User Manual

ApoAlert™ Caspase Fluorescent Assay Kits User Manual

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PT3191-1 (051415)
Published May 2015

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I. Introduction

The **ApoAlert Caspase Fluorescent Assay Kits** provide a simple means for detecting the activity of key caspases in mammalian cells: caspase-3 and caspase-9. These caspases are part of a large family of cysteine proteases that mediate programmed cell death—apoptosis. Upon activation, they disable cellular homeostatic and repair processes, and cleave important structural components in the cell, causing morphological and functional changes to cells undergoing apoptosis.

Background

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 Fluorescent Assay Kits allow you to detect the activity of a specific caspase—caspase-3 or caspase-9/6—which becomes active at different stages and/or different pathways of the apoptotic process.

Caspase-3 is an active cell-death protease involved in the execution phase of apoptosis, during which 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, treatment with radiation and pharmacological agents (Zou *et al.*, 1999), as well as to other upstream caspases, such as caspase-8 and caspase-9.

Caspase-9, an upstream caspase in the CD95 apoptotic pathway, is activated via the mitochondrial release of cytochrome c to the cytosol. Released cytochrome c binds to the apoptotic protease activating factor (Apaf-1) in the cytosol, forming a complex that activates procaspase-9 (Zou *et al.*, 1999; Cain *et al.*, 1999; and Hu *et al.*, 1999). Active caspase-9 initiates a protease cascade that also activates caspase-3 (Fernandes-Alnemri *et al.*, 1994) and other downstream caspases.

I. Introduction *continued*

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

Figure 1 illustrates how to detect protease activity using the ApoAlert Caspase Fluorescent Assay Kits. The Caspase-3 Fluorescent Assay Kit detects the emission shift of 7-amino-4-trifluoromethyl coumarin (AFC). The acetylated AFC-substrate conjugate usually emits **blue** light (λ_{max}=400 nm); however, cleavage of the substrate by the appropriate caspase liberates AFC, which fluoresces **yellow-green** (λ_{max}=505 nm). Similarly, the Caspase-9/6 Fluorescent Assay Kit detects the emission shift of 7-amino-4-methyl coumarin (AMC). The acetylated LEHD-AMC conjugate emits in the UV range (λ_{max}=380 nm); however, free AMC fluoresces **blue-green** at 440 nm upon liberation by caspase-9/6.

Each Caspase Fluorescent Assay Kit includes a specific, synthetic caspase inhibitor. When you are assaying whole cell lysates, the inhibitor can be used as a negative control. Inhibitors are also available separately for investigating the overall roles of proteases in the apoptotic process.

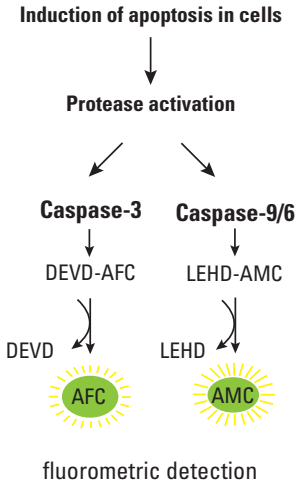


Figure 1. Detecting protease activity using ApoAlert Caspase Fluorescent Assay Kits. Fluorometric detection for caspase-3 is performed using a 400-nm excitation filter and 505-nm emission filter. To detect caspase-9/6, fluorometric detection is performed using a 380-nm excitation filter and 460-nm emission filter.

II. List of Components

Store Cell Lysis Buffer and 2X Reaction Buffer at 4°C after opening. Free AFC may be stored at 4°C after first use. Store substrate, inhibitor, and DTT at –20°C.

Caspase-3 Fluorescent Assay Kit (Cat. No. 630215; 100 assays)

- 3 x 44 ml **Cell Lysis Buffer**
- 5 ml **2X Reaction Buffer**
- 1 ml **DTT** (1 M)
- 500 µl **Caspase-3 Substrate, DEVD-AFC** (1 mM)
- 10 µl **Caspase-3 Inhibitor, DEVD-CHO** (1 mM)
- 4 ml **AFC** (80 µM)

Caspase-9/6 Fluorescent Assay Kit (Cat. No. 630212; 100 assays)

- 125 ml **Cell Lysis Buffer**
- 5 ml **2X Reaction Buffer**
- 1 ml **DTT** (1 M)
- 500 µl **Caspase-9/6 Substrate, LEHD-AMC** (5 mM)
- 40 µl **Caspase-9/6 Inhibitor, LEHD-CHO** (5 mM)
- 150 µl **DMSO**
- 4 ml **AMC** (80 µM)

III. Additional Materials Required

The following materials are required but not supplied.

- **Centrifuge** for collecting cells
- **Reaction tubes (0.5–1.5 ml)** or **96-well plates**
- **DMSO** for Caspase-3 assay
- **Fluorometer** or **96-well plate reader**

IV. ApoAlert Caspase-3 Assay Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE BEGINNING

A. General Considerations

A relatively high concentration of DTT (10 mM) is required for full activity of the enzymes. 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.

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: add 10 µl of 1 M DTT stock per 1 ml of 2X Reaction Buffer. Do not add DTT to stock 2X Reaction Buffer.
- Turbidity or particulate materials in samples can decrease assay precision.
- Ensure that all buffers are completely in solution. You may need to heat free AFC to dissolve precipitates that can form during cold storage.

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 induction. You may treat your own cells with a known inducer. At Clontech, we use Staurosporine (500 nM, for 4 hr) or human anti-Fas monoclonal antibody (clone CH-11; 500 ng/ml final concentration, for 4–12 hr). Anti-Fas clone DX2 is **not** recommended for use with this kit.
- Comparing the emission of an apoptotic sample with an uninduced control allows determination of the fold-increase in protease activity. Units of protease activity can also be quantitated accurately and reproducibly using a standard curve established with the appropriate free fluorescent molecule (Section VII).

Cells:

You may perform the assay in 0.5–1.5-ml reaction tubes or 96-well plates. Use at least 1 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. Assay Procedure

1. Induce apoptosis in cells by 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 1 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.

   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-CHO) 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-AFC; 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 in a fluorometer with a 400-nm excitation filter and 505-nm emission filter. For plate reading, transfer samples to a 96-well plate. For quantification of protease activity, proceed to Section VII.

V. ApoAlert Caspase-9/6 Assay Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE BEGINNING.

A. General Considerations

A relatively high concentration of DTT (10 mM) is required for full activity of the enzymes. 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.

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: add 10 µl of 1 M DTT stock per 1 ml of 2X Reaction Buffer. Do not add DTT to stock 2X Reaction Buffer.
- Turbidity or particulate materials in samples can decrease assay precision.
- Ensure that all buffers are completely in solution before use.

Controls:

- We recommend performing three control reactions:
  1) a negative control on uninduced cells (Step VI.B.1)
  2) a control on induced cells treated with Caspase-9/6 Inhibitor (Step VI.B.6)
  3) a positive control for Caspase-9 induction. You may treat your own cells with a known inducer. At Clontech, we use Staurosporine (500 nM, for 4 hr) or human anti-Fas monoclonal antibody (clone CH-11; 500 ng/ml final concentration, for 4–12 hr). Anti-Fas clone DX2 is **not** recommended for use with this kit.
- Comparing the emission of an apoptotic sample with an uninduced control allows determination of the fold-increase in protease activity.

Cells:

You may perform the assay in 0.5–1.5-ml reaction tubes or 96-well plates. Use at least 1 x 10^6 cells per sample. Fewer cells may reduce the observed increase in protease activity.

V. ApoAlert Caspase-9/6 Assay Protocol *continued*

B. Assay Procedure

1. Induce apoptosis in cells by 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 1 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 supernatants to new microcentrifuge tubes.

   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-9, incubate an induced sample with Caspase-9/6 Inhibitor before adding substrate. This reaction verifies that the signal detected by the kit is due to protease activity.

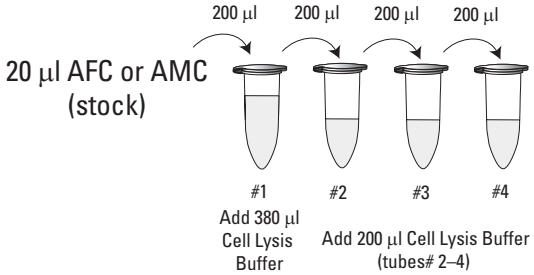
   Add 50 µl of **2X Reaction Buffer/DTT Mix** (Section VI.A) and 2 µl of Caspase-9/6 Inhibitor (LEHD-CHO) to 50 µl of supernatant from a sample obtained in Step 5. Incubate on ice for 30 min together with the other samples. Proceed to Step 7, where you will add reaction buffer to the remaining samples.

7. Add 50 µl of **2X Reaction Buffer/DTT Mix** (Section VI.A) to each reaction. If you are using the Caspase-9/6 Inhibitor as a negative control, add 2 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 the 5 mM Caspase-9/6 Substrate (LEHD-AMC; 250 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 in a fluorometer with a 380-nm excitation filter and 460-nm emission filter. For plate reading, transfer samples to a 96-well plate. For quantification of protease activity, proceed to Section VII.

VI. Quantification of protease activity for Caspase-3 & -9/6

AFC (Caspase-3) or AMC (Caspase-9/6) Calibration Curve

1. Label four 0.5-ml microcentrifuge tubes, as shown in the diagram below.
2. Prepare 4 µM of AFC or AMC solution in 1X Cell Lysis Buffer: Add 380 µl 1X Cell Lysis Buffer to tube #1, then add 20 µl of AFC or AMC stock solution (provided). Mix well.
3. To the remaining tubes, add 200 µl 1X Cell Lysis Buffer, and perform a serial dilution: From tube #1, add 200 µl into tube #2 and mix well. Then from tube #2, add 200 µl into tube #3, and mix well. Do the same for tube #4.



4. Measure the samples in a fluorometer (AFC: 400 nm Ex., 505 nm Em.; AMC: 380 Ex, 460 Em.) using cell lysis buffer (without AFC or AMC) for background calibration.
5. Prepare a calibration curve with x = µM AFC or AMC and y = fluorescence units (FU).
6. Use the slope (ΔFU/ΔµM AFC or AMC) of the curve to calculate caspase activity with the following formula:

$$\text{Caspase activity} = (\Delta\text{FU/hr}) \times \frac{1}{\text{curve slope}}$$

ΔFU/hr= the difference in FU between an uninduced control and an induced sample.

VII. References

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