Instruction Manual
for

Caspase 3/7 Assay Kit
(Ac-DEVD-AMC Substrate)
Fluorescent Microplate Detection

Catalog # 17-367

Sufficient reagents for 192 assays per kit.

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NOT RECOMMENDED OR INTENDED FOR DIAGNOSIS OF DISEASE IN HUMANS.

DO NOT USE IN HUMANS.

I. STORAGE AND STABILITY

Storage: Upon receipt, store individual components at recommended temperatures. Store the 96 well white plates at room temperature. Store all other components at -20ºC.

Stability: Components stable for 6 months from date of shipment if stored and handled correctly.

II. ASSAY OVERVIEW

Apoptosis, or programmed cell death, is a process for the neat and tidy disposal of unwanted cells. Apoptosis is intended to remove cells while preventing the development of an inflammatory response, which is often associated with necrotic cell death. Programmed cell death is a critical process during development, and is fundamental to homeostasis in nearly all multicellular organisms. Disruption of an appropriate apoptotic response is implicated in the development of many disease states, including cancer (lack of apoptosis) or one of several degenerative diseases (enhanced apoptosis).

One group of proteins fundamental to the apoptotic process is the Caspase (cysteinyl aspartate-specific protease) Family. Some caspases are necessary for the transmission of the early signals that send a cell into the apoptotic program while others are critical for the execution of that program. For example, aggregation of Fas by Fas ligand results in the formation of an intracellular complex that results in activation of caspase 8. Active caspase 8 then activates other proteins which perpetuate the apoptotic signal. Caspase 8 also activates caspase 3, which is responsible for the cleavage of many cellular proteins which will later be packaged into apoptotic bodies prior to disposal by phagocytes.

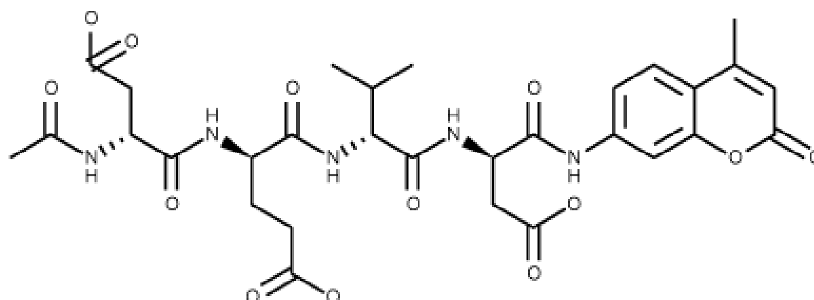
At least 14 caspases have been identified. Some have a role as initiators of apoptosis (caspase 2, 8, 9, 10, and 12), some are effectors (caspase 3, 6 and 7), and others have highly specialized roles in specific cell types (for example, the cytokine activators (caspase 1, 4, 5, 11, and 13). All caspases are produced and maintained as an inactive precursor; induction of apoptosis results in proteolytic processing to produce two polypeptides, one usually of 17-25kDa and the other of 10-12kDa, which dimerize to form the active caspase. Active caspases cleave targets bearing the appropriate target sequence, usually a four or five amino acid sequence ending in aspartic acid. Unfortunately, there are many recognition sequences and the caspases are fairly promiscuous, as most caspases can recognize more than one sequence, and there are many sequences that are recognized by more than one caspase (Table 1).

The most popular method for assaying caspase activity involves the incorporation of a caspase recognition sequence peptide into a coumarin-based fluorescent compound, such aminomethyl coumarin (AMC). The intact peptide-coumarin conjugate is non-fluorescent, but when the peptide is cleaved off by an active caspase, the resulting cleavage product is fluorescent (Fig. 1). Upstate's Caspase 3/7 Assay Kit (Ac-DEVD-AMC Substrate) is a microplate-based fluorescent assay which utilizes the preferred substrate for caspase 3 & 7, DEVD, conjugated to AMC. The assay kit is highly sensitive and detects the activated form of both caspase 3 and caspase 7. The kit may be used to measure the relative amount of activated caspase in cell lysates, or used with purified caspase to identify compounds which may inhibit or enhance caspase activity. Each kit comes with two assay microplates and sufficient reagents for 192 assays.

Table 1: Caspase Properties

				Caspase Recognition Peptide Sequence											
Caspase	**Alternate Name**	**Pro-Caspase Size (kDa)**	**Active Caspase Size (kDa)**	**YVAD**	**YVED**	**WEHD**	**DEHD**	**VDVAD**	**DEVD**	**LEHD**	**LEVD**	**VEHD**	**VEID**	**IETD**	**LETD**
1	ICE	45	20/10	X	X	X									
2	Ich-1, Nedd-2	49	19/12				X	X		X					
3	Apopain, CPP32, prICE, Yama	34	17/12						X						
4	Ich-2, ICErel-II, TX	43	19/10			X				X	X				
5	Ich-3, ICErel-III, TY	48	22/10			X				X					
6	Mch-2	34	18/11									X	X	X	
7	Mch-3, ICE-LAP3, CMH-1	37	20/12						X						
8	Mch-5, FLICE, MACH	55	20/11											X	X
9	Mch-6, ICE-LAP-6	46	20/12							X					
10	Mch-4, FLICE 2	59	18/12							X				X	
11	Ich-3	43	23/10			X									
12	-	50	19/10												
13	ERICE	43	25/13												
14	MICE	31	18/11	X					X	X					

Figure 1: Caspase Substrate Diagram



III. SYSTEM COMPONENTS

A. Provided Kit Components

White flat bottom 96-well plate
Two 96 well white plates.

2X Caspase Buffer
Catalog # 20-274
One vial containing **10.5ml** of 40mM PIPES, pH 7.2, 150mM NaCl, 5mM EDTA, 0.2% CHAPS and 15% sucrose.

Dithiothreitol
Catalog # 20-273
Two vials, each containing **15.4mg** of lyophilized dithiothreitol.

Caspase Substrate
Catalog # 12-541
One vial containing **200µl** of a proprietary cocktail of Ac-DEVD-AMC substrate in DMSO.
Protect Caspase Substrate solution from light.

Caspase 3, active
Catalog # 14-264
One vial containing **20µg** of recombinant full length protein in **200µl** of phosphate buffered saline (PBS) containing 50% glycerol.

B. Required Materials Not Provided

- Enzyme preparation or cell extract containing active caspase 3 or caspase 7
- Caspase 3/7 inhibitor (optional)
- Ice bucket
- Timer
- Sterile tubes
- Variable volume (5-200µl) pipet + tips
- Variable volume (5-200µl) multichannel pipet + tips
- Reagent troughs for multichannel pipettes
- Microplate incubator
- Fluorescence 96-well plate reader capable of detecting 7-amino-4-methylcoumarin (AMC) (Excitation maximum (Ex max.) of ~346nm, and Emission maximum (Em max.) of ~442nm).

IV. Caspase3/7 ASSAY PROCEDURE (Fluorometric Detection)

Safety Warnings and Precautions: The Caspase 3/7 Assay kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

A. General Notes

1. During shipment, small volumes of product may accumulate in the vial cap. For maximum recovery of product, centrifuge the vial prior to removing the cap.

2. The reconstituted Caspase Substrate Solution should be protected from light.

3. Allow all reagents, except the Caspase 3 (Catalog # 14-264), to warm to room temperature prior to use.

4. The source of caspase 3/7 activity may be an extract or purified enzyme.

5. We recommend performing the following experimental controls in duplicate:
   - A ‘no enzyme’ negative control.
   - A positive control, using active Caspase 3 (Catalog # 14-264) provided as a source of Caspase activity.
   - A control or test sample treated with a Caspase inhibitor.
   - A ‘no substrate’ control.

6. DTT is required for maximum Caspase activity. Reconstitute DTT and add it to the 1.5X Caspase Buffer immediately prior to use.

7. Equilibrate the Caspase 3 (Catalog # 14-264) to room temperature immediately before use to maintain caspase activity. To avoid future temperature fluctuations, aliquot the enzyme upon initial use. The end user should determine the optimum incubation time and assay temperature for their source of caspase activity.

8. **Filter Selection:** The AMC dye has an Ex max. of ~346nm, and an Em max. of ~442nm. In general, the excitation filter wavelength and emission filter wavelength chosen should be as close as possible to the maxima for the dye for optimal results. Additionally, a narrower bandpass filter is preferred to a larger bandpass filter. Using filters with a larger bandpass could result in lower signal to background ratios. *It should be noted that bandpass definitions might vary depending on the filter manufacturer.*

B. Preparation of Assay Solutions

Prepare sufficient volume of each solution based on the number of assays to be performed, plus a slight overage to account for pipetting inaccuracies (either 10% extra or one extra assay point is generally sufficient).

1. **1.5X Caspase Buffer:** Ensure the 2X Caspase Buffer is warmed to room temperature and the solution is not cloudy. A brief 30°C incubation will help clear the solution if it has not warmed sufficiently. For each plate remove 5ml of the **2X Caspase Buffer** (Catalog # 20-274) and transfer to a sterile tube. Add 0.8 ml of dH_2O to one vial of DTT. Gently mix and transfer the contents of the reconstituted DTT to the 5ml of 2X Caspase Buffer. Wash the DTT vial with an additional 0.8ml of dH_2O and transfer to 5.8ml of Caspase Buffer. Reconstitute the DTT immediately prior to preparing 1.5X Caspase Buffer. Unused 1.5X buffer may be stored at -20ºC for 1-2 months. Avoid freeze-thaw cycles.

2. **Caspase3/7 inhibitor (optional):** Dilute a Caspase 3/7 inhibitor in 1.5X Caspase Buffer (not provided).

3. **3X Caspase Substrate:** For each plate add 75μl of **Caspase Substrate** (Catalog # 12-541) to 2.425ml of 1.5X Caspase Buffer. The caspase substrate is in DMSO. Rapidly warm to room temperature prior to pipetting. Protect the diluted substrate from light.

4. **Test Sample:** Prepare test compounds or samples at 3X their final reaction concentrations. The assay can tolerate a compound prepared in up to 20% DMSO. Most commonly used sample buffers will not interfere with the assay. Prepare a sufficient volume of diluted sample for the number of assay points. Each assay point requires 25μl. Store on ice. Particulate matter or cloudy solutions may interfere with the assay.

5. **Caspase 3 (positive control):** Equilibrate the **Caspase 3, active** (Catalog # 14-264) to room temperature and dilute 10-fold using H_2O or test sample buffer. Prepare a sufficient volume of diluted Caspase 3 for the number of assay points. Each assay point requires 25μl. Store on ice.

C. Protocol

1. Prepare the Assay Solutions (see Section IV-B).
2. Pipet 25μl of 1.5X Caspase Assay Buffer, or 1.5X Caspase Assay Buffer containing a caspase 3/7 inhibitor into each well.
3. Add 25μl of test sample, or 25μl of the diluted caspase 3 (positive control), or 25μl of sample diluent (negative control). Allow the wells to equilibrate to assay temperature (30°C or 37°C).
4. Add 25μl of the 3X Caspase Assay Substrate and mix thoroughly.
5. Incubate the microtiter plate at 37°C for 15-60 minutes. The optimum incubation time (15-60 minutes) and temperature (30°C or 37°C) should be determined by the end user. The plate can be read at multiple time points to determine the linear range of the assay.
6. Read the plate in a fluorescence plate reader capable of detecting AMC (Ex max. of ~346nm, and Em max. of ~442nm).

V. REFERENCES

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VI. INSTRUMENT SETTINGS APPENDIX

As an example, settings used to read this assay on a Molecular Devices Analyst AD™ plate reader are provided in Table 2 and settings used to read this assay on a Wallac Victor^2 1420 (PerkinElmer) plate reader are provided in Table 3.

Table 2: Molecular Devices Analyst AD™ Settings

Parameter	Setting
Mode	Fluorescence intensity
Excitation filters	360-35nm
Emission filters	460-35nm
Dichroic mirror	430nm
Z-height	3mm
Attenuator	High
Integration time	100,000μsec
Lamp	Continuous
Readings per well	One
PMT setup	SmartRead sensitivity 0
Units	Counts/sec

Table 3: Wallac Victor^2 1420 (PerkinElmer) Settings

Parameter	Setting
Mode	Fluorescence intensity
Excitation filters	380nm
Emission filters	460nm
CW Lamp Energy	1000
Readings per well	One
Integration time	0.1 sec
Units	Fluorescent Units