

## DeadEnd™ Colorimetric Apoptosis Detection System for the Analysis of Retinal Apoptosis

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### Introduction

Apoptosis is currently one of the most intensively researched areas of science, and numerous ways exist to measure the process. Among the morphological, biochemical and molecular changes occurring in the dying cells, DNA fragmentation is widely recognized as a hallmark of apoptosis, and its detection is the most common analysis for damage to chromatin and cleavage of DNA into oligonucleosomal-length fragments. The standard technique uses the TdT-mediated dUTP nick-end labeling (TUNEL) assay for the detection of DNA fragmentation in individual cultured cells or in tissue sections. Here we present data on the in situ detection of apoptotic cells in retinal sections using the DeadEnd™ Colorimetric Apoptosis Detection System, a modified TUNEL method, which provides an easy, rapid and

accurate analysis of apoptotic cells. In the present study, apoptosis was induced after a brief exposure of the isolated and intact retinas to NMDA, which is known to trigger excitotoxic-mediated delayed neuronal cell death in an apoptotic fashion in brain as well as in retina (1,2).

### In Situ DNA Nick-End Labeling

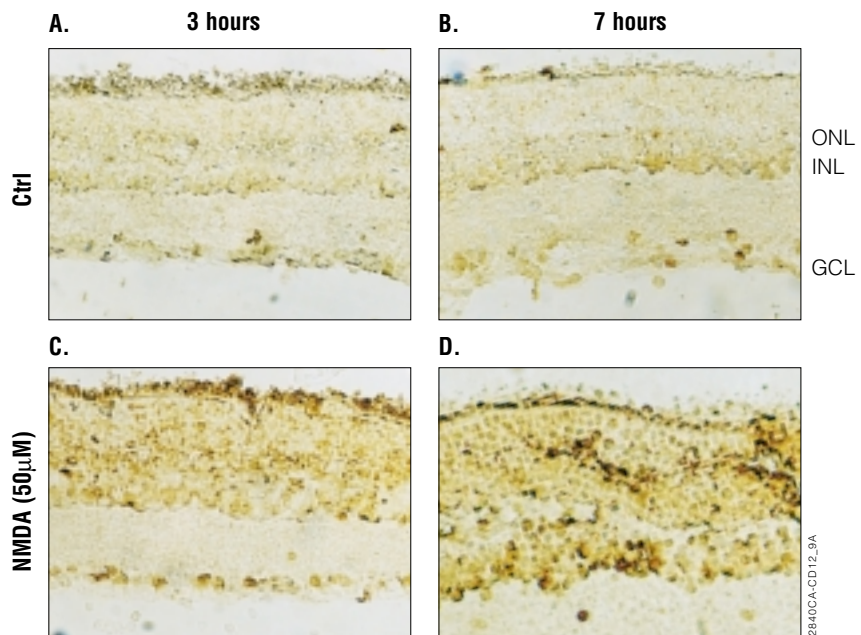
Isolated and intact retinas were exposed to a brief NMDA pulse as previously described (3), and DNA fragmentation was detected after 3 and 7 hours.

### Methods

Three or seven hours after the excitotoxin pulse, the retinas were fixed overnight with 4% paraformaldehyde solution in 0.1M PBS (pH 7.4) and embedded in paraffin. Five micrometer-thick sections were mounted on poly-L-lysine coated slides, subsequently deparaffinized by immersion in fresh xylene for 5 minutes at room temperature, then rehydrated in a graded alcohol series. The in situ detection of fragmented DNA using the DeadEnd™ Colorimetric Apoptosis Detection System was performed according to the manufacturer's instructions (4) with slight modifications. Briefly, sections were treated with 20µg/ml proteinase K and incubated for 10 minutes at room temperature, washed in PBS and refixed in 4% paraformaldehyde. DNA was end-labeled at the 3' end using biotinylated dUTP in TdT buffer at 37°C for 90

**Figure 1.** ▶

Retinal apoptosis detected by DeadEnd™ Colorimetric Apoptosis Detection System (Cat.# G7130), a modified TUNEL method. Representative photographs showing DNA fragmentation 3 and 7 hours after exposing retinas to a 30-minute pulse of 50µM NMDA (Panels C and D). Control retinas (Panels A and B): no labeled cells are found after 3 hours of incubation (Panel A), and only a few scattered cells are TUNEL-positive after 7 hours (Panel B). Exposure to NMDA (Panels C and D) triggers apoptosis: after 3 hours, DNA-fragmented cells are seen in INL and GCL (Panel C); at 7 hours, the number of TUNEL-positive cells is markedly increased in the same layers and few positive cells are also seen in ONL (Panel D). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Magnification: 200X.




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minutes. After washing, 0.3% hydrogen peroxidase was used to block endogenous peroxidase. Streptavidin HRP (1:500 in PBS) was added for 30 minutes at room temperature. DNA strand breaks were visualized by using the hydrogen peroxidase and the chromogen 3,3'-diaminobenzidine tetrahydrochloride. Positive controls were performed by incubating the slides with DNase I (1 unit/ml) for 10 minutes at room temperature; negative controls were performed by omitting TdT enzyme.

**Results**

Following the 50µM NMDA pulse, TUNEL-positive cells appeared dark brown (as a function of the length of incubation) as shown in Figure 1. TUNEL-positive cells were scattered throughout the inner nuclear layer (INL) and ganglion cell layer (GCL) at three hours (Panel C), and much more intense and numerous in the outer nuclear layer (ONL), INL and GCL at seven hours (Panel D). As TUNEL staining rarely labels intact nuclei (see control retinas, Panels A and B), the labeling we detected appeared to be specific for nuclear DNA strand breaks.

**Summary**

Evidence is presented on apoptotic cell death induced by NMDA receptors in retinal tissue as demonstrated by use of the DeadEnd™ Colorimetric Apoptosis Detection System. TUNEL-positive cells were identified in specific retinal layers predominantly affected by excitotoxic insults. 

**References**

1. Lipton, S.A. and Nicotera, P. (1998) *The Neuroscientist* **4**, 345.
2. Lam, T.T. et al. (1999) *Invest. Ophthalmol. Vis. Sci.* **40**, 2391.
3. Guarneri, P. et al. (1998) *Eur. J. Neurosci.* **10**, 1752.
4. *DeadEnd™ Colorimetric Apoptosis Detection System Technical Bulletin #TB199*, Promega Corporation.

**Ordering Information**

Product	Size	Cat.#
DeadEnd™ Colorimetric Apoptosis Detection System	40 reactions	G7130
	20 reactions	G7360

**Live/Dead Assay**


**In situ labeling of apoptotic neurons with CaspACE™ FITC-VAD-FMK Marker**

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Embryonic (day 18) rat brain cortical neurons were plated at a density of 250,000 cells in 35mm glass bottom multiwell dishes and grown for 4 days in DMEM/F12 with N2 supplements. On day 4 some of the cultures were exposed to staurosporine (0.5µM) for a period of 24 hours to induce apoptosis. The cell-permeable fluorescein isothiocyanate (FITC) conjugate of the caspase inhibitor VAD-FMK (CaspACE™ FITC-VAD-FMK In Situ Marker, Cat.# G7461/G7462) was used to detect apoptotic cells. This inhibitor irreversibly binds to activated caspase, allowing for the in situ labeling of cells in which the caspase activation cascade has been initiated. Following incubation of the primary neurons with staurosporine, the CaspACE™ FITC-VAD-FMK In Situ

Marker (10µM) was added to the culture dishes and incubated at 37°C for 30 minutes. The medium was removed, and the cells were rinsed with PBS.

The dishes were placed on the stage of a Nikon® TE 200 microscope with fluorescence and DIC capabilities. The images were captured using a DAGE video camera and processed with an imaging system in Adobe® Photoshop™. Phase contrast images of the cells were obtained using DIC optics, and the fluorescence was detected at 450–490nm excitation/515nm emission. The fluorescence images were superimposed on the phase contrast images to permit both the viable and the apoptotic neurons to be observed in a single field. The total number of neurons and the percentage of apoptotic cells were easily quantified by cell counting. Primary neuronal cultures contain a small number of nonviable cells, but exposure to staurosporine markedly enhanced the number of apoptotic cells. 

**Ordering Information**

Product	Size	Cat.#
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl	G7461
	125µl	G7462