



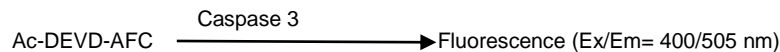
Caspase-3 Activity Assay Kit II (Fluorometric)

rev 03/19

(Catalog # K533-100; 100 assays; Store at -20°C)

I. Introduction:

Caspase-3 (3.4.22.56) is a cysteine protease that cleaves the C-terminal of an Aspartate residue at the P1 position. The cleavage event is a critical step in the downstream signaling and amplification of apoptosis, or programmed cell death. Apoptosis is a tightly regulated event that plays a central role in the development and homeostasis of multi-cellular organisms. BioVision's Caspase-3 Fluorometric Activity Assay Kit II allows the detection and quantification of Caspase-3 Activity by using a synthetic substrate DEVD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin), which upon cleavage by Caspase-3 will emit a strong, stable fluorometric signal (Ex/Em= 400/505 nm). The assay is sensitive, fast and allows the high-throughput quantification of Caspase-3 Activity in cell lysate or adherent cells. It can be used to evaluate induction of apoptosis and/or evaluation of apoptosis inhibitors. As little as 11.25 mU of activity per well and less than 500 apoptotic cells can be detected.



II. Applications:

- Quantification of Caspase-3 Enzymatic activity in mammalian cells
- Side-by-side comparison of inducers of apoptosis with Staurosporine (included)
- High-throughput screening of inducers or inhibitors in biological samples

III. Sample Type:

- Adherent or suspension mammalian cell line grown in a 96-well plate or in a Falcon flask can be used

IV. Kit Contents:

Components	K533-100	Cap Code	Part Number
Caspase Cell Lysis Buffer	100 ml	NM	K533-100-1
Caspase 2X Reaction Buffer	20 ml	WM	K533-100-2
Ac-DEVD-AFC (1 mM)	500 µl	Amber	K533-100-3
DTT (1 M)	1 ml	Green	K533-100-4
AFC Standard (1 mM)	100 µl	Yellow	K533-100-5
Staurosporine (1 mM)	200 µl	Violet	K533-100-6
Caspase-3 (Positive Control)	1 vial	Red	K533-100-7

V. User Supplied Reagents and Equipment:

- 96-well plate white microplate with a clear, flat bottom
- 96-well plate white microplate with an opaque, flat bottom
- Phenol-red free media (PRFM), DMSO, Fetal Calf Serum (FCS)
- Multi-well spectrophotometer (ELISA reader)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **Cell Lysis Buffer & 2X Reaction Buffer:** Warm to room temperature before use. Store at either 4°C or -20°C.
- **Ac-DEVD-AFC, AFC Standard, DTT & Staurosporine:** Aliquot and store at -20°C.
- **Caspase-3:** Reconstitute in 15 µl dH₂O. Aliquot 5 µl into microfuge tubes and store at -80°C.

VII. Caspase 3 Activity Assay Protocol:

1. Sample Preparation:

Intact Cells: Day 1: Seed 1X10⁶ cells/well in a white, clear bottom 96-well plate. Grow overnight to confluency in 100 µl of media + 10% FCS. **Day 2:** Prepare the following reagent: Phenol-red free media w/5% FCS: 47.5 ml PRFM + 2.5 ml FCS). Also prepare 100 µM Staurosporine (apoptosis-inducer). Add 25 µl of Staurosporine stock solution to 225 µl of PRFM (+5% FCS). Next, remove media and gently wash cells with 100 µl of FCS-free PRFM. Gently rock plate back and forth, and then remove FCS-free, PRFM. Next, add 90 µl of PRFM (+5% FCS) to each well. Add 10 µl of 100 µM working-concentration staurosporine (or other desired compound) to each well, for 100 µl final volume and 10 µM final concentration. Incubate cells with Staurosporine for 4.5 hours.

Cell Lysate: Grow cells in T-25 Falcon flasks to confluency. *If you want to test another compound in parallel with Staurosporine, grow two T-25 flasks.* Remove media, and then wash cells in flask with PRFM and FCS-free media. Rock flask back and forth and then remove media. Add 4950 µl of PRFM (+5% FCS) and add 50 µl stock Staurosporine for a 10 µM final concentration. Incubate at 37°C for 4.5 hours. At the end of the incubation, remove and save PRFM (+5% FCS). See Note 1 for evaluation of incubation media. Wash cells in flask 3 times with ice-cold PBS. **Cell lysate preparation:** add 1 ml of cold Caspase Cell Lysis Buffer. Allow flask to sit on ice for 10 minutes. Scrape cells and collect cell debris and lysate, transfer to clean Eppendorf tube. Spin for 10 minutes at 10,000 x g at 4 °C. Remove supernatant and transfer to a clean Eppendorf tube. For initial evaluation, prepare three dilutions: 1:1, 1:5 and 1:10. Add 100 µl of each lysate dilution per well in a white-walled 96 well plate. *Reserve 100 µl of each dilution for determination of protein concentration using the BCA II Protein Determination Assay (Cat. # K813).*



Positive Control: Prepare a 1:10 dilution by adding 2 µl of Caspase-3 to 18 µl of Caspase Cell Lysis Buffer. Continue diluting 5-fold by adding 2 µl of positive control to 8 µl diluted of Caspase Cell Lysis Buffer for a 1:100 dilution. Add 4 µl of diluted enzyme to well(s) designated as positive control. Bring volume to 100 µl with Caspase Cell Lysis Buffer.

Controls: Include the following controls in each experiment: Blank (includes Caspase Reagent Reaction Mix (CRRM), Vehicle, and cell culture medium/lysis buffer without cells or cell lysate; Negative control (CRRM and vehicle-treated cells in cell media/lysis buffer); Assay control (CRRM and treated cells in cell media/lysis buffer); and Positive Control (Caspase-3 in Caspase Cell Lysis Buffer and CRRM).

Notes:

- For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.
 - For samples exhibiting significant background, prepare parallel sample well(s) as background controls.
 - If Caspase-3 Activity within the cell lysate is low, you may wish to also evaluate the Caspase-3 Activity within the incubating media. Simply prepare dilutions and evaluate as previously described for lysate.
- AFC Standard Curve:** Dilute the 1 mM AFC working to 50 µM by combining 10 µl + 190 µl dH₂O. Add 0, 2, 4, 6, 8, 10 µl of 50 µM AFC Standard to each well to generate 0, 100, 200, 300, 400, 500 pmol AFC/well. Bring to 100 µl with Caspase Cell Lysis Buffer.
 - Reaction Mix:** Determine the number of wells of sample, AFC Standard Curve and Controls. For each well, prepare the following:

	Reaction Mix	Reaction Mix for Standards
2X Reaction Buffer	94 µl	99 µl
Substrate	5 µl	---
DTT	1 µl	1 µl

Mix well and add 100 µl of the Reaction Mix to each well containing the Samples, Controls, and Positive Control wells. Add 100 µl of the Reaction Mix for Standards to each well containing the Standards. Mix well.

- Measurement:** Measure fluorescence immediately at Ex/Em= 400/505 nm. Record fluorescence in kinetic mode by recording every minute for 1-2 hours at room temperature. Depending on microplate selected, use the appropriate method of measurement (i.e. if using white microplate, opaque bottom: plate reader should measure fluorescence by scanning the top. If using a white microplate, clear bottom, plate reader should measure fluorescence from bottom)

Note: Incubation time depends on the Caspase-3 activity in samples. We recommend measuring the OD in kinetic mode, and choosing two time points (t_1 & t_2) in the linear range to calculate the Caspase-3 activity of the samples. The AFC Standard Curve can be read in Endpoint mode (i.e., at the end of the incubation time).

- Calculation:** Subtract 0 Standard reading from all readings. Plot AFC Standard Curve. If sample background control reading is significant, subtract the background control reading from its paired sample reading. Calculate activity of the test sample: $\Delta RFU = RFU_2 - RFU_1$. Apply the ΔRFU to the AFC Standard Curve to get B pmol of AFC generated during the reaction time ($\Delta t = t_2 - t_1$).

$$\text{Sample Caspase-3 Activity} = \frac{B}{(\Delta t \times V)} \times D = \text{pmol/min/ml} = \text{mU/ml}$$

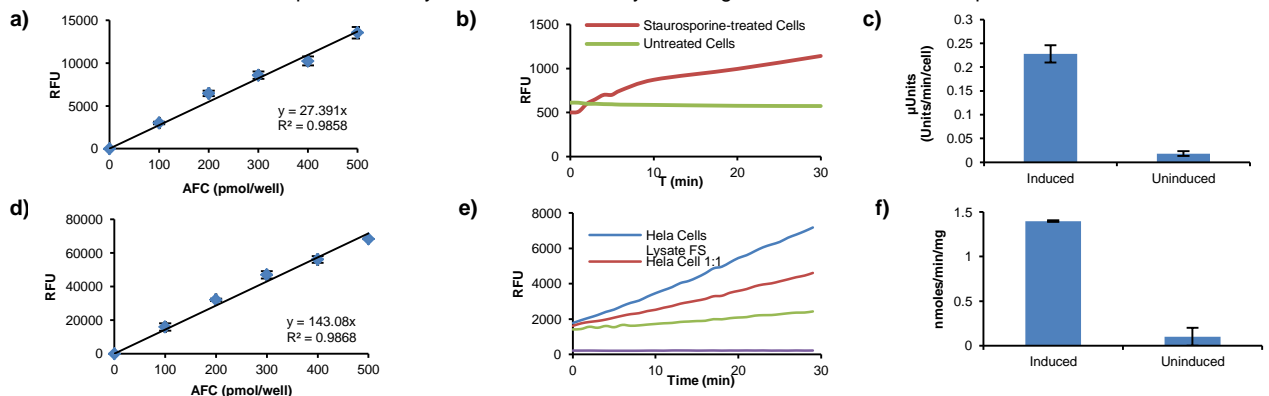
Where: **B** = AFC amount from Standard Curve (pmol).

Δt = reaction time (min.)

V = sample volume added into the reaction well (ml).

D = Dilution Factor

Unit Definition: One Unit of Caspase-3 Activity is the amount of enzyme that generates 1 nmole of AFC per minute at 37°C.



Figures. Experiments using white, clear bottom microplate (A-C). **A.** AFC Standard Curve **B.** Kinetics of Caspase-3 Activity in intact HeLa cells. Apoptosis was induced using Staurosporine ([10 µM]; 4.5 hours) **C.** Specific Activity of Caspase 3 in HeLa cells. Staurosporine-induced: 0.227 ± 0.01 µU/cell; Untreated: 0.018 ± 0.004 µU/cell. **D-F:** Experiments using opaque white microplate **D.** AFC Standard Curve (top read). **E.** Kinetics of Caspase-3 Activity using HeLa lysate. Apoptosis was induced using Staurosporine ([10 µM]; 4.5 hours). **F.** Specific Activity of Caspase-3 in HeLa cells. Staurosporine-treated: 1.39 ± 0.01 U/mg; Untreated $<0.1 \pm 0.10$ U.

VIII. RELATED PRODUCTS:

Caspase-3 Inhibitor Drug Screening Kit (K153)

Caspase-3 Colorimetric Assay Kit (K106)

FOR RESEARCH USE ONLY! Not to be used on humans.