IMMUNOHISTOCHEMICAL STAINING USING PROMEGA ANTI-ACTIVE® AND APOPTOSIS ANTIBODIES

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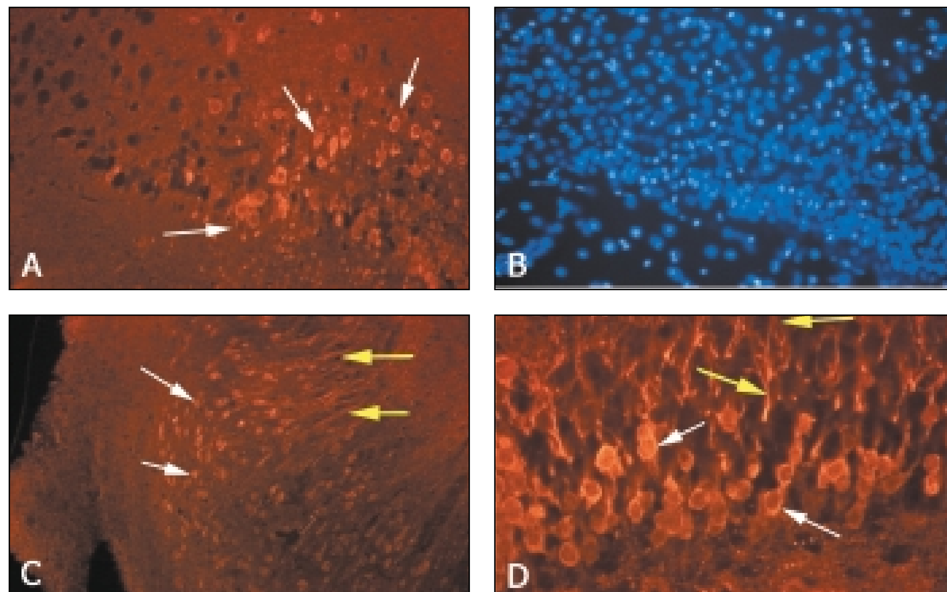
This article describes protocols for tissue staining with Promega polyclonal antibodies in mouse and rat systems. Protocols are provided for Anti-ACTIVE® MAPK, Anti-ACTIVE® JNK, Anti-ACTIVE® p38, and Anti-ACTIVE® CaM KII pAbs. In addition Promega protocols using polyclonal antibodies useful for the study of apoptosis (Anti-PARP p85 Fragment pAb, Anti-ACTIVE® Caspase-3 pAb, and Anti-pS^{473} Akt pAb) are described. Immunohistochemical data are shown for each of the above antibodies.

Introduction

Protein expression, as monitored by in situ cellular and subcellular localization, has become exceedingly important in the post genome sequencing era. Immunohistochemistry, coupled with traditional molecular and biochemical techniques, continues to be a powerful tool for studying gene expression. By utilizing robust antibodies capable of detecting antigens in fixed

Method for staining adult rat brain frozen hippocampal sections (see Figures 1–4).

1. Remove brain and place in 4% paraformaldehyde overnight at 4°C.
2. Wash 3 times for 10 minutes in PBS.
3. Cryoprotect at 4°C overnight in 25% sucrose plus 5% glycerin in PBS.
4. Freeze tissue and cut 30 micron sections on a freezing/sliding microtome.
5. Wash sections 3 times for 10 minutes in PBS.
6. Block 90 minutes in 5% donkey serum + 0.01% Triton® X-100 in PBS.
7. Incubate overnight at 4°C in primary antibody diluted in 1% donkey serum + 0.01% Triton® X-100 in PBS. (Recommended dilutions: Anti-ACTIVE® MAPK, 1:500; Anti-ACTIVE® CaM KII, 1:500; Anti-ACTIVE® JNK, 1:500; Anti-ACTIVE® p38, 1:500; Anti-ERK 1/2, 1:500; Anti-pS^{473} Akt, 1:50; Anti-GFAP diluted to 2.0µg/ml).
8. Wash 3 times for 10 minutes in PBS.
9. Incubate in donkey anti-rabbit Cy™3-conjugated pAb (Jackson ImmunoResearch) diluted 1:1,000 in PBS for 60 minutes at 22–25°C.
10. Wash 3 times for 10 minutes in PBS.
11. Mount in Vectashield® + DAPI anti-fade reagent (Vector Laboratories).



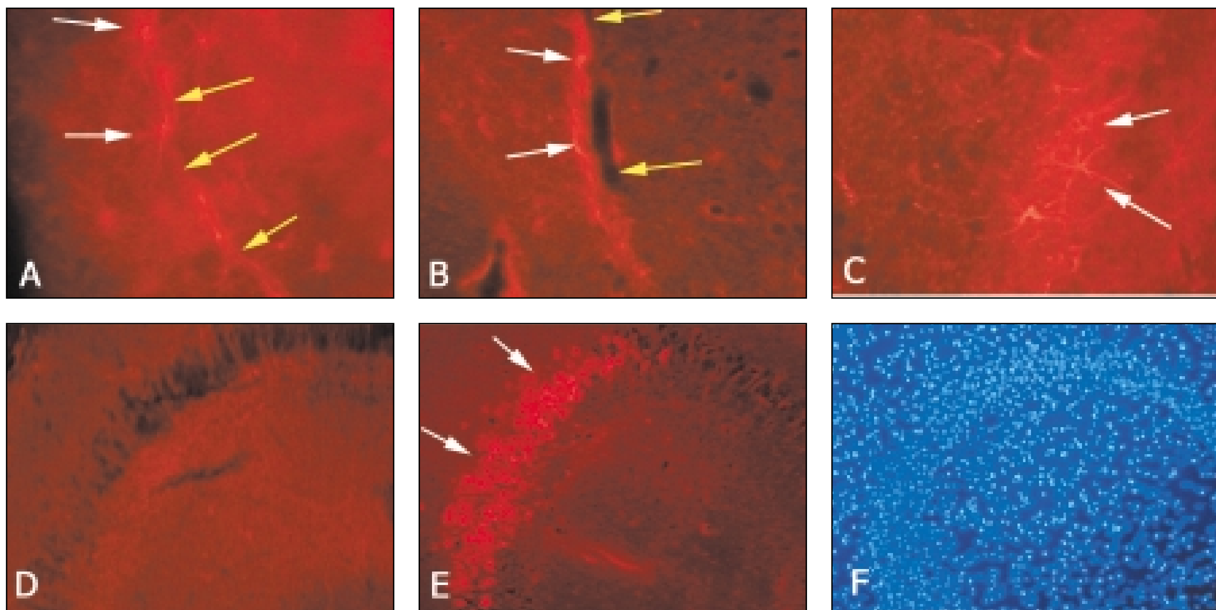
▲ Figure 1. Adult rat brain frozen hippocampal sections stained with Anti-ACTIVE® CaM KII pAb (Cat.# V1111) and visualized with anti-rabbit Cy™3 conjugate. All four images are taken from the same section. Panel A. Labeling in this region is specific for a subset of pyramidal neurons (arrows). Panel B. DAPI nuclear stain in the same field as A, further demonstrating the Anti-ACTIVE® CaM KII localization in a cellular subset. Panel C. Low power image of a different area showing neuronal cell bodies (white arrows) and streaming dendrites (yellow arrows) labeled with Anti-ACTIVE® CaM KII. Panel D. High power image of dentate granule cells showing intense labeling of neuronal cell bodies (white arrows) and dendrites (yellow arrows).

tissue sections, scientists can delineate gene expression patterns under various experimental conditions.

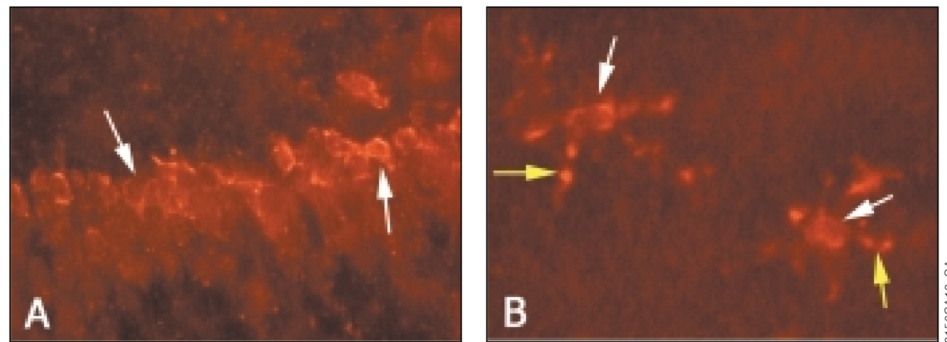
Promega has developed many polyclonal antibodies useful for the study of eukaryotic signal transduction. Anti-ACTIVE® MAPK, Anti-ACTIVE® JNK, Anti-ACTIVE® p38 and Anti-ACTIVE® CaM KII Antibodies are affinity-purified rabbit polyclonal antibodies that are specific for the active, phosphorylated forms of these kinases. All four antibodies have been shown to be specific to the phosphorylated kinases in both Western analysis and immunocytochemistry. These Anti-ACTIVE® Antibodies detect phosphokinases in activated PC12 cells but not in unstimulated control cells (1). Specificity in ICC was demonstrated by blocking the antibody binding after preincubation with the phosphopeptide immunogen and by absence of such blocking after preincubation with the nonphosphopeptide (2).

Promega has also developed polyclonal antibodies useful for the study of apoptosis. Anti-PARP p85 Fragment pAb[(a)], Anti-ACTIVE® Caspase-3 pAb, and Anti-pS^{473} Akt pAb are affinity-purified rabbit polyclonal antibodies specific for caspase-cleaved PARP, the active form of caspase-3, and phosphorylated Akt, respectively. PARP, poly (ADP-ribose) polymerase, is a known substrate for caspase-3 and caspase-7 (3–5). Western blots and immunocytochemistry have demonstrated that the Anti-PARP p85 Fragment pAb recognizes only the caspase-cleaved form of PARP (p85 fragment) (6,7). Caspase-3, like most caspases, is activated upon cleavage of the proform and tetramerization of its subunits. The Anti-ACTIVE® Caspase-3 pAb only recognizes the activated form of the enzyme (8). These two antibodies are specific and sensitive markers for apoptotic cells. In contrast, the Anti-pS^{473} Akt is a marker for cell survival. Anti-pS^{473} Akt pAb is specific for the phosphorylated form of Akt (9); this activated Akt then phosphorylates a variety of targets that ultimately promotes cell survival.

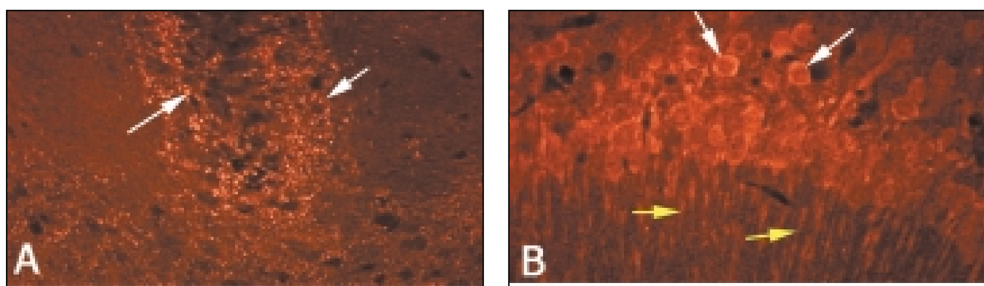
The purpose of this article is to demonstrate the utility of these antibodies in fixed tissue sections from two different rodent species.



▲ Figure 2. Adult rat brain frozen hippocampal sections stained with Anti-ACTIVE® MAPK pAb (Cat.# V8031) or Anti-ERK 1/2 pAb (Cat.# V1141) and visualized with an anti-rabbit Cy™3 conjugate. Panel A. Anti-ACTIVE® MAPK pAb-labeled section. Labeling appears to be specific to astrocytes in this area of the hippocampus. Note the typical astrocyte morphology of the Anti-ACTIVE® MAPK positive cells (white arrows) surrounding the blood vessel (yellow arrows). Anti-ACTIVE® MAPK also labeled neurons in the hippocampal molecular layer (data not shown). Panel B. Astrocytic cells are labeled with Anti-ERK 1/2 in an adjacent section. Anti-ERK 1/2 labels both phosphorylated and nonphosphorylated forms of MAPK. These astrocytes (white arrows) are also seen in close association with a blood vessel (yellow arrows). Panel C. Anti-GFAP (Glial Fibrillary Acidic Protein, an astrocyte-specific marker Cat.# G5601) labeling (white arrows) in an adjacent section showing typical astrocyte morphology. Only a subset of astrocytes is labeled with Anti-ACTIVE® MAPK and Anti-ERK 1/2 (data not shown). Panel D. Low power image of CA3 region stained with Anti-ACTIVE® MAPK. Note the absence of neuronal staining. Panel E. CA3 region labeled with Anti-ERK 1/2 showing a subset of pyramidal cells (arrows) not labeled with Anti-ACTIVE® MAPK. These neurons are expressing MAPK, but there is no detectable phosphorylation at this stage. Panel F. DAPI image of E showing all nuclei.



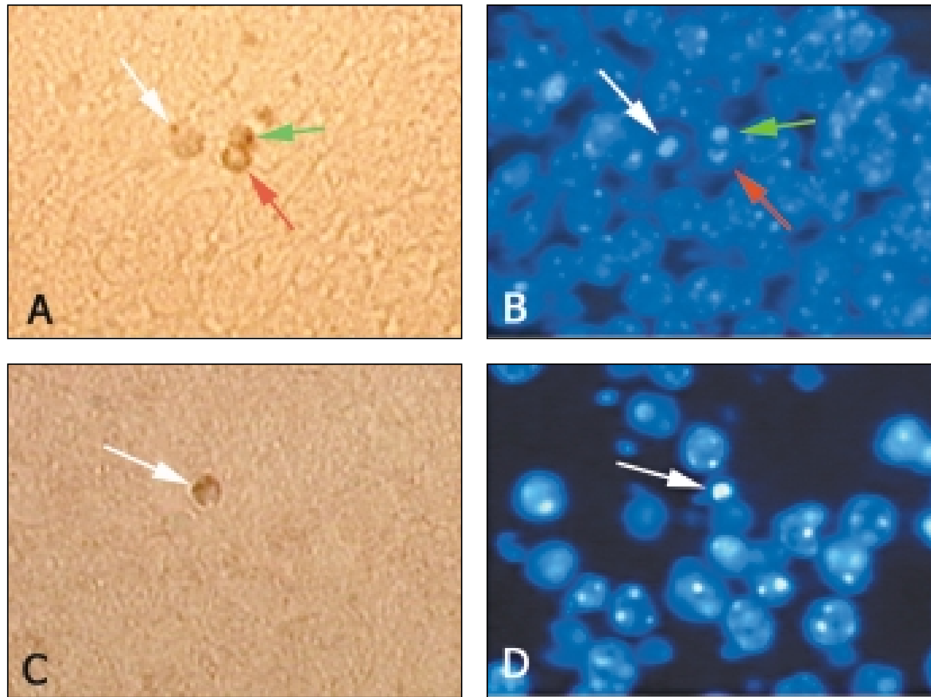
▲ Figure 3. Adult rat brain frozen hippocampal sections stained with Anti-ACTIVE® p38 pAb (Cat.# V1211) and Anti-ACTIVE® JNK pAb (Cat.# V7931) and visualized with an anti-rabbit Cy™3 conjugate. Panel A. Anti-ACTIVE® p38 pAb stained section demonstrating specific labeling in the polymorphic region lining the pyramidal neuronal layer (arrows). Staining was limited to these small cells throughout the hippocampus. Panel B. Anti-ACTIVE® JNK pAb labeling in the same region demonstrates a unique labeling pattern in unidentified multiprocess cells resembling glial cells. Note the ring of staining surrounding the cell body (white arrows) and staining present in the processes (yellow arrows).



▲ Figure 4. Adult rat brain frozen hippocampal sections stained with Anti-pS473 Akt pAb (Cat.# G7441) and visualized with an anit-rabbit Cy™3 conjugate. Panel A. Low power image from the CA4 region showing staining specific for dendritic termini (arrows). Panel B. High power image showing dentate granule cell staining in both the cell body (white arrows) and streaming dendrites (yellow arrows).

Method for staining postnatal day 0 mouse brain, paraffin-embedded sections. (See Figure 5. All steps are performed at room temperature.)

1. Embed tissue in paraffin after fixation in 4% paraformaldehyde. Six micron sections are used for this protocol.
   Note: Best results will be obtained if the animal is perfused with fix and postfixed after dissection.
2. Deparaffinize by washing tissue 3 times for 5 minutes each in Hemo De™ (Fisher Scientific) or xylene. Rinse tissue sections for 2 minutes in 100% ethanol. Transfer sections to 95% ethanol for 2 minutes, then transfer them to 70% ethanol for 2 minutes. Finally, rinse tissue sections 2 times for 2 minutes each in PBS.
3. Permeabilize for 10 minutes in PBS + 0.1% Triton® X-100.
4. Wash sections 2 times for 5 minutes each in PBS.
5. Block endogenous peroxide activity by incubating sections in 0.3% H_2O_2 in PBS for 30 minutes.
6. Wash sections 2 times for 5 minutes each in PBS.
7. Block for 45 minutes in PBS + 5% donkey serum.
8. Incubate with Anti-ACTIVE® Caspase-3 pAb diluted 1:125 or Anti-PARP p85 Fragment pAb diluted 1:50 in PBS + 1.0 % donkey serum for 60 minutes.
9. Wash sections 3 times for 5 minutes each in PBS.
10. Incubate with biotin conjugated donkey anti-rabbit pAb (Jackson ImmunoResearch) diluted 1:500 in PBS for 60 minutes.
11. Wash sections 3 times for 5 minutes each in PBS.
12. Incubate in RTU (Ready To Use) ABC reagent (Vector Laboratories) for 60 minutes.
13. Wash sections 3 times for 5 minutes each in PBS.
14. Develop with DAB substrate kit (Vector Laboratories) for 10 minutes.
15. Wash 3 times for 5 minutes each in water.
16. Mount in Vectashield® + DAPI anti-fade reagent (Vector Laboratories).



▲ Figure 5. Demonstration of Anti-ACTIVE® Caspase-3 and Anti-PARP p85 Fragment pAb positive cells in postnatal day 0 (P0) mouse brain paraffin-embedded sections. Panel A. Three Anti-ACTIVE® Caspase-3 pAb positive cells (colored arrows) and, Panel B, their corresponding DAPI- stained nuclei. Note the correspondence of Anti-ACTIVE® Caspase-3 pAb label with the typical apoptotic condensed nuclear morphology in Panel B. Panel C. The Anti-PARP p85 Fragment pAb positive cell (white arrow) corresponds to an apoptotic DAPI-stained nucleus in Panel D. (white arrow).

References

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Acknowledgments

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Ordering Information

Product	Size	Cat.#
Anti-ACTIVE® MAPK pAb, Rabbit, (pTEpY)	40µl	V8031
Anti-ACTIVE® JNK pAb, Rabbit, (pTPpY)	40µl	V7931
	120µl	V7932
Anti-ACTIVE® p38 pAb, Rabbit, (pTGpY)	100µl	V1211
Anti-ACTIVE® CaM KII pAb, Rabbit, (pT286)	40µl	V1111
Anti-PARP p85 Fragment pAb[(a)]	50µl	G7341
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Anti-pS473 Akt pAb	40µl	G7441
Anti-GFAP pAb	100µg	G5601
Anti-ERK 1/2 pAb, Rabbit	40µl	V1141

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(a) Patent Pending.