

Immunostaining with the New Anti-ACTIVE® p38 Antibody



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p38 is one of the stress-induced kinases in the MAP Kinase superfamily. Active p38 kinase has been found in neuronal abnormalities such as Alzheimer's disease. The Anti-ACTIVE® p38 pAb is a useful tool for detecting active, dually phosphorylated p38 in both Western blotting and fluorescent immunostaining applications.

INTRODUCTION

Mitogen-Activated Protein (MAP) Kinases play an important role in signal transduction in eukaryotic cells where they modulate many cellular events (1,2). The MAP Kinase superfamily includes the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 kinases that are found in three interwoven signal transduction cascades. The p38 pathway also dovetails with apoptotic signaling in the form of Fas ligand expression (3). These kinases phosphorylate and, thus, activate transcription factors in response to mitogens, growth factors, or various forms of stress. ERK, JNK and p38, when activated by MAP Kinase kinases, known as MEKs, undergo phosphorylation on the threonine and tyrosine residues in the sequence pTXpY. Thus, phosphorylation on both threonine and tyrosine is necessary for activation of ERK, JNK and p38. When phosphorylated, the ERKs form homodimers; however, this is not true of JNK or p38 kinase (4).

The p38 pathway is activated by ultraviolet light, cytokines, osmotic shock agents (e.g., sorbitol) and inhibitors of protein synthesis (e.g., anisomycin). This spectrum of regulators suggests that the pathway is a transducer of many stress responses. These signals are transmitted from cell membrane-bound receptors through a variety of small GTP-binding proteins to the level of the MEK Kinases (MEKK1/2/3). A MEKK then activates MEK3/6, which in turn, activates the p38 kinase, which phosphorylates transcription factors (e.g., ATF-2). In fact, there are four isoforms of p38: α , β , γ and δ . The α and γ isoforms are activated in PC12 cells by the stress of hypoxia (5).

The Anti-ACTIVE® p38 pAb, which is made against a synthetic peptide encompassing residues pTGpY of p38 (6), is a reformulation of a previous p38 antibody in the Anti-ACTIVE® pAb series. Anti-ACTIVE® p38 pAb is made by injecting rabbits with a dually phosphorylated peptide containing the pTGpY motif of human p38 (5) and has been affinity-purified by a two-step procedure to deplete the product of antibodies reacting with the nonphosphorylated form of the peptide. Thus, the final antibody preparation is specific to the pTGpY sequence of the active kinase and will detect the α , γ and δ isoforms. This new antibody performs well for both Western blotting and fluorescent immunostaining applications. In addition, each lot of Anti-ACTIVE® p38 pAb is qualified for robust performance through testing at Promega in immunocytochemical and Western blotting assays with PC12 cells and extracts, respectively.

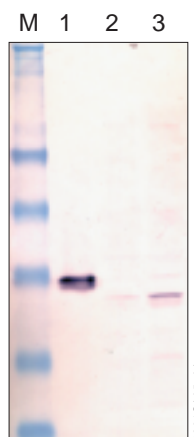
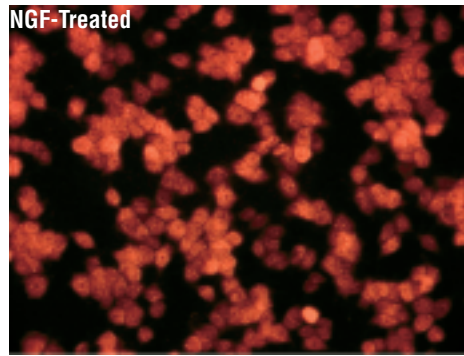
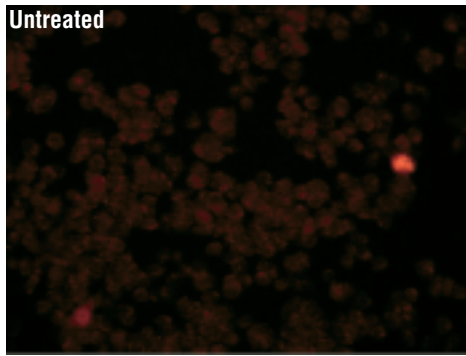


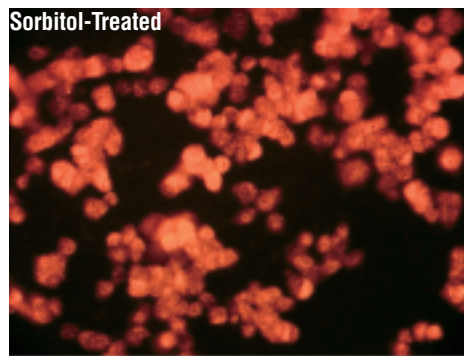
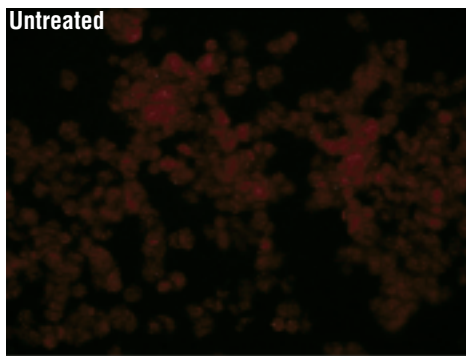
Figure 1. Western blotting with Anti-ACTIVE® p38 pAb (Cat.# V1211). Proteins were electrotransferred from a 10% polyacrylamide SDS gel onto a nitrocellulose membrane. Lane 1, recombinant, active p38 kinase (5ng); lane 2, Untreated PC12 Cell Extract (20 μ g); lane 3, Sorbitol-Treated PC12 Cell Extract (Cat.# V8100) (20 μ g). Lane M, molecular weight marker standards. The membrane was probed with the new Anti-ACTIVE® p38 pAb at a 1:2,000 dilution. The secondary antibody, Donkey Anti-Rabbit IgG (H+L), AP (Cat.# V7971), was used at a 1:10,000 dilution, and colorimetric development was performed using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841). The recombinant enzyme has an expression tag. Protocols developed and performed at Promega.

How is p38 involved with signaling in neuronal cells and tissues? A few examples are given here from Alzheimer's disease (AD) research and inflammation research. The p38 kinase is activated in the human brain during Alzheimer's disease, specifically in "neuritic plaques, neuropil threads, and neurofibrillary tangle-bearing neurons" (7). Furthermore, fibrillar forms of β -amyloid (a hallmark of AD) have been shown to induce transient activation of p38 kinase in the cultured microglia of neonatal rats (8). Another factor in neurological aging is oxidative stress. In the CA1 region of the hippocampus of Syrian hamsters, it was found that transient ischemia induced p38 activation in microglia (9). Similarly, electroconvulsive shock activated p38 in the hippocampus and cerebellum of rats (10). However, p38 does not seem to be involved in long-term spatial memory in rats, because the p38 inhibitor SB203580, when infused into the CA1 region of the hippocampus, did not disrupt performance in the Morris water maze (11). It is worth noting that many of these studies used antibodies directed against active, phosphorylated forms of p38 kinase.

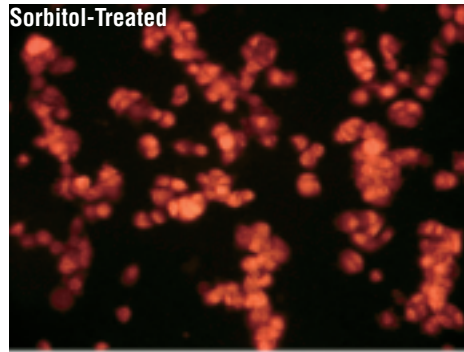
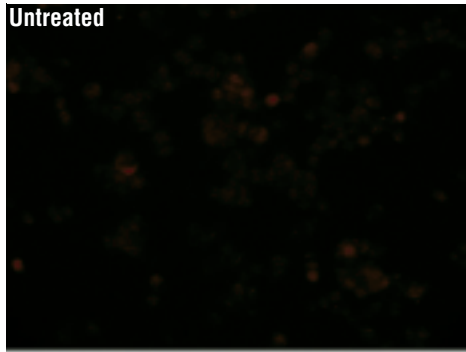
A. Anti-ACTIVE® MAPK pAb



B. Anti-ACTIVE® JNK pAb



C. Anti-ACTIVE® p38 pAb



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Figure 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 CyTM3 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. Protocol is described in Table 1.

PERFORMANCE IN WESTERN BLOTTING

Figure 1 shows Western blotting using the Anti-ACTIVE® p38 pAb. This antibody, used at a 1:2,000 dilution, detects 5ng of recombinant, active p38 kinase (lane 1) and active p38 in 20µg of Sorbitol-Treated PC12 Cell Extract (Cat.# V8100) (lane 3). In contrast, there is little p38 detected in 20µg of Untreated PC12 Cell Extract (lane 2). The addition of sorbitol to the PC12 cells acted as an osmotic shock agent, triggering phosphorylation of p38 kinase (as well as JNK; data not shown).

PERFORMANCE IN FLUORESCENT IMMUNOSTAINING

Figure 2 shows immunostaining using our Anti-ACTIVE® antibodies. Panel C shows immunostaining by Anti-ACTIVE® p38 pAb used at a 1:500 dilution. The left panel was produced using untreated PC12 cells and reveals only trace fluorescent staining. The right panel was generated using sorbitol-treated (1M for 30 minutes) PC12 cells and shows abundant immunofluorescence. A protocol for immunostaining is in Table 1.

**Table 1. PC12 Cell Growth and Immunocytochemistry Procedures.**

1. Precoat 4-chambered slides (Fisher 12-565-21) with rat tail collagen, 6µg/cm², for 1 hour.
2. Grow PC12 cells for 1 or 2 days in chambered slides.
3. Leave two chambers untreated. In the remaining two chambers, activate the cells as follows.
4. For sorbitol treatment, to activate p38 kinase on the day before immunocytochemistry (ICC), add fresh medium without serum. On the day of ICC, add sorbitol to the medium (to a final concentration of 1M) for 30 minutes at 37°C.
5. Wash once with cold PBS.
6. Fix cells with 10% paraformaldehyde for 30 minutes at room temperature.
7. Wash 3X, 5 minutes each time, with PBS.
8. Permeabilize the cells with cold methanol at -20°C for 10 minutes.
9. Wash 3X, 5 minutes each time, with PBS.
10. Block with blocking buffer (1% BSA, 5% donkey serum in PBS) for 3 hours at room temperature.
11. Wash once for 5 minutes with PBS.
12. Incubate with Anti-ACTIVE® p38 pAb in antibody dilution buffer (1% BSA, 1% donkey serum in PBS) overnight at 4°C. Recommended dilution for Anti-ACTIVE® p38 pAb is 1:500.
13. Wash 5X, 15 minutes each time, in PBS.
14. Incubate with donkey anti-rabbit CyTM3 conjugate (Jackson ImmunoResearch) at a 1:1,000 final dilution for 90 minutes at room temperature in the dark.
15. Wash 5X, 15 minutes each time, with PBS in the dark. Remove grid and mount the slides with Vectashield® (Vector Labs) containing DAPI.
16. View by fluorescence microscopy.

PC12 cells (ATCC) are grown in tissue culture flasks that were pre-coated with rat tail collagen, 6µg/cm², for 1 hour at 37°C. During growth at 37°C in 5% CO₂, the medium needs to be changed every other day until the cells reach ~80% confluence. The medium consists of RPMI 1640 with 25mM HEPES and 300mg/L of L-glutamine, 10% horse serum, 5% fetal bovine serum and 0.5mM EGTA.

Anti-ACTIVE® p38 pAb is qualified for use in immunocytochemistry applications. Thus, each lot is tested to ensure that it performs as demonstrated in Figure 2 for stimulated and unstimulated PC12 cells. In addition, the Anti-ACTIVE® MAPK pAb (Cat.# V8031) and Anti-ACTIVE® JNK pAb (Cat.# V7931, V7932) are also qualified for immunostaining.

REFERENCES

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Ordering Information

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

Related Products

Product	Size	Cat.#
Anti-ACTIVE® MAPK pAb, Rabbit, (pTEpY)	40µl	V8031
Anti-ACTIVE® JNK pAb, Rabbit, (pTPpY)	40µl	V7931
	120µl	V7932
SB 203580	1mg	V1161
MEK Inhibitor U0126	5mg	V1121
PD 98059	5mg	V1191
Donkey Anti-Rabbit IgG, (H+L) AP	60µl	V7971
Donkey Anti-Rabbit IgG, (H+L) HRP	60µl	V7951
Western Blue® Stabilized Substrate for Alkaline Phosphatase	100ml	S3841
PC12 Cell Extracts, Western Controls		
Sorbitol/Untreated	10 blots	V8100
NGF/Untreated	10 blots	V8110
Anti-pT ¹⁸³ MAPK pAb, Rabbit	50µl	V8081
Anti-ERK 1/2 pAb, Rabbit	40µl	V1141
Blot-Qualified BSA	10g	W3841

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