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ab252897 Caspase-3 Activity Assay Kit (Fluorometric)

View Caspase-3 Activity Assay Kit (Fluorometric) datasheet:

http://www.abcam.com/ab252897

[use http://www.abcam.cn/ab252897 for China, or http://www.abcam.co.jp/ab252897 for Japan)

For the quantification of Caspase-3 Enzymatic activity in mammalian cells for studying apoptosis and identifying inhibitors/inducers.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Caspase-3 Activity Assay Kit (Fluorometric) (ab252897) 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.

2. Protocol Summary

Prepare Samples, Standards, Positive Control and Background controls as directed.

Prepare Reaction Mix and Reaction Mix for Standards.

Add Reaction Mix (100 µL) to Samples, Controls and Positive Controls (100 µL each). Add Reaction Mix for Standards (100 µL) to Standards (100 µL).

Measure fluorescence immediately at Ex/Em= 400/505 nm. Record fluorescence in kinetic mode by recording every minute for 1-2 hrs at room temperature. Standards may be read in end-point mode.

3. Materials Supplied and Storage

Store kit at -20°C in the dark immediately on receipt and check below for storage for individual components.

Kit can be stored for 1 year from receipt, if components have not been reconstituted.

Avoid repeated freeze-thaws of reagents.

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Ac-DEVD-AFC (1 mM)	500 µL	-20°C	-20°C
AFC Standard (1 mM)	100 µL	-20°C	-20°C
Caspase 2X Reaction Buffer	20 mL	-20°C	-20°C or 4°C
Caspase Cell Lysis Buffer	100 mL	-20°C	-20°C or 4°C
Caspase-3 (Positive Control)	1 vial	-20°C	-80°C
DTT (1 mM)	1 mL	-20°C	-20°C
Staurosporine (1 mM)	200 µL	-20°C	-20°C

4. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Fluorescent microplate reader capable of measuring Ex/Em = 400/505 nm.
- 96-well plate white microplate with a clear, flat bottom.
- 96-well plate white microplate with an opaque, flat bottom.
- Phenol-red free media.
- DMSO.
- Fetal calf serum.

5. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

6. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

6.1 Ac-DEVD-AFC (1 mM)

Ready to use as supplied. Aliquot and store at -20ºC.

6.2 AFC Standard (1 mM)

Ready to use as supplied. Aliquot and store at -20ºC.

6.3 Caspase 2X Reaction Buffer

Ready to use as supplied. Warm to room temperature before use. Store at either 4ºC or -20ºC.

6.4 Caspase Cell Lysis Buffer

Ready to use as supplied. Warm to room temperature before use. Store at either 4ºC or -20ºC.

6.5 Caspase-3 (Positive Control)

Reconstitute in 15 µL dH_2O. Aliquot 5 µL into microfuge tubes and store at -80ºC.

6.6 DTT (1 mM)

Ready to use as supplied. Aliquot and store at -20ºC.

6.7 Staurosporine (1 mM)

Ready to use as supplied. Aliquot and store at -20ºC.

7. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

7.1 Dilute the 1 mM AFC Standard to 50 µM by combining 10 µL and 190 µL dH_2O.

7.2 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.

7.3 Bring to 100 µL with Caspase Cell Lysis Buffer.

Standard #	50 µM AMC Standard (µL)	Caspase Cell Lysis Buffer (µL)	Final volume standard in well (µL)	End amount AMC in well (pmol/well)
1	0	200	100	0
2	4	196	100	100
3	8	192	100	200
4	12	188	100	300
5	16	184	100	400
6	20	180	100	500

Each dilution has enough amount of standard to set up duplicate readings (2 x 100 µL).

8. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

1 Intact Cells:

8.1.1 Day 1: Seed $1X10^6$ cells/well in a white, clear bottom 96-well plate. Grow overnight to confluency in 100 µL of media plus 10% FCS.

8.1.2 Day 2: Prepare the following reagent: Phenol-red free media w/5% FCS: 47.5 mL PRFM plus 2.5 mL FCS). Also prepare 100 µM Staurosporine (apoptosis-inducer).

8.1.3 Add 25 µL of Staurosporine stock solution to 225 µL of PRFM (plus 5% FCS).

8.1.4 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.

8.1.5 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.

8.1.6 Incubate cells with Staurosporine for 4.5 hours.

8.2 Cell Lysate:

8.2.1 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.

8.2.2 Remove media, and then wash cells in flask with PRFM and FCS-free media. Rock flask back and forth and then remove media.

8.2.3 Add 4950 µL of PRFM (plus 5% FCS) and add 50 µL stock Staurosporine for a 10 µM final concentration. Incubate at 37°C for 4.5 hours.

8.2.4 At the end of the incubation, remove and save PRFM (plus 5% FCS).

8.2.5 Wash cells in flask 3 times with ice-cold PBS.

8.2.6 Cell lysate preparation: add 1 mL of cold Caspase Cell Lysis Buffer. Allow flask to sit on ice for 10 mins.

8.2.7 Scrape cells and collect cell debris and lysate, transfer to clean Eppendorf tube. Spin for 10 mins at 10,000 x *g* at 4°C. Remove supernatant and transfer to a clean Eppendorf tube.

8.2.8 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.

8.2.9 Reserve 100 µL of each dilution for determination of protein concentration using the BCA II Protein Determination Assay.

Δ Note: For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.

Δ Note: For samples exhibiting significant background, prepare parallel sample well(s) as background controls.

Δ Note: 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.

9. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

9.1 Reaction mix preparation:

1. Mix enough reagents for the number of assays to be performed. For each well, prepare a total 100 µL Mix containing:

Component	Reaction Mix (µL)	Reaction Mix for Standards (µL)
Caspase 2X reaction Buffer	94	99
Ac-DEVD-AFC (1 mM)	5	---
DTT (1 mM)	1	1

2. Mix well and add 100 µL of the Reaction Mix to each well containing the Samples, Controls, and Positive Control.
3. Add 100 µL of the Reaction Mix for Standards to each well containing the Standards. Mix well.
4. Caspase-3 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 diluted Positive Control to 8 µL 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.
5. 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).

9.2 Measurement:

1. 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.
2. 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).

Δ Note: Incubation time depends on the Caspase-3 activity in samples. We recommend measuring the fluorescence in kinetic mode, and choosing two time points (t1 & t2) in the linear range to calculate the Caspase-3 activity of the samples.
Δ Note: The AFC Standard Curve can be read in endpoint mode (i.e. at the end of incubation time).

9.3 Calculation:

1. Subtract 0 Standard reading from all readings.
2. Plot the AFC Standard curve.
3. Choose two time points (t1 and t2) within the linear portion of graph.
4. If sample background control reading is significant, subtract the background control reading from its paired sample reading.
5. Calculate activity of the test sample: ΔRFU = RFU2 – RFU1.
6. Apply the ΔRFU to the AFC Standard Curve to get B pmol of AFC generated during the reaction time ($\Delta t = t2 - t1$).

Caspase-3 Activity = B/(Δt x V) x D = pmol/min/mL (mU/mL)

Where:
B = AFC amount in the sample well from Standard Curve (pmol).
Δt = reaction time (mins).
V = sample volume added into the reaction well (mL).
D = sample dilution factor (D = 1, if undiluted).

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

10. Typical Data

Data provided **for demonstration purposes only.**

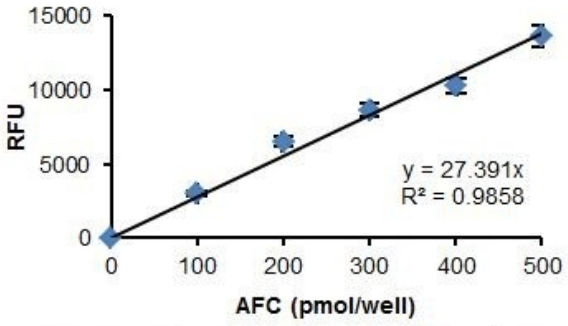

Figure 1. AFC standard curve. White, clear bottom microtitre plate.

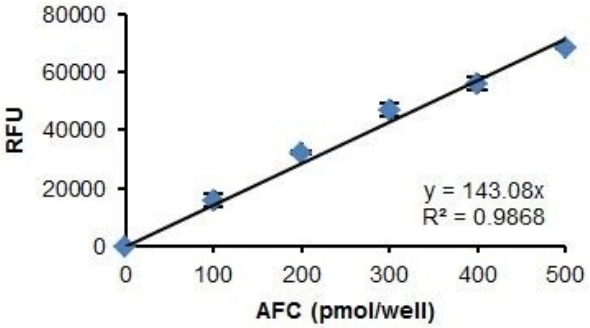

Figure 2. AFC standard curve. White, opaque microtitre plate.

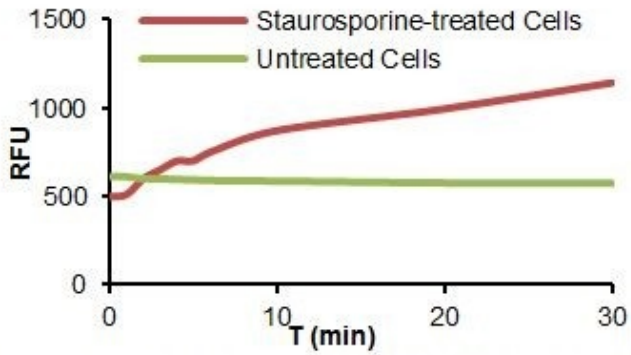

Figure 3. Kinetics of Caspase-3 activity in intact HeLa cells**.** Apoptosis was induced using Staurosporine ([10 µM]; 4.5 hours). White, clear bottom microtiter plate.

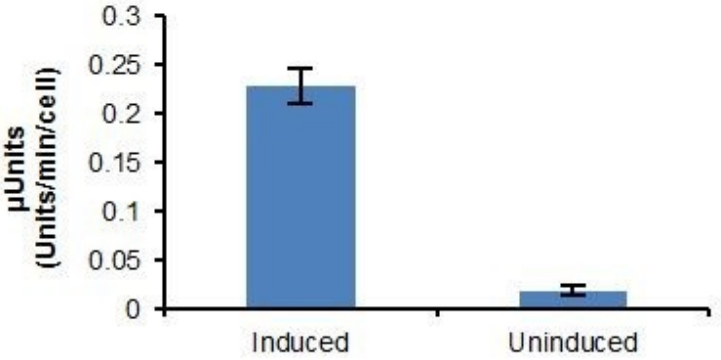

Figure 4. Specific activity of Caspase 3 in HeLa cells. Staurosporine-induced: 0.227 ± 0.01 µU/cell; Untreated: 0.018 ± 0.004 µU/cell. White, clear bottom microtiter plate.

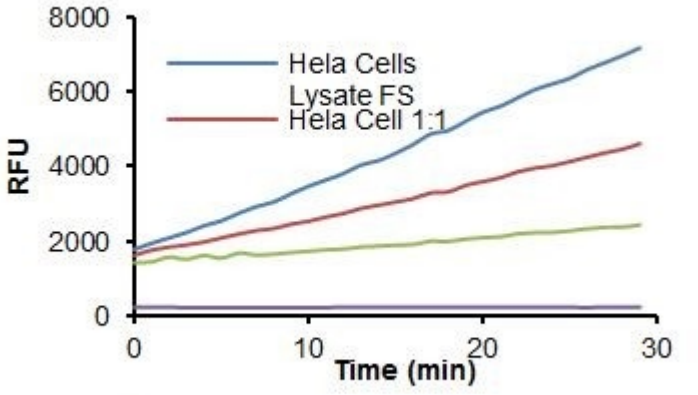

Figure 5. Kinetics of Caspase-3 activity using HeLa lysate. Apoptosis was induced using Staurosporine ([10 µM]; 4.5 hours). White, opaque microtitre plate.

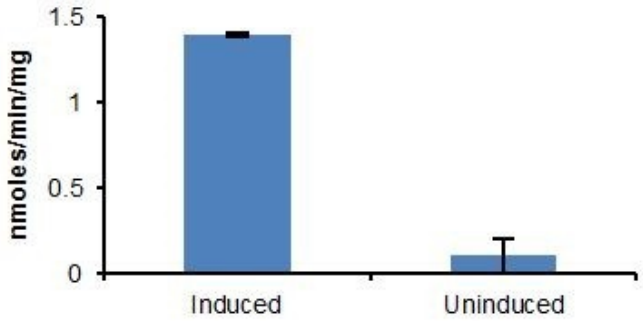

Figure 6. Specific activity of Caspase-3 in HeLa cells. Staurosporine-treated: 1.39 ± 0.01 U/mg; Untreated. White, opaque microtitre plate.

11. Notes

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