User Manual

ApoAlert™ Caspase Colorimetric Assay Kits User Manual

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I. Introduction

The ApoAlert Caspase-3 Colorimetric Assay Kit provides a simple means for detecting the activity of Caspase 3 in mammalian cells.This caspase is part of a large family of cysteine proteases that mediate apoptosis, or programmed cell death. Upon activation, caspases cleave a variety of target proteins in the cell, causing morphological and functional changes to cells undergoing apoptosis (Lazebnik *et al.*,1994; Casciola-Rosen *et al.*, 1996).

Background

Apoptosis plays a fundamental role in many normal biological processes as well as in several disease states (Thompson, 1995). Apoptosis can be induced by various stimuli that all produce the same end result: systematic and orderly cell death.

Caspases cleave a variety of cellular substrates after aspartic acid residues—a characteristic that is central to their role in mammalian apoptosis. Caspases are synthesized in the cytosol of mammalian cells as inactive zymogens, which become active through intracellular caspase cascades (Cohen, 1997). The ApoAlert Caspase-3 Colormetric Assay Kit allows you to detect the activity of caspase-3, which becomes active via different pathways of the apoptotic process.

Caspase-3 is an active cell-death protease involved in the execution phase of apoptosis, where cells undergo morphological changes such as DNA fragmentation, chromatin condensation, and apoptotic body formation (Porter & Janicke, 1999; Zou *et al.*, 1999). Caspase-3 is activated in response to serum withdrawal, activation of Fas, and treatment with radiation and pharmacological agents (Zou *et al.*, 1999).

For a review of caspases and apoptosis, see Green & Reed, 1998.

I. Introduction *continued*

The ApoAlert Caspase-3 Colorimetric Assay Kit provides a simple means for assaying caspase-3 activity in mammalian cell lines. Figure 1 shows the cleavage reaction. The colorimetric assay uses the spectrophotometric detection of the chromophore p-nitroaniline (pNA) after its cleavage by caspases from the labeled caspase-specific substrates.

Inhibitors for Caspase-3 are available for investigating the overall roles of proteases in the apoptotic process. Caspase-3 is specifically inhibited by the synthetic tetrapeptides DEVD-CHO and DEVD-fmk. DEVD-CHO is a reversible inhibitor; DEVD-fmk is irreversible.

Comparing the reading of an apoptotic sample with an uninduced control allows determination of the fold-increase in protease activity. Units of protease activity can also be quantified accurately and reproducibly using a standard curve established with free pNA (Section V).

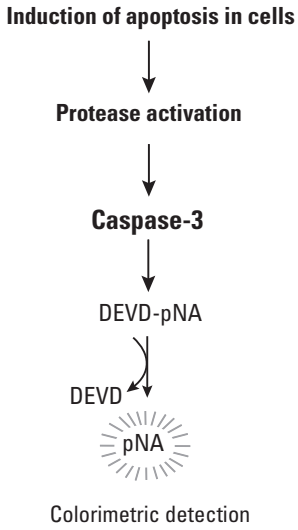

Figure 1. Detecting protease activity using the ApoAlert Caspase-3 Colormetric Assay Kit. Colormetric detection for caspase-3 is performed using a spectrophotometer at 405 nm.

II. List of Components

Store Cell Lysis Buffer and 2X Reaction Buffer at 4°C after opening.
Free pNA may be stored at 4°C after first use.
Store all other components at –20°C.

Caspase-3 Colorimetric Assay Kits

25 assays (630216)		100 assays (630217)		
• 32	ml	125	ml	Cell Lysis Buffer
• 1.25	ml	5	ml	2X Reaction Buffer
• 250	µl	1	ml	DTT (1 M)
• 125	µl	500	µl	Caspase-3 Substrate, DEVD-pNA (1 mM)
• 15	µl	50	µl	Caspase-3 Inhibitor, DEVD-fmk (1 mM)
• 50	µl	200	µl	pNA (100 mM)

III. Additional Materials Required

The following materials are required but not supplied:

- Microcentrifuge for collecting cells
- Reaction tubes (0.5–1.5 ml) or 96-well plates
- Spectrophotometer and cuvettes, or microplate reader capable of measurement at 400–405 nm.
- DMSO

IV. ApoAlert Caspase-3 Assay Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE BEGINNING

A. General Considerations

- A relatively high concentration of DTT (10 mM) is required for full enzyme activity. Ensure that DTT is added to the Reaction Buffer when the assay is performed. Otherwise, unexpected low caspase activity will occur. Turbidity or particulate materials in samples can decrease assay precision.
- If not analyzed immediately, samples should be stored at –20°C or lower.
- Protect DEVD-pNA from light.
- Cover or cap all reagents when not in use, and do not mix reagents from various kits.

Reagents

- Aliquot a sufficient volume of 2X Reaction Buffer (50 µl/assay) for the number of assays you will perform. **Immediately before use,** add DTT to the aliquotted 2X Reaction Buffer to obtain a final concentration of 10 mM (1:100 dilution of stock DTT solution). **Do not** add DTT to stock 2X Reaction Buffer.
- Ensure that all buffers are completely in solution before use. After thawing, store the Cell Lysis Buffer and 2X Reaction Buffer at 4°C.

Controls

- We recommend performing three control reactions:
  1) a negative control on uninduced cells (Step IV.B.1)
  2) a control on induced cells treated with Caspase-3 Inhibitor (Step IV.B.6)
  3) a positive control for Caspase-3 using a known apoptosis inducer.

Cells

You may perform the assay in 0.5–1.5-ml reaction tubes or 96-well plates. Use at least 2 x 10^6 cells per sample. Using fewer cells may reduce the observed increase in protease activity.

IV. ApoAlert Caspase-3 Assay Protocol *continued*

B. Caspase-3 Assay Procedure

1. Induce apoptosis in cells using the desired method. Remember to incubate a concurrent control culture without induction.

   Set up duplicate cell plates for the following samples: induced, uninduced (negative control), induced plus inhibitor (Step 6; optional), and induced without substrate (Step 8).

2. Count cells and centrifuge 2 x 10^6 cells at 400 x g for 5 min.

   Note: After removing the supernatant, you may freeze the cell pellets at –70°C and assay at a later time.

3. Resuspend cells in 50 µl of chilled Cell Lysis Buffer.
4. Incubate cells on ice for 10 min.
5. Centrifuge cell lysates in a microcentrifuge at maximum speed for 10 min at 4°C to precipitate cellular debris. Transfer the supernatants to new microcentrifuge tubes and place them on ice.

   Note: At this point, samples may be assayed immediately or frozen for assay at a later time.

6. **[Optional]** In the absence of a positive control reaction with purified Caspase-3, incubate an induced sample with Caspase-3 Inhibitor before adding substrate. This reaction verifies that the signal detected by the kit is due to protease activity.

   Add 50 µl of 2X Reaction Buffer/DTT Mix (Section IV.A) and 1 µl of Caspase-3 Inhibitor (DEVD-fmk) to 50 µl of supernatant from a sample obtained in Step 5. Incubate on ice for 30 min together with the other samples. Proceed to Step 7, where you will add reaction buffer to the remaining samples.

7. Add 50 µl of 2X Reaction Buffer/DTT Mix (Section IV.A) to each reaction. If you are using the Caspase-3 Inhibitor as a negative control, add 1 µl DMSO per 50 µl of 2X Reaction Buffer to samples without inhibitor to ensure that all samples are tested under similar conditions.
8. Add 5 µl of 1 mM Caspase-3 Substrate (DEVD-pNA; 50 µM final conc.) to each tube. Incubate at 37°C for 1 hr (or up to 3 hours maximum) in a water bath. Set up a parallel control reaction that does not contain conjugated substrate.
9. Read samples at 405 nm in a microplate reader or spectrophotometer using a 100-µl quartz cuvette. Alternatively, you may dilute the samples to 1 ml with PBS and use a standard cuvette, although doing so will weaken the signal. For quantification of protease activity, proceed to Section VI.

   Note: Subtract the background reading from cell lysates and buffers from the reading of induced and uninduced samples before calculating the fold-increase in Caspase-3 activity.

V. Colorimetric Quantification of Protease Activity

pNA Calibration Curve

1. Generate a pNA calibration curve:
   a. Dilute 100 mM pNA solution in DMSO or in Cell Lysis Buffer to make 0, 0.5, 1, 2, and 4 mM stock solutions.
   b. To 5 µl of each stock solution, add 95 µl of Cell Lysis Buffer to give these final concentrations:

      5 µl 0 mMpNA + 95 µl of Buffer = 0 nmole pNA

      5 µl 0.5 mM pNA + 95 µl of Buffer = 2.5 nmole pNA (25 µM)

      5 µl 1.0 mM pNA + 95 µl of Buffer = 5.0 nmole pNA (50 µM)

      5 µl 2.0 mM pNA + 95 µl of Buffer = 10 nmole pNA (100 µM)

      5 µl 4.0 mM pNA + 95 µl of Buffer = 20 nmole pNA (200 µM)

   c. Measure the five dilutions with a spectrophotometer at 405 nm and prepare a calibration curve with x = nmole pNA and y = O.D. Units (ODU).

      Sample results (read at 405 nm; your results may vary):

nmole pNA	ODU
0	0.001
2.5	0.060
5.0	0.138
10.0	0.237
20.0	0.489

2. Use the slope (ΔODU/Δnmol pNA) of this curve to calculate units of caspase activity with the following formula:

   $$\text{Units caspase activity} = \Delta\text{ODU} \times \frac{1}{\text{curve slope}}$$

   ΔODU= the difference in ODU between an uninduced control and its corresponding induced sample.

VI. References

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