Anti-ACTIVE® Caspase-3 pAb for the Detection of Apoptosis

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Promega has recently introduced the Anti-ACTIVE® Caspase-3 pAb, which is intended for use as an in situ marker for the detection of apoptosis. The antibody detects the active form of caspase-3 that occurs in apoptotic cells and thus provides a simple method for detection of apoptosis in situ. Here we describe this new Anti-ACTIVE® antibody and discuss the advantages of this method in the context of other technologies available for verifying apoptosis.

Introduction

Apoptosis is a complex process that involves a variety of different signaling pathways and results in a multitude of changes in the dying cell. Many of the events that occur during apoptosis are mediated by a family of cysteine proteases called caspases (1). Caspases exert their action at several levels of signaling during apoptosis, ranging from responding to external factors at the transmembrane receptor to proteolytic breakdown of cellular components. Their proteolytic activity results ultimately in the demise of the cell and has led to caspases being called the central executioners of apoptosis. Among the group of 11 caspases identified to date in humans, caspase-3 has been recognized as a central player in mediating apoptosis and is the most widely studied.

Caspases are synthesized and exist mostly in the cytoplasm of viable cells as an inactive pro-enzyme. Activation of caspase zymogens is an early event in the process of apoptosis. Caspase activation precedes phosphatidyl serine exposure on the external leaflet of the lipid bilayer as indicated by annexin V binding (2,3), which has historically been promoted as an early marker of apoptosis. In response to early upstream apoptotic signaling events, pro-caspases are processed by enzymatic cleavage to generate active enzymes.

The p32 pro-enzyme of caspase-3 first undergoes cleavage at the C-terminal side of Asp^{28} to remove the amino terminal pro-domain. Additional cleavage at the C-terminal side of Asp^{175} results in the generation of the p17 and p12 fragments (4). The X-ray crystal structure of caspase-3 shows the formation of a tetramer composed of two small and two large subunits (5). The tetramer has been suggested to be the catalytically active form. A diagram of the pro-enzyme showing processing sites is shown in Figure 1.

The results of enzymatic activation of pro-caspases include conformational changes and the generation of neo-epitopes. Both of these changes can be selectively detected by specific antibodies. We have taken advantage of the unveiling of neo-epitopes during caspase processing to generate antibodies that serve as specific markers of apoptosis.

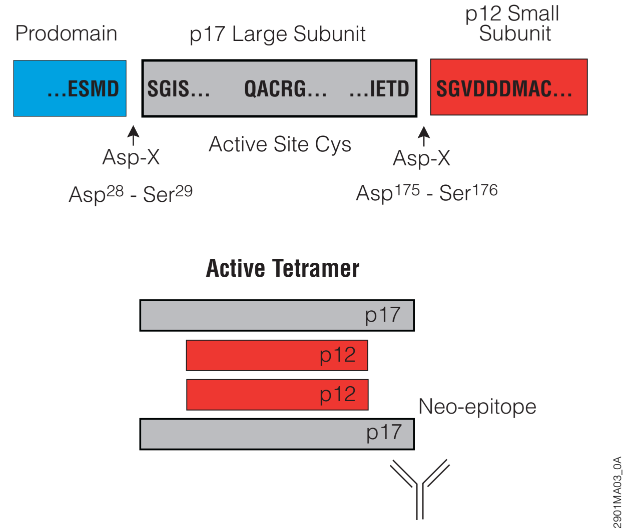

Figure 1. Pro-caspase-3 domains and cleavage sites. All caspases contain a conserved pentapeptide QACXG at their active site.

Anti-ACTIVE® Caspase-3

Anti-ACTIVE® Caspase-3 pAb (Cat.# G7481) is an affinity purified rabbit polyclonal antibody directed against a peptide from the p17 fragment of human caspase-3. Anti-ACTIVE® Caspase-3 pAb detects the active form of the enzyme that occurs in apoptotic cells, thus making the antibody useful as an in situ marker for apoptosis. The antibody was generated using an antigenic peptide that has an identical amino acid sequence among the human, mouse, rat and hamster proteins, thus predicting broad species reactivity. Western blot data indicate that the Anti-ACTIVE® Caspase-3 pAb may also detect the large subunit of recombinant active caspase-7. Although reactivity is much lower than with active caspase-3, limited cross reactivity is not surprising as caspase-3 and caspase-7 are in the same caspase sub-family and have similarities in their amino acid sequences.

The flow diagram in Figure 2 illustrates an abbreviated staining protocol for using Anti-ACTIVE® Caspase-3 pAb. This general protocol can be used with either fluorescent or light microscopy. A detailed protocol is available from the Promega web site at www.promega.com. Because of sample variation, as with any antibody preparation, a titration of the primary antibody concentration is recommended for optimal performance.

E-mail *techserv@promega.com* for technical questions

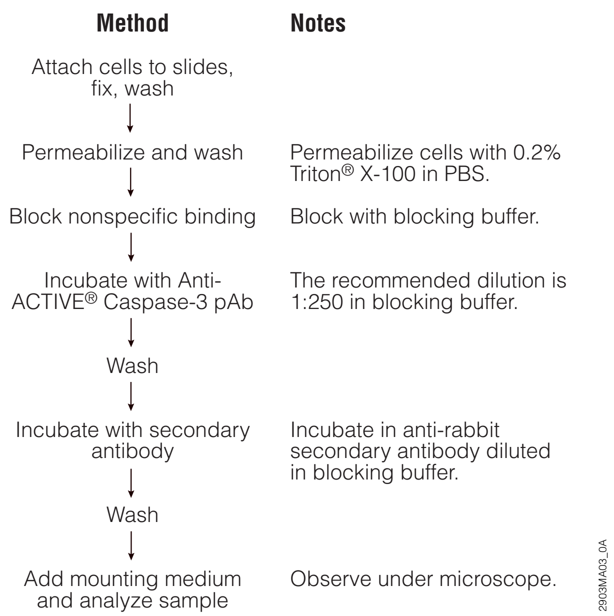

Figure 2. General immunocytochemical detection method using Anti-ACTIVE® Caspase-3 pAb.

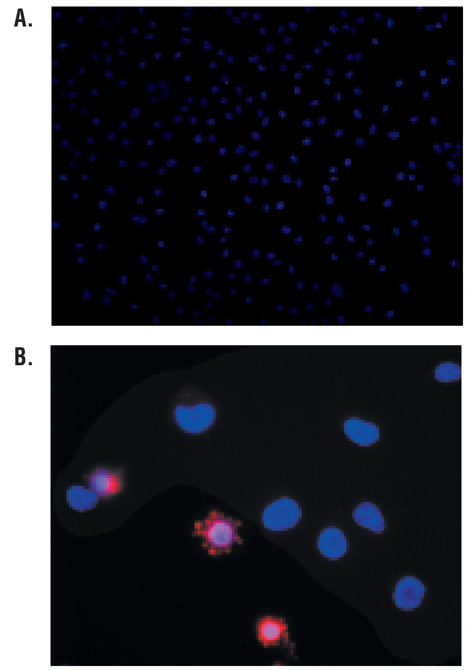

Figure 3. Anti-ACTIVE® Caspase-3 pAb labeling of apoptotic murine fibroblasts. Murine L929 cells were cultured on collagen coated slides and induced to undergo apoptosis by treating with TNFα in the presence of actinomycin D for 5.5 hours. Slides were then washed, formalin fixed and labeled with Anti-ACTIVE® Caspase-3 pAb and counterstained with DAPI. **Panel A:** Untreated control cells, photographed at 200X. **Panel B:** Apoptotic cells, photographed at 630X. Cells in Panel A show no antibody staining while cells showing apoptotic morphology in Panel B are stained with the antibody.

Figure 3 illustrates specific immunofluorescent staining of apoptotic murine fibroblasts using Anti-ACTIVE® Caspase-3 pAb. The sample treated to undergo apoptosis shows distinct staining of cells exhibiting apoptotic morphological characteristics (Panel B). The background staining is low in control cells (Panel A). Anti-ACTIVE® Caspase-3 staining of apoptotic human neuroblastoma cells is illustrated in Figure 4. Again, strong staining of apoptotic cells is apparent (Panel B), while untreated cells (Panel A) or cells treated with the caspase inhibitor Z-VAD-FMK, which also binds to active caspases, (Panel C) show minimal staining with the Anti-ACTIVE® Caspase-3 pAb.

Other apoptosis detection methods

A similar neo-epitope strategy has been used to develop antibodies that specifically recognize products resulting from caspase cleavage of substrates. Approximately 70 caspase substrates have been identified that could be potential candidates for biomarkers (6,7). Neo-epitopes generated by caspase proteolysis of substrates have been used as antigens for generating antibodies specific for immunodetection of the cleaved product without recognition of the intact substrate (8,9). Some caspase substrates such as poly-ADP ribose polymerase (PARP) are ubiquitous and good candidates for general markers of apoptosis whereas other substrates may be poor candidates (e.g., cytokeratin 18 where expression is somewhat limited to epithelial cells; 10–12). Promega previously has developed the Anti-PARP p85 Fragment pAb[(a)] (Cat.# G7341) and its usefulness as a marker for apoptosis has been demonstrated (13–15). Additional applications for the Anti-PARP antibody have been demonstrated using flow cytometry (16) and immunohistochemical staining of neurons in rabbit trigeminal ganglia (17).

Staining with Anti-PARP antibody has been shown to precede DNA fragmentation as measured using the TUNEL assay (13).

Monitoring downstream apoptotic events such as DNA fragmentation (using TUNEL), exposure of cell surface phosphatidyl serine (using annexin V labeling), and antibodies against cleavage products have been used to indirectly monitor caspase-3 mediated activity and apoptosis.

Detection of active caspase-3 in situ may be a more direct indicator of apoptosis than detection of a secondary process such as DNA fragmentation or cleavage of a caspase substrate.

Summary

A variety of methods currently are available for detecting apoptosis. The two most common methods reported in the literature are observation of morphology and detection of DNA fragmentation. Although these methods have proven useful, the results from both assays can have problems of interpretation and the possibility of artifacts. Apoptosis was originally defined based on morphological characteristics that appear upon cell death (18). Cell shrinkage and the appearance of condensed nuclei stained with nucleic acid dyes are commonly used as evidence of apoptosis. However, major drawbacks in relying on morphology as the only detection method include the potential loss of characteristic features during tissue processing and the requirement for a highly skilled observer to reproducibly score morphological criteria. The use of marker antibodies for the active form of caspase-3 or for the p85 fragment of PARP provide additional methods for verifying apoptosis that overcome the drawbacks associated with detection methods that rely solely on morphological analysis.

A.

B.

C.

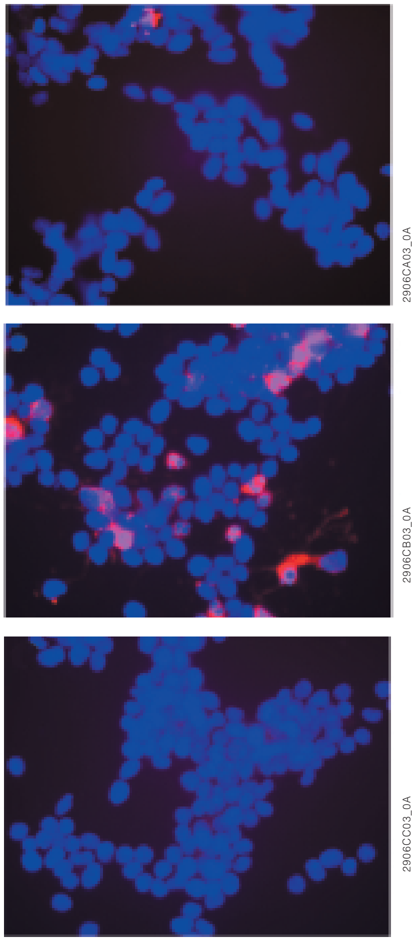

Figure 4. Anti-ACTIVE® Caspase-3 pAb labeling of apoptotic human neuroblastoma cells. Human SH-SY5Y cells were cultured on chamber slides and induced to undergo apoptosis by treatment with the protein kinase inhibitor, staurosporine for 5 hours. Slides were then washed, formalin fixed and labeled with Anti-ACTIVE® Caspase-3 pAb followed by an anti-rabbit Cy™3 conjugated secondary antibody and counterstained with DAPI. **Panel A:** untreated controls. **Panels B:** staurosporine treated apoptotic cells. **Panel C:** cells pre-treated with the caspase inhibitor, Z-VAD-FMK (50µM) before staurosporine treatment. Caspase activation is inhibited by Z-VAD-FMK.

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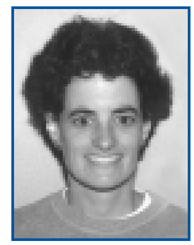

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Ordering Information

Product	Size	Cat.#
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Anti-PARP p85 Fragment pAb	50µl	G7341

Related Products

Product	Size	Cat.#
CaspACE™ Assay System, Colorimetric	100 reactions	G7220
	50 reactions	G7351
CaspACE™ Assay System, Fluorometric	160 assays	G3540

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(a)Patent Pending.