Instructions for Using Caspase Substrates and Inhibitors

Catalog Numbers
170-3170 to 170-3194
and
170-3196 to 170-3198

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4006151 Rev A

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Section 1
Introduction

Measurements of caspase[1] activity are simplified using synthetic oligopeptide substrates and inhibitors. Substrates are made by modifying the caspase cleavage site (C-terminal aspartic acid) with 7-amino-4-trifluoromethyl coumarin (AFC). When liberated from the peptide, AFC produces an optical change that can be detected by either fluorescence or absorbance.[2] Inhibitors are made by modifying the C-terminal aspartic acid with fluoromethyl ketone (FMK). The FMK derivatives are cell permeant and irreversible. Table 1 lists optimized peptide recognition sequences[3,4] for caspases 1–10. The molecular weights (MW) include salts and esters.

Table 1. Caspase Substrates and Inhibitors

Caspase (Alias)	Optimal Peptide Sequence	Substrate MW Ac-peptide-AFC	Inhibitor MW Z-peptide-FMK
1 (ICE)	WEHD	952	877
4 (ICErel-II, TX, ICH-2)	"	"	"
5 (ICErel-III, TY)	"	"	"
2 (ICH-1, NEDD2)	VDVAD	770	695
3 (apopain, CPP32, Yama)	DEVD	729	668
7 (Mch3, ICE-LAP3, CMH-1)	"	"	"
10 (Mch4)	"	"	"
6 (Mch2)	VEID	727	652
8 (MACH, FLICE, Mch5)	LETD	729	724
9 (ICE-LAP6, Mch6)	LEHD	879	804
general inhibitor	Boc-D-FMK	n/a	263
general inhibitor	Z-VAD-FMK	n/a	468

Section 2
Measuring Caspase Activity

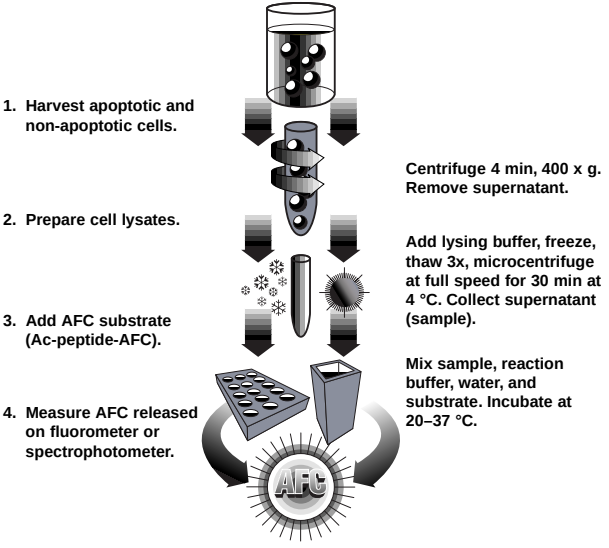

Fig. 1. The general method.

2.1 Prepare Reagents, Sample and Equipment

- Prepare the stock solutions: substrate, inhibitor, reaction buffer and cell lysis buffer—refer to Section 3.
- Prepare the cell lysate—refer to Section 4.
- Prepare the detection system—refer to Section 2.3.

2.2 Mix the Reaction Components

Mix the reaction components to prepare a Blank and the Samples to be measured. Total volumes can be adjusted for any cuvette or microtiter plate format. In any case, the component proportions should remain constant according to the following guide.

Blank

Component	Volume	Examples Total = 200 µl	Total = 1 ml
Water	0.95 x total	190 µl	950 µl
Reaction Buffer (25x)	0.04 x total	8 µl	40 µl
Substrate (100x)	0.01 x total	2 µl	10 µl

Sample

Component	Volume	Examples Total = 200 µl	Total = 1 ml
Water	(0.95 x total) - L	185 µl	930 µl
Reaction Buffer (25x)	0.04 x total	8 µl	40 µl
Substrate (100x)	0.01 x total	2 µl	10 µl
Cell Lysate (sample)	*L	5 µl	20 µl

* The cell lysate volume (L) is arbitrary and determines the volume of water to be added. Enough lysate should be used to detect a measurable activity within 3 hours after mixing the components. For example, begin with 10 µl lysate (from 10^6 cells) per ml of reaction solution.

- Caspase activity can be blocked using peptide-FMK inhibitors added directly to live cells. Dilute the inhibitor stock solution at least 1000-fold into the cell culture media, *e.g.*, add 5 µl of inhibitor stock to 5 ml cell media and incubate at least 15–30 minutes prior to preparing lysates. Alternatively, dilute the inhibitor stock at least 1,000-fold into samples containing crude cell extracts or purified enzyme and incubate for 15 minutes prior to adding AFC substrates. Read Section 3.5 for using inhibitors with purified enzyme.

- Positive control samples can be made by using recombinant enzyme or lysates from cells induced to undergo apoptosis by a variety of methods—see Section 5, "Inducing Apoptosis".

2.3 Measure the Enzyme Activity

Prepare an absorbance or fluorescence detector according to the manufacturers directions. AFC fluorescence measurements are at least 10-fold more sensitive than absorbance.[2,5] In either case, the rate of signal change is proportional to the enzyme activity.

- To measure AFC fluorescence, select 390–400 nm excitation and 510–540 nm emission. Set the instrument gain so the signal is about 20% full scale using the "Blank" (see Section 2.2). For example, if the instrument's maximum signal = 10,000 units, set the gain so the signal reads 2000 for the blank. If subsequent sample measurements indicate very low activity, increasing the instrument gain should improve the sensitivity.
- To measure AFC absorbance, select 390–400 nm. Zero the instrument using a buffer blank without substrate.
- Record the time and signal for the blank and enzyme sample immediately following mixing of the reaction components. Repeat measurements every 15–30 minutes until the enzyme sample signal nears the instrument maximum or until 3 hours have elapsed. Because the reaction rates will vary with temperature, keep the sample temperature constant, *e.g.* 37 °C, between readings and use the same temperature for samples that will be compared. Cover the samples to avoid evaporation.

2.4 Analyze the Data

Compare the fluorescence or absorbance rate of change ($\Delta S/\Delta t$) to obtain a measure of relative activity for different samples measured under equivalent conditions. Plot ΔS versus Δt and calculate the slope ($\Delta S/\Delta t$).

$$\Delta S=[S(t_i)–B(t_i)]–[S(t_o)–B(t_o)], \qquad \Delta t = (t_i–t_o),$$

S = sample signal at time t, and B = blank signal at time t. t_i = time of measurement, t_o = time of initial measurement. The blank measurement is required to remove signal caused by baseline substrate hydrolysis and instrument drift.

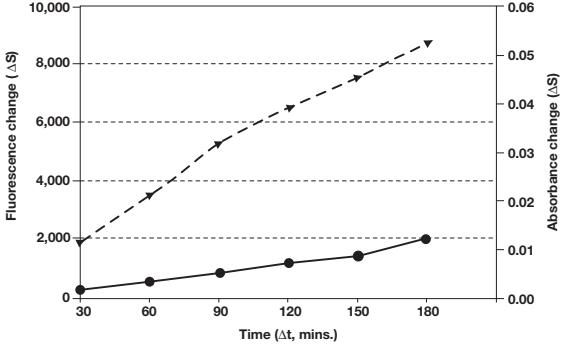

Fig. 2. Caspase 3 activity $\Delta S/\Delta t$. Jurkat tumor cells were stimulated with 500 ng/ml anti-Fas for 2 hours and assayed for the presence of Caspase 3 (Δ). Baseline levels are also shown for unstimulated cells (●).

Section 3
Solution Recipes

3.1 Additional Reagents Required

DMSO	[dimethyl sulfoxide] high grade
HEPES	[N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)]
EDTA	[ethylenediaminetetraacetic acid]
CHAPS	[3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfonate]
DTT	[dithiothreitol]
PMSF	[phenylmethylsulfonylfluoride]

Pepstatin A, Aprotinin, Leupeptin

3.2 Substrate Stock Solutions, 100x

Add 0.25 ml of DMSO to a 1 mg vial of substrate, molar concentration = 4/MW (see Table 1). Thoroughly dissolve the contents of the vial. Store DMSO stock solutions at -20 °C.

3.3 Inhibitor Stock Solutions, 1000x

Add 1.0 ml of DMSO to a 1 mg vial of inhibitor, molar concentration = 1/MW (see Table 1). Thoroughly dissolve the contents of the vial. Store DMSO stock solutions at -20 °C.

3.4 Buffers

A. Reaction Buffer, 25x

[250 mM HEPES, pH 7.4, 50 mM EDTA, 2.5% CHAPS,125 mM DTT]

1. Dissolve the following components in filtered, deionized water and adjust to pH 7.4. V = volume (ml) of water, FW = formula weight.

Component	Mass (grams)	Example (V = 10 mL)
HEPES	V x FW x $250x10^{-6}$	650 mg (Na salt FW=260)
EDTA	V x FW x $50x10^{-6}$	186 mg (Na_2 salt•$2H_20$ FW = 372)
CHAPS	25 mg/ml	250 mg
DTT	19 mg/ml	193 mg

2. Keep the solution on ice while preparing experiments. Store the solution at -20 °C.

B. Cell Lysis Buffer

1. Prepare 100x stock solutions of the following proteinase inhibitors:

PMSF	35 mg/ml
pepstatin A	1 mg/ml
aprotinin	1 mg/ml
leupeptin	2 mg/ml

2. Dilute to 1x the proteinase inhibitors and reaction buffer in deionized water, *e.g.*, to 920 µl water, mix 40 µl reaction buffer (25x), 10 µl each of PMSF, pepstatin A, aprotinin, leupeptin.
3. Keep the solutions on ice while preparing experiments. Store the buffer solutions at -20 °C.

3.5 FMK Inhibitors Used With Purified Enzyme

Peptide-FMK inhibitors contain O-Me esters to facilitate entry into live cells. When peptide FMK inhibitors are used with purified caspases the ester groups are not rapidly hydrolyzed and may reduce the inhibitor-enzyme reaction rate. The ester groups can be removed by pretreating with esterase. For example, dilute the FMK inhibitor stock (or DMSO only for a control) 10-fold into 10 mM borate buffer, pH 8.0, containing 1 unit of esterase (*e.g.*, carboxylic acid ester hydrolase, Boehringer-Mannheim) and incubate for 15 minutes on ice. Then dilute an additional 100-fold into the final reaction mixture and incubate again for 15 minutes prior to adding the substrate.

Section 4
Preparation of Cell Lysates

4.1 Cell Lysates From Suspension Cultures

1. Obtain sufficient cells to prepare approximately 10^5 cells per 1 µl of lysate, *e.g.*, 10^7 cells for 100 µl of lysate.
2. Transfer cells into a 15-ml centrifuge tube and centrifuge for 4 minutes at 400 x g.
3. Decant the supernatant and resuspend cells in 1 ml of PBS.
4. Transfer the cells into a microcentrifuge tube and centrifuge for one minute at full speed.
5. Decant the supernatant and dissociate the cell pellets by agitating the bottom of the tube.
6. Add 100 µl lysis buffer per 10^7 cells and vortex gently.

7. Freeze and thaw the sample three or four times by transferring from an isopropanol-dry ice bath to a 37 °C water bath; do not vortex between freeze thaw steps.
8. Centrifuge lysed cells at 4 °C for 30 minutes at full speed.
9. Transfer the supernatant to a clean tube and keep on ice if the assay is to be performed within one hour, otherwise, store cell extracts at -70 °C and minimize freeze-thaw cycles.

4.2 Cell Lysates From Adherent Cultures

1. Adherent cells can be grown in 6-well microtiter plates or in 100-mm tissue culture plates. Obtain sufficient cells to prepare approximately 10^5 cells per 1 µl of lysate, *e.g.*, 10^7 cells for 100 µl of lysate.
2. Transfer media containing suspended cells from the plates to a 15-ml tube. Cells undergoing apoptosis may release from the plate surface, but can be recovered for the assay.
3. Add a small volume of PBS to the plates to rinse adherent cells.
4. Centrifuge suspended cells in the 15-ml tube for 4 minutes at 400 x g, decant the supernatant, resuspend in a small volume of PBS and transfer to a microcentrifuge tube.
5. Again pellet the cells in a microcentrifuge, discard the supernatant, add 100 µl of lysis buffer to the tube and agitate gently.
6. Decant the PBS wash from the dish containing the adherent cells and transfer the lysis solution from the microcentrifuge tube to the plate. Use 100 µl per 10^7 cells.
7. Rock the plate to coat the entire surface and release the cells using a rubber policeman.

8. Transfer the cells and lysis solution back to the microcentrifuge tube.
9. Proceed to the "freeze and thaw" step 7 in Section 4.1 and complete the preparation.

Section 5
Inducing Apoptosis

Not all caspases are activated by every method of inducing apoptosis, *e.g.*, Fas-mediated apoptosis may not activate caspases 1, 4, or 5. Also, not all cell types are affected the same by a given stimulus. Consult published methods for applying a particular stimulus with a particular cell type. Triggers of apoptosis in mammalian cells[6] include:

- Receptor factors: Fas ligand (FasL), anti-Fas, tumor necrosis factor (TNF)[7-9]
- Lytic factors: perforin combined with granzymes[10]
- Drugs: camptothecin, etoposide, vincristine, Taxol, doxorubicin, dexamethasone[11-16]
- UV irradiation[17,18]

5.1 Example Procedure Using Anti-Fas

Multivalent Fas ligand (FasL) or anti-Fas can cause aggregation of Fas receptors and induce apoptosis.[7] Not all cell types that express Fas undergo apoptosis by this method of induction. Human cell lines responsive to anti-human Fas, include Jurkat, U937, HeLa and HepG2. Mouse cell lines responsive to anti-mouse Fas, include BALB/c thymocytes. The following protocol can be used as a guide to inducing apoptosis with anti-Fas.

Materials

Cells: human or mouse origin, sensitive to Fas-mediated apoptosis

Culture Medium: RPMI-1640, 10% heat inactivated FBS, 1% L-glutamine, 1% pen/strep.

Antibodies: anti-human Fas (clone DX2) or anti-mouse Fas (clone Jo2) monoclonal antibody

Flasks: T25 tissue culture flasks

Procedure

a) Using culture medium, adjust cell density to 1–2 x 10^6 cells/ml in a T25 flask.
b) Add anti-human Fas (final 0.5–1.0 µg/ml), or antimouse Fas (final 2–10 µg/ml).
c) Incubate cells for 2–12 hours at 37 °C and 5% CO_2.
d) Proceed to Section 4 "Preparation of cell lysates".

Section 6
Storage

Substrates and inhibitors will gradually hydrolyze on exposure to water. Store unopened reagents at room temperature, desiccated and out of direct sunlight. Stock solutions should be prepared in high grade dimethyl sulfoxide (DMSO) and stored at -20 °C. To avoid contamination with moisture, bring frozen stock solutions to room temperature before opening the vials. Freeze-thaw cycles will not damage the reagents. **Do not store aqueous solutions of substrates and inhibitors**.

Section 7
References

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Section 8
Product Information

		Catalog Numbers	
Substrate	**Caspase**	**1 mg**	**5 x 1 mg**
Ac-WEHD-AFC	1,4,5	170-3170	170-3171
Ac-VDVAD-AFC	2	170-3174	170-3175
Ac-DEVD-AFC	3,7,10	170-3178	170-3179
Ac-VEID-AFC	6	170-3182	170-3183
Ac-LETD-AFC	8	170-3186	170-3187
Ac-LEHD-AFC	9	170-3190	170-3191
Inhibitor	**Caspase**	**1 mg**	**3 x 1 mg**
Z-WEHD-FMK	1,4,5	170-3172	170-3173
Z-VDVAD-FMK	2	170-3176	170-3177
Z-DEVD-FMK	3,7,10	170-3180	170-3181
Z-VEID-FMK	6	170-3184	170-3185
Z-LETD-FMK	8	170-3188	170-3189
Z-LEHD-FMK	9	170-3192	170-3193
Boc-D-FMK	general	170-3194	170-3198
Z-VAD-FMK	general	170-3196	170-3197