKs, the mono- and dual-specificity protein phosphatases and the subcellular localization of each enzyme to elicit the appropriate physiological response. This level of complexity emphasizes the need for reagents that can accurately detect the active, dually phosphorylated forms of these enzymes.

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Promega Product Citations

Anti-ACTIVE® MAPK pAb

Ackerley, S. *et al.* (2000) Glutamate slows axonal transport of neurofilaments in transfected neurons. *J. Cell Biol.* **150**, 165.

Atwal, J.K. *et al.* (2000) The TrkB-Shc site signals neuronal survival and local axon growth via MEK and PI3-kinase. *Neuron* **27**, 265.

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Promega Resources

Protocol

Anti-ACTIVE® MAPK, JNK and p38 Polyclonal Antibodies and Anti-ACTIVE® Qualified Secondary Antibody Conjugates**TB262**

Publications

Jarvis, B.W. and Moravec, R. (1998) New Anti-ACTIVE® MAPK and 'pan ERK 1/2' Antibodies for Western analysis. *Promega Notes* **69**, 9.

Curtin, M. (2000) Technically Speaking: Anti-ACTIVE® Antibodies and MAPK signaling pathways. *Promega Notes* **76**, 23.

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Anti-ACTIVE® Antibodies

Anti-ACTIVE® MAPK pAb, Rabbit, (pTEpY)

Product	Size	Catalog #
Anti-ACTIVE® MAPK pAb, Rabbit, (pTEpY)	40µl	V8031

Description: Anti-ACTIVE® MAPK pAb, Rabbit, is an affinity-purified polyclonal antibody that specifically recognizes the dually phosphorylated, active form of MAPK (also known as p44/ERK1 and p42/ERK2) enzymes. Anti-ACTIVE® MAPK pAb is raised against a dually phosphorylated peptide sequence representing the catalytic core of the active ERK enzyme. The phosphorylated amino acid residues correspond to Thr 183 and Tyr 185 of the p42/ERK2 enzyme. The recommended dilution of Anti-ACTIVE® MAPK pAb for Western blot analysis is 1:5,000.

Features

- **Specificity:** The Anti-ACTIVE® MAPK pAb recognizes the active forms of ERK1, ERK2 and ERK7 (1).
- **ICC-Qualified:** Each lot is tested in immunocytochemical applications using PC12 cells.

References

1. Abe, M.K. *et al.* (1999) *Mol. Cell. Biol.* **19**, 1301.

Anti-ACTIVE® JNK pAb, Rabbit, (pTPpY)

Product	Size	Catalog #
Anti-ACTIVE® JNK pAb, Rabbit, (pTPpY)	40µl	V7931
	120µl	V7932

Description: Anti-ACTIVE® JNK pAb, Rabbit is an affinity-purified polyclonal antibody that recognizes the dually phosphorylated, active form of JNK (c-Jun N-terminal protein kinase), also known as SAPK (Stress-Activated Protein Kinase). Anti-ACTIVE® JNK pAb is raised against a dually phosphorylated peptide sequence representing the catalytic core of the active JNK enzyme. The phosphorylated amino acid residues correspond to Thr 183 and Tyr 185 of the JNK2 enzyme. The recommended dilution of Anti-ACTIVE® JNK pAb for Western blot analysis is 1:5,000.

Features

- **Specificity:** The Anti-ACTIVE® JNK pAb recognizes the active forms of JNK1, JNK2 and JNK3 isoforms.
- **ICC-Qualified:** Each lot is tested in immunocytochemical applications using PC12 cells.

Anti-ACTIVE® p38 pAb, Rabbit, (pTGpY)

Product	Size	Catalog #
Anti-ACTIVE® p38 pAb, Rabbit, (pTGpY)	100µl	V1211

Description: Anti-ACTIVE® p38 pAb, Rabbit, is an affinity-purified polyclonal antibody that recognizes the active form of p38 kinase. The Anti-ACTIVE® p38 pAb is raised against the dually phosphorylated peptide sequence representing the catalytic core of the active p38 enzyme. The phosphorylated amino acid residues correspond to Thr 180 and Tyr 182 of the p38 enzyme. The recommended dilution of Anti-ACTIVE® p38 pAb for Western blot analysis is 1:2,000.

Features

- **Specificity:** The Anti-ACTIVE® p38 pAb recognizes the active forms of p38 α , γ and δ isoforms.
- **ICC-Qualified:** Each lot is tested in immunocytochemical applications using PC12 cells.

A. Anti-ACTIVE® MAPK pAb

Untreated



NGF-Treated

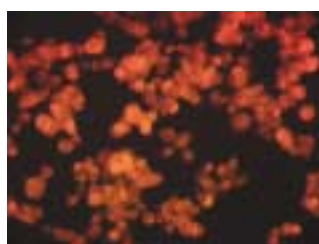


B. Anti-ACTIVE® JNK pAb

Untreated



Sorbitol-Treated



C. Anti-ACTIVE® p38 pAb

Untreated



Sorbitol-Treated



Figure 1.2. Detection of activated MAPK, JNK and p38 in PC12 cells by immunocytochemistry (ICC). Cells were either untreated or treated with 200ng/ml NGF or 1M sorbitol. Anti-ACTIVE® pAbs were used at the following dilutions: MAPK, 1:500 (Panel A); JNK, 1:1,000 (Panel B); p38, 1:500 (Panel C). The secondary antibody conjugate was donkey anti-rabbit Cy[™]3 conjugate (Jackson ImmunoResearch) at a 1:1,000 dilution. The images were visualized using a Zeiss® fluorescence microscope and captured using an Optronics digital camera at 1/15-second exposure.

Promega Product Citations

Anti-ACTIVE® JNK pAb

Ackerley, S. *et al.* (2000) Glutamate slows axonal transport of neurofilaments in transfected neurons. *J. Cell Biol.* **150**, 165.

Davis, P.K. and Johnson, G.V.W. (1999) The microtubule binding of Tau and high molecular weight Tau in apoptotic PC12 cells is impaired because of altered phosphorylation. *J. Biol. Chem.* **274**, 35686.

Halfter, H. *et al.* (2000) Oncostatin M-mediated growth inhibition of human glioblastoma cells does not depend on Stat3 or on mitogen-activated protein kinase activation. *J. Neurochem.* **75**, 973.

Limatola, C. *et al.* (1999) The growth-related gene product beta induces sphingomyelin hydrolysis and activation of c-jun N-terminal kinase in rat cerebellar granule neurons. *J. Biol. Chem.* **274**, 36537.

Sugino, T., Nozaki, K. and Hashimoto, N. (2000) Activation of mitogen-activated protein kinases in gerbil hippocampus with ischemic tolerance induced by 3-nitropropionic acid. *Neurosci. Lett.* **278**, 101.

Tisay, K.T. and Key, B. (1999) The extracellular matrix modulates olfactory neurite outgrowth on ensheathing cells. *J. Neurosci.* **19**, 9890.

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Anti-ACTIVE® MAPK, JNK and p38 Polyclonal Antibodies and Anti-ACTIVE® Qualified Secondary Antibody Conjugates.....**TB262**

Publications

Jarvis, B.W. and O'Brien, M. (1999) PC12 cell extracts for use with the new Anti-pT¹⁸³ MAPK and Anti-ACTIVE® Antibodies. *Promega Notes* **72**, 10.

Curtin, M. (2000) Technically Speaking: Anti-ACTIVE® Antibodies and MAPK signaling pathways. *Promega Notes* **76**, 23.

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Promega Product Citations

Anti-ACTIVE® p38 pAb

Katagiri, T. *et al.* (2000) Protein-tyrosine kinase Pyk2 is involved in interleukin-2 production by Jurkat T cells via its tyrosine 402. *J. Biol. Chem.* **275**, 19645.

Sugino, T. *et al.* (2000) Activation of mitogen-activated protein kinases after transient forebrain ischemia in gerbil hippocampus. *J. Neurosci.* **20**, 4506.

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Anti-ACTIVE® MAPK, JNK and p38 Polyclonal Antibodies and Anti-ACTIVE® Qualified Secondary Antibody Conjugates.....**TB262**

Publications

Jarvis, B.W. and Huang, S.-C. (2000) Immunostaining with the new Anti-ACTIVE® p38 Antibody. *Promega Notes* **76**, 3.

Curtin, M. (2000) Technically speaking: Anti-ACTIVE® Antibodies and MAPK signaling pathways. *Promega Notes* **76**, 23.

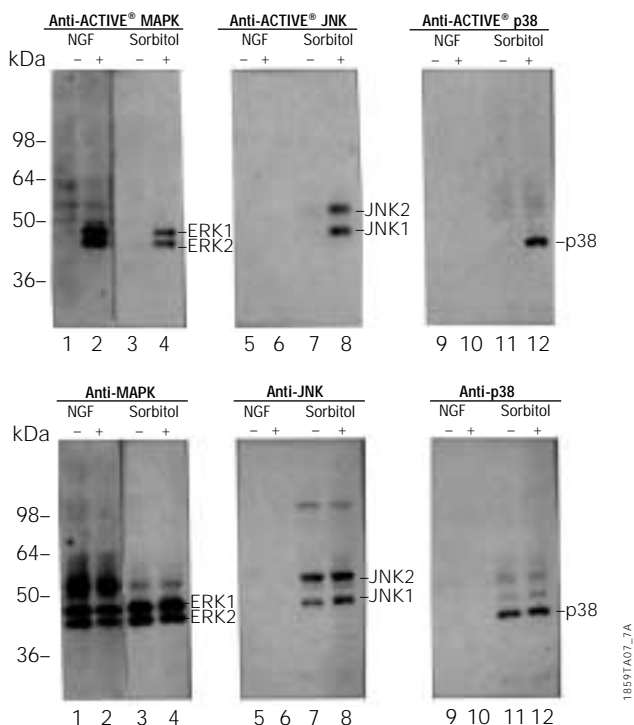


Figure 1.3. Detection of activated MAPK, JNK and p38 in PC12 cell extracts by Western blot analysis using Anti-ACTIVE® MAPK, JNK and p38 Polyclonal Antibodies. PC12 cells were grown to 60–80% confluence in RPMI 1640 medium supplemented with 25mM HEPES, 0.5mM EGTA, 10% horse serum and 5% fetal bovine serum. Cells were either untreated, treated with 50ng/ml of nerve growth factor (NGF) for 5 minutes, or 0.5M sorbitol for 5 minutes, as indicated. Cells were harvested, homogenized and subjected to high-speed centrifugation. The resulting supernatants were stored at –70°C. Aliquots of each extract were analyzed by SDS-PAGE (10% gel, under reducing conditions) and transferred to nitrocellulose membranes. The membranes were probed with either the indicated Anti-ACTIVE® pAb (upper row) or with the corresponding antibodies that recognize both active and inactive forms of each subfamily of kinases (lower row). For JNK, the antibody that detects both forms was generated against whole JNK enzyme from rat, while the corresponding antibodies for p38 and ERK1 and ERK2 were generated against synthetic peptides derived from the deduced amino acid sequence of each protein. The secondary antibody used was Promega's Donkey Anti-Rabbit IgG (H+L), AP, Anti-ACTIVE® Qualified Conjugate (Cat.# V7971). Chemiluminescent detection was performed using a Tropix Western-Star™ Kit and Kodak® BioMax® film, as described by the manufacturer. Lanes: lanes 1, 5 and 9, 2µg of unstimulated PC12 cell extract; lanes 2, 6 and 10, 2µg of NGF-stimulated PC12 cell extract; lanes 3, 7 and 11, 20µg of unstimulated PC12 cell extract; lanes 4, 8 and 12, 20µg of sorbitol-treated PC12 cell extract.

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Other Antibodies

Anti-ERK 1/2 pAb, Rabbit

Product	Size	Catalog #
Anti-ERK 1/2 pAb, Rabbit	40µl	V1141

Description: Anti-ERK 1/2 pAb is a polyclonal antibody purified from rabbit serum. The antibody is affinity-purified using a peptide sequence in human/rat ERK1. This antibody detects both the active and inactive forms of ERK1 and ERK2.

Anti-pT¹⁸³ MAPK pAb, Rabbit

Product	Size	Catalog #
Anti-pT ¹⁸³ MAPK pAb, Rabbit	50µl	V8081

Description: Anti-pT¹⁸³ MAPK pAb is a polyclonal antibody purified from rabbit serum. The antibody is affinity-purified using a monophosphorylated peptide (mono pT¹⁸³ peptide) corresponding to the monophospho-threonine form of ERK1 and ERK2.

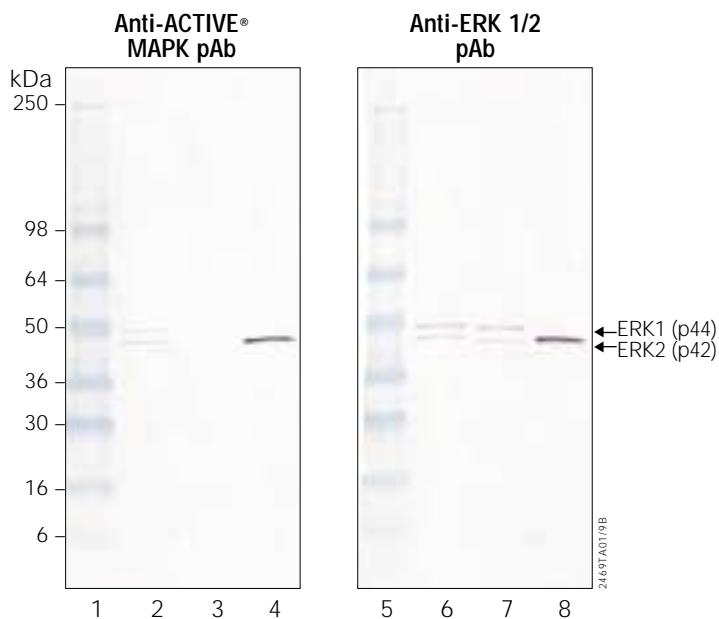


Figure 1.4. Western blot analysis of recombinant, active ERK2 and PC12 cell extracts using Anti-ACTIVE[®] MAPK pAb and Anti-ERK 1/2 pAb. Proteins were separated by SDS-PAGE on a NOVEX[®] 4–20% Tris-glycine gel followed by electrotransfer to nitrocellulose. Blots were then probed with Anti-ACTIVE[®] MAPK pAb (Cat.# V8031) at a 1:5,000 dilution (Panel A) or with Anti-ERK 1/2 pAb (Cat.# V1141) at a 1:5,000 dilution (Panel B). Detection was performed using Donkey Anti-Rabbit IgG (H+L), AP, Anti-ACTIVE[®] Qualified secondary antibody (Cat.# V7971) and Western Blue[®] Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841). Lanes 1 and 5 contain prestained molecular weight marker standards; lanes 2 and 6 contain 1µg of NGF-stimulated PC12 cell extract; lane 3 contains 10µg of unstimulated PC12 extract; lane 7 contains 1µg of unstimulated PC12 extract; lanes 4 and 8 contain 1ng of recombinant, active ERK2 enzyme.

Promega Product Citations

Anti-ERK 1/2 pAb

McWhinney, C. *et al.* (2000) Constitutively active mutants of the alpha1a- and the alpha1b-adrenergic receptor subtypes reveal coupling to different signaling pathways and physiological responses in rat cardiac myocytes. *J. Biol. Chem.* **275**, 2087.

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Anti-ERK 1/2 pAb, Rabbit, Promega Product Information Sheet**9PIV114**

Publications

Jarvis, B.W. and Moravec, R. (1998) New Anti-ACTIVE[®] MAPK & 'pan ERK 1/2' Antibodies for Western analysis. *Promega Notes* **69**, 9.

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Promega Product Citations

Donkey Anti-Rabbit IgG (H+L), AP/HRP, Anti-ACTIVE® Qualified

Durham, P. L. and Russo, A.F. (1998) Serotonergic repression of mitogen-activated protein kinase control of the calcitonin gene-related peptide enhancer. *Mol. Endocrinol.* **12**, 1002.

Sugino, T. *et al.* (2000) Activation of mitogen-activated protein kinases after transient forebrain ischemia in gerbil hippocampus. *J. Neurosci.* **20**, 4506.

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Anti-ACTIVE® MAPK, JNK and p38 Polyclonal Antibodies and Anti-ACTIVE® Qualified Secondary Antibody Conjugates**TB262**

Publications

Curtin, M. (2000) Technically Speaking: Anti-ACTIVE® Antibodies and MAPK signaling pathways. *Promega Notes* **76**, 23.

Donkey Anti-Rabbit IgG (H+L), HRP, Anti-ACTIVE® Qualified

Product	Size	Catalog #
Donkey Anti-Rabbit IgG (H+L), HRP	60µl	V7951

Description: Donkey Anti-Rabbit IgG (H+L), HRP, is an affinity-purified horseradish peroxidase (HRP) -conjugated secondary antibody for use with the Anti-ACTIVE® pAbs. It is qualified for use in Western blot analysis using chemiluminescent and colorimetric detection methods. This antibody conjugate exhibits minimal cross-reactivity to goat, mouse and sheep IgG, bovine serum albumin (BSA) and proteins in mammalian cell extracts. This antibody conjugate provides low backgrounds and highly specific signals when used at the recommended 1:5,000 dilution of conjugate with Anti-ACTIVE® MAPK pAb, and 1:10,000 dilution of conjugate with Anti-ACTIVE® JNK and Anti-ACTIVE® p38 pAbs.

Feature

- **Optimized:** Provides specific detection and low background when used in Western blot analysis with Promega's Anti-ACTIVE® rabbit polyclonal antibodies.

Donkey Anti-Rabbit IgG (H+L), AP, Anti-ACTIVE® Qualified

Product	Size	Catalog #
Donkey Anti-Rabbit IgG (H+L), AP	60µl	V7971

Description: Donkey Anti-Rabbit IgG (H+L), AP, is an affinity-purified alkaline phosphatase (AP) -conjugated secondary antibody for use with the Anti-ACTIVE® pAbs. It is qualified for use in Western blot analysis using chemiluminescent and colorimetric detection methods. This antibody conjugate exhibits minimal cross-reactivity to goat, mouse and sheep IgG, bovine serum albumin (BSA) and proteins in mammalian cell extracts. This antibody conjugate provides low background and highly specific signals when used at the recommended 1:5,000 dilution of conjugate with Anti-ACTIVE® MAPK pAb, and 1:10,000 dilution of conjugate with Anti-ACTIVE® JNK and Anti-ACTIVE® p38 pAbs.

Feature

- **Optimized:** Provides specific detection and low background when used in Western blot analysis with Promega's Anti-ACTIVE® rabbit polyclonal antibodies.

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Control PC12 Cell Extracts

PC12 Cell Extracts, Western Controls-NGF/Untreated

Product	Size	Catalog #
PC12 Cell Extracts, Western Controls-NGF/Untreated	10 blots	V8110

Description: Control cell extracts for use with Anti-ACTIVE® MAPK, Anti-pT¹⁸³ MAPK and Anti-ERK 1/2 polyclonal antibodies in Western blot analysis. PC12 Cell Extracts, Western Controls contain both treated and untreated cell extracts for use as positive and negative controls in Western blot analysis. The NGF (nerve growth factor)-treated cell extract is recommended as a positive control for the detection of activated ERK1 and ERK2. The untreated extract is recommended for use as a negative control for the detection of the activated ERK1 and ERK2 enzymes but can serve as a positive control when detecting total ERK1 and ERK2 enzymes using the Anti-ERK 1/2 pAb ("pan"). Each kit contains sufficient extract to run 10 lanes each of the positive and negative controls.

PC12 Cell Extracts, Western Controls-Sorbitol/Untreated

Product	Size	Catalog #
PC12 Cell Extracts, Western Controls-Sorbitol/Untreated	10 blots	V8100

Description: Control cell extracts for use with Anti-ACTIVE® JNK and p38 polyclonal antibodies in Western blot analysis. PC12 Cell Extracts, Western Controls contain both treated and untreated cell extracts for use as positive and negative controls in Western blot analysis. The sorbitol-treated cell extract is recommended as a positive control for the detection of activated JNK1 and JNK2 as well as p38 kinase. The untreated extract is recommended for use as a negative control for these enzymes. Each kit contains sufficient extract to run 10 lanes each of the positive and negative controls.

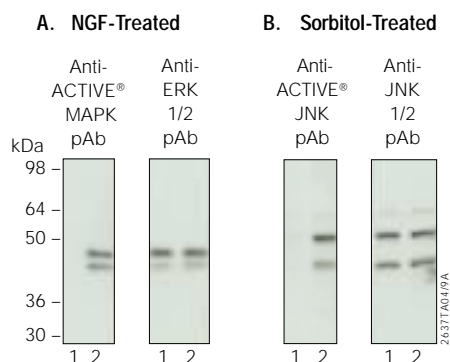


Figure 1.5. Western blots of PC12 Cell Extracts probed with Anti-ACTIVE® and anti-pan antibodies. Panel A: Lanes contained 2µg of Untreated (lanes 1) or NGF-Treated PC12 Cell Extract (lanes 2). Blots were probed with Anti-ACTIVE® MAPK pAb (Cat.# V8031) or Anti-ERK 1/2 pAb (Cat.# V1141) as indicated. **Panel B:** Lanes contained 10µg of Untreated (lanes 1) or Sorbitol-Treated PC12 Cell Extract (lanes 2). Blots were probed with Anti-ACTIVE® JNK pAb (Cat.# V7931) or anti-pan JNK 1 pAb (Santa Cruz Biotech), as indicated. The secondary antibody was Donkey Anti-Rabbit IgG (H+L), AP, Anti-ACTIVE® Qualified (Cat.# V7971). Chemiluminescent detection was performed using a Western-Star™ kit (Tropix). Proteins were transferred to nitrocellulose from 10% SDS-polyacrylamide minigels.

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Publications

Jarvis, B.W. and O'Brien, M. (1999) PC12 Cell Extracts for use with the new Anti-pT¹⁸³ MAPK and Anti-ACTIVE® Antibodies. *Promega Notes* **72**, 10.

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Promega Product Citations

MEK Inhibitor U0126

Tolwinski, N.S. *et al.* (1999) Nuclear localization of mitogen-activated protein kinase kinase 1 (MKK1) is promoted by serum stimulation and G2-M progression. *J. Biol. Chem.* **274**, 6168.

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Promega Resources

Protocol

MEK Inhibitor Promega Product Information Sheet**9PIV112**

Publications

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Ahn, N.G. *et al.* (1999) U0126: An inhibitor of MKK/ERK signal transduction in mammalian cells. *Promega Notes* **71**, 4.

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Inhibitors

MEK Inhibitor U0126

Product	Size	Catalog #
MEK Inhibitor U0126	5mg	V1121

Description: MEK Inhibitor U0126 is a potent, cell-permeable inhibitor of MAPK (ERK 1/2) activation by inhibiting the kinase activity of MAP Kinase Kinase (MAPKK or MEK 1/2). U0126 inhibits MEK1 with a IC₅₀ of 0.5µM in vitro (1). It has been used in both in vivo and in vitro studies of MEK (1-4).

References

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PD 98059

Product	Size	Catalog #
PD 98059	5mg	V1191

Description: PD 98059 (2'-amino-3'-methoxyflavone) is a potent, cell permeable and selective inhibitor of MAPK/ERK kinase 1 (MAP kinase kinase 1 or MEK1). It blocks the activation of MEK1, therefore inhibiting the subsequent phosphorylation and activation of MAP kinase. IC₅₀ values are in the 1-20µM range (1-4).

References

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4. Waters, S.B. *et al.* (1995) *J. Biol. Chem.* **270**, 20883.

SB 203580

Product	Size	Catalog #
SB 203580	1mg	V1161

Description: SB 203580 is a specific, cell-permeable inhibitor of the stress- and inflammatory cytokine-activated MAP kinase homologues p38α, p38β and p38β2. SB 203580 acts as a competitive inhibitor of ATP binding. Reported IC₅₀ values for p38 activity range from 21nM to 1µM. SB 203580 has no significant effect on the activities of ERKs, JNKs, p38γ or p38δ (1-4).

References

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LY 294002

Product	Size	Catalog #
LY 294002	5mg	V1201

Description: LY 294002 [2-(4-Morpholinyl)-8-phenyl-4 H-1-benzopyran-4-one] is a potent and specific cell-permeable inhibitor of phosphatidylinositol 3-kinases (PI 3-kinases) with IC₅₀ values in the 1-50µM range. LY 294002 competitively inhibits ATP binding to the catalytic subunit of PI 3-kinases and does not inhibit PI 4-kinase, DAG-kinase, PKC, PKA, MAPK, S6 kinase, EGFR or c-src tyrosine kinases and rabbit kidney ATPase (1,2).

References

1. Rameh, L.E. and Cantley, L.C. (1999) *J. Biol. Chem.* **274**, 8347.
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