TECHNICAL BULLETIN

Apo-ONE® Homogeneous Caspase-3/7 Assay

Instructions for use of Products
G7790, G7791 and G7792

Revised 12/18
TB295

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Apo-ONE® Homogeneous Caspase-3/7 Assay

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1. Description

The Apo-ONE® Homogeneous Caspase-3/7 Assay provides the necessary reagents for fast and sensitive measurement of the activities of caspase-3 and -7 in cell-based and biochemical applications. These members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in mammalian cells (1–4). The Apo-ONE® Homogeneous Caspase-3/7 Assay provides a profluorescent substrate with an optimized bifunctional cell lysis/activity buffer for caspase-3/7 (DEVDase) activity assays. This assay can be flexibly configured for use in high-throughput systems. Figure 1 illustrates the simple “add, mix and read” format of this assay.

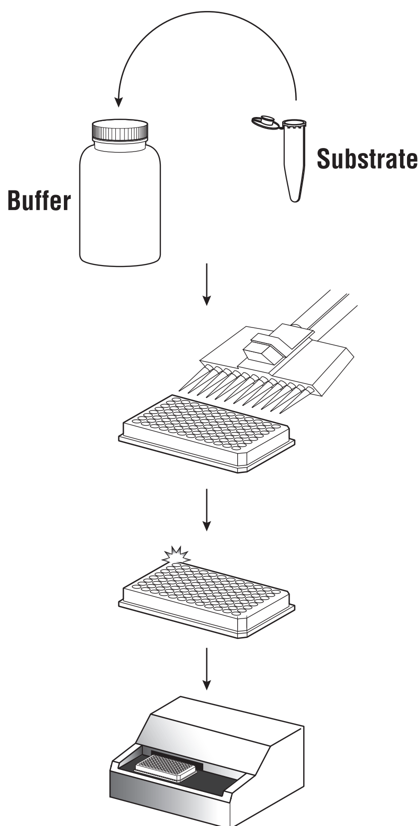

Figure 1. Schematic overview of the Apo-ONE® Homogeneous Caspase-3/7 Assay protocol. This assay is easily adaptable to a 384-well format.

Note: Extended mixing times using a plate shaker are often unnecessary but may be beneficial particularly when using cell-based systems.

Assay Principle

The Apo-ONE® Homogeneous Caspase-3/7 Buffer rapidly and efficiently lyses/permeabilizes cultured mammalian cells and supports optimal caspase-3/7 enzymatic activity. The caspase-3/7 substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110), exists as a profluorescent substrate prior to the assay. To perform the Apo-ONE® Homogeneous Caspase-3/7 Assay, the Buffer and Substrate are mixed and added to the sample. Upon sequential cleavage and removal of the DEVD peptides by caspase-3/7 activity and excitation at 499nm, the rhodamine 110 leaving group becomes intensely fluorescent (Figure 2). The emission maximum is 521nm (5).

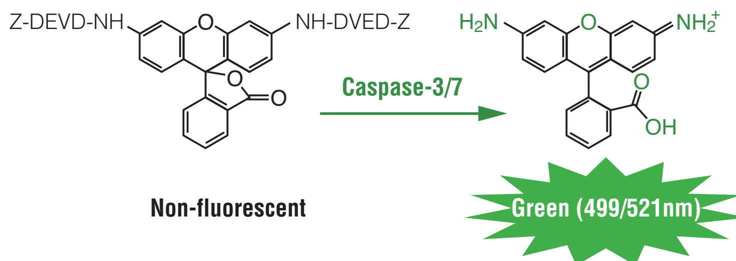

Figure 2. Cleavage of the non-fluorescent Caspase Substrate Z-DEVD-R110 by Caspase-3/7 to create the fluorescent Rhodamine 110.

The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample. The Apo-ONE® Assay using the Z-DEVD-R110 Substrate is more sensitive over short incubation times than assays using the common Ac-DEVD-AMC substrate (Figure 3). The fluorescent product may be quantitated by comparison with standard concentrations of a rhodamine 110 reference standard (Sigma Cat.# 83695). Specific inhibition of caspase-3/7 activity may be achieved by the addition of caspase inhibitor Ac-DEVD-CHO.

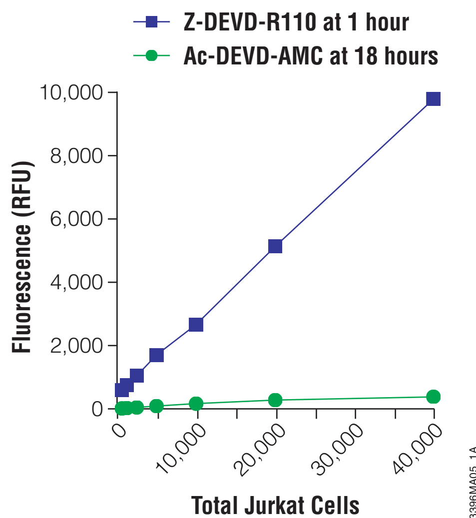

Figure 3. Sensitivity of the Apo-ONE® Homogeneous Caspase-3/7 Assay. Apoptosis was induced in Jurkat cells by treatment with anti-Fas receptor antibody for 5 hours. Cells were serially diluted twofold into a 96-well plate. Apo-ONE® Homogeneous Caspase-3/7 Buffer containing either Z-DEVD-R110 or Ac-DEVD-AMC was added to the cells and incubated at room temperature. Z-DEVD-R110 data were collected on a fluorometer 1 hour post-addition. Ac-DEVD-AMC data were collected 18 hours post-addition. Because of inherent differences in the fluor signals, significantly different incubation times were required for the two substrates.

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1. Description (continued)

The ability to perform multiple assays on the same sample well provides valuable internal control data and saves the time and cost of duplicating cell culture setup. The Apo-ONE® Homogeneous Caspase-3/7 Assay can be multiplexed with the CellTiter-Blue® Cell Viability Assay or the CytoTox-ONE™ (LDH) Homogeneous Membrane Integrity Assay. The CellTiter-Blue® Assay is a fluorescent, homogeneous cell viability assay based on the reducing ability of cells to convert resazurin to resorufin. It does not lyse cells, and the excitation and emission wavelengths used ($560_{Ex}/590_{Em}$) are different than for the Apo-ONE® Assay, so it is possible to do both assays in the same well with only a modest reduction in Apo-ONE® Assay fluorescence signal (Figure 4). The CytoTox-ONE™ (LDH) Membrane Integrity Assay detects the release of lactate dehydrogenase from compromised cells and can be used as an indicator of necrosis. This assay can be performed using an aliquot of culture supernatant transferred to a second plate, while performing the Apo-ONE® Assay using the remaining cells.

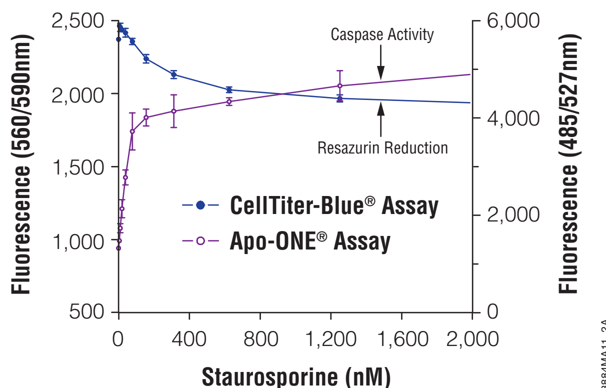

Figure 4. Multiplexing two assays in the same well. Jurkat cells were treated with various concentrations of staurosporine. CellTiter-Blue® Reagent (20µl) was added to each well immediately after drug addition for a final volume of 120µl, and the cells were incubated for 5 hours prior to recording fluorescence ($560_{Ex}/590_{Em}$). Caspase activity was then measured in the same wells by adding 120µl of the Apo-ONE® Caspase-3/7 Assay Reagent. Cells were incubated for an additional hour at ambient temperature prior to recording fluorescence ($485_{Ex}/527_{Em}$).

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Advantages of the Apo-ONE® Homogeneous Caspase-3/7 Assay

Simple: A proprietary bifunctional cell lysis/activity buffer combined with the profluorescent substrate Z-DEVD-R110 results in a sensitive, single-reagent assay that is easily automated (Figure 1).

Fast: Requires shorter incubation and no sample preparation compared to other apoptosis assays; incubate for as little as 30 minutes.

Sensitive: Detect caspase activity from as few as several hundred cells in a single assay (Figure 3).

Robust: The Z´-factor is a statistical value that compares the dynamic range of an assay to data variation in order to assess assay quality. Z´ factor values greater than 0.5 indicate excellent assay quality (6). The Apo-ONE® Homogeneous Caspase-3/7 Assay delivers excellent Z´-factors in cell and purified enzyme models (Figure 5).

Scalable: Simply maintain a 1:1 ratio of assay reagent volume to sample volume to perform from one to thousands of assays.

Flexible: Use with purified caspase enzyme (Figure 8); adherent, suspension or primary cell cultures (Figures 3, 4 and 7); or fresh tissue (7). Perform assays in a cuvette or in 96- or 384-well plates.

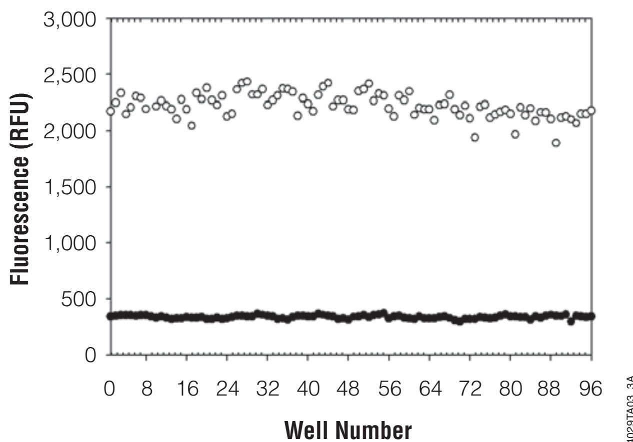


Figure 5. Z´-Factor analysis. HepG2 cells were plated in a 96-well plate at 20,000 cells per well and treated with staurosporine. A Z´-factor of >0.83 was obtained for the Apo-ONE® Assay.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Apo-ONE® Homogeneous Caspase-3/7 Assay	**1ml**	**G7792**

Each system contains sufficient reagents to make 1ml of Apo-ONE® Caspase-3/7 Reagent (10 assays of 100µl/well in a 96-well plate or 40 assays of 25µl/well in a 384-well plate). Includes:

- 10µl Caspase Substrate Z-DEVD-R110 (100X)
- 1ml Apo-ONE® Homogeneous Caspase-3/7 Buffer

PRODUCT	SIZE	CAT.#
Apo-ONE® Homogeneous Caspase-3/7 Assay	**10ml**	**G7790**

Each system contains sufficient reagents to make 10ml of Apo-ONE® Caspase-3/7 Reagent (100 assays of 100µl/well in a 96-well plate or 400 assays of 25µl/well in a 384-well plate). Includes:

- 100µl Caspase Substrate Z-DEVD-R110 (100X)
- 10ml Apo-ONE® Homogeneous Caspase-3/7 Buffer

PRODUCT	SIZE	CAT.#
Apo-ONE® Homogeneous Caspase-3/7 Assay	**100ml**	**G7791**

Each system contains sufficient reagents to make 100ml of Apo-ONE® Caspase-3/7 Reagent (1,000 assays of 100µl/well in a 96-well plate or 4,000 assays of 25µl/well in a 384-well plate). Includes:

- 1ml Caspase Substrate Z-DEVD-R110 (100X)
- 100ml Apo-ONE® Homogeneous Caspase-3/7 Buffer (also available separately, Cat.# G7781)

Storage and Stability: Store the Apo-ONE® Homogeneous Caspase-3/7 Assay, protected from light and moisture, at –20°C. Avoid multiple freeze-thaw cycles. Homogeneous Caspase-3/7 Reagent (Substrate diluted in Buffer) may be stored, protected from light, at 4°C for up to 24 hours.

3. Reagent Preparation

Please read the protocol thoroughly before beginning. Directions are given for performing the assay in a total volume of 200µl using 96-well plates and a fluorescence plate reader. However, the assay can be easily adapted to different volumes providing that the 1:1 ratio of Homogeneous Caspase-3/7 Reagent volume to sample volume is preserved (e.g., 25µl of sample + 25µl Apo-ONE® Caspase-3/7 Reagent). This assay is easily adaptable to a 384-well format.

Materials to be Supplied by the User

- 96- or 384-well opaque white or black plate suitable for cell culture (Nalge Nunc International has FluoroNunc™ Products for such applications)
- fluorescent plate reader (e.g., GloMax® Discover Multimode Microplate Reader, Cat.# GM3000)
- single and multichannel pipettors
- plate shaker
- (optional) caspase inhibitor Ac-DEVD-CHO (MilliporeSigma Cat.# 235420)

Thaw the 100X Substrate and Buffer to room temperature. Mix by inversion or vortexing. Dilute the Substrate 1:100 with the Buffer to obtain the desired volume of Apo-ONE® Caspase-3/7 Reagent (e.g., 100µl of 100X Substrate to 9,900µl Buffer). Store the Apo-ONE® Homogeneous Caspase-3/7 Reagent, protected from light, at 4°C for up to 24 hours until use. Do not freeze and store the Apo-ONE® Caspase-3/7 Reagent. Avoid multiple freeze-thaw cycles of the Substrate and Buffer.

4. Detection of Caspase-3/7 Activity in Cell Culture

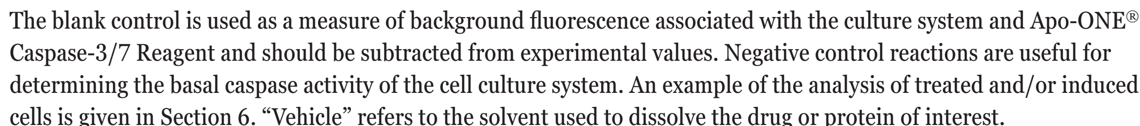
4.A. Assay Conditions

Prepare the following reactions to detect caspase-3/7 activity in cell culture:

Blank: Apo-ONE® Caspase-3/7 Reagent + cell culture medium without cells.

Negative Control: Apo-ONE® Caspase-3/7 Reagent + vehicle-treated cell culture.

Assay: Apo-ONE® Caspase-3/7 Reagent + treated cell culture.



The blank control is used as a measure of background fluorescence associated with the culture system and Apo-ONE® Caspase-3/7 Reagent and should be subtracted from experimental values. Negative control reactions are useful for determining the basal caspase activity of the cell culture system. An example of the analysis of treated and/or induced cells is given in Section 6. "Vehicle" refers to the solvent used to dissolve the drug or protein of interest.

Notes:

1. Prior to starting the assay, prepare the Apo-ONE® Caspase-3/7 Reagent as described in Section 3 and mix thoroughly.
2. For best results, empirical determination of the optimal cell number, apoptosis induction treatment and incubation period for the cell culture system may be necessary.
3. Use identical cell numbers and volumes for the assay and the negative control samples.
4. Do not mix Apo-ONE® Caspase-3/7 Reagent and samples by manual pipetting. Mixing in this manner is unnecessary and may create bubbles that interfere with fluorescence readings or cross-contaminate the samples. Gentle mixing may be performed using a plate shaker.
5. Total incubation time for the assay depends upon the amount of caspase-3/7 present in the sample. Minimal apoptotic induction and low cell number may require an extended incubation period. Maximum recommended incubation time is 18 hours.
6. The Apo-ONE® Caspase-3/7 Reagent was formulated to mediate cellular lysis and support optimal caspase-3/7 activity. In rare instances, the reagent does not affect complete lysis of cultured cells. In such cases, lysis is enhanced by a freeze-thaw cycle. For best results, freeze at –70°C and then thaw at room temperature. After equilibration, mix to homogeneity and incubate until measurable fluorescence is achieved.

4.B. Standard Assay (96-well, 200µl final reaction volume)

1. Add 100µl of Apo-ONE® Caspase-3/7 Reagent to each well of a white or black 96-well plate containing 100µl of blank, control or cells in culture. If reusing tips, be careful not to touch pipette tips to the wells containing samples to avoid cross-contamination. Perform blank and negative controls in triplicate. Cover the plate with a plate sealer if incubating for extended periods (>4 hours).

   Note: To perform this assay in a 384-well plate, simply maintain the 1:1 volume ratio of Apo-ONE® Caspase-3/7 Reagent to sample. Adjust the total reaction volume such that the bottom of the well is covered but liquid does not splash out of the well during the assay.

2. Gently mix contents of wells using a plate shaker at 300–500rpm from 30 seconds up to read time. Incubate at room temperature for 30 minutes to 18 hours depending upon expected level of apoptosis (and thus caspase-3/7 activity) in the cells analyzed. The optimal incubation period should be determined empirically.

3. Measure the fluorescence of each well. The optimal excitation wavelength for detection is 499nm with emission maximum at a wavelength of 521nm (5). This protocol was developed using a spectrofluorometer configured to detect caspase-3/7 activity at an excitation wavelength range of 485 ± 20nm and an emission wavelength range of 530 ± 25nm. Fluorescence measurements should be determined empirically and should be completed within 18 hours.

5. Detection of Caspase-3 or -7 Activity in Purified Caspase Preparations

5.A. Assay Conditions

Prepare the following reactions to detect caspase-3 or -7 activity (or inhibition of activity) in purified enzyme preparations:

Blank: Apo-ONE® Caspase-3/7 Reagent + vehicle control for enzyme treatment agent or inhibitor, if used.

Positive Control: Apo-ONE® Caspase-3/7 Reagent + vehicle control + purified caspase-3 or -7 enzyme.

Assay: Apo-ONE® Caspase-3/7 Reagent + treatment agent + purified caspase-3 or -7 enzyme.

The blank is used as a measure of any background fluorescence associated with the treatment agent vehicle and Apo-ONE® Caspase-3/7 Reagent and should be subtracted from experimental values. The positive control is used as a maximal fluorescence (rate or endpoint relative fluorescent unit, RFU) obtainable with the purified enzyme system. An example of the analysis of purified enzyme activity and inhibitor treatment is given in Section 7. “Vehicle” refers to the solvent used to dissolve the inhibitor or treatment agent used in the study.

Notes:

1. Prepare the Apo-ONE® Caspase-3/7 Reagent as described in Section 3 and mix thoroughly prior to starting the assay.
2. Caspase-specific activities and unit definitions can vary widely depending on the manufacturer. Figure 6 shows a 160-fold difference in activity using enzymes from two different suppliers even though the specific activities of the enzymes are the same based on the units listed on the product labels. Assays using caspase-3 or -7 will need to be optimized depending on the unit definition.
3. Use identical enzyme concentrations for the assay and positive control reactions.
4. Do not mix Apo-ONE® Caspase-3/7 Reagent by manual pipetting upon addition to sample. Mixing in this manner is unnecessary and may create bubbles that interfere with fluorescence readings or cross-contaminate the samples. Gentle mixing may be done using a plate shaker.
5. Total incubation time for the assay will be dependent upon the amount of purified active caspase-3 or -7 present in the sample. Exceedingly low enzyme concentrations may require an extended incubation period.

5.B. Standard Assay (96-well, 200µl final reaction volume)

1. Add 100µl of Apo-ONE® Homogeneous Caspase-3/7 Reagent to each well of a white or black 96-well plate containing 100µl of blank, control or assay treatment. If reusing tips, be careful not to touch pipette tips to the wells containing samples to avoid cross-contamination. Perform blank and positive controls in triplicate. If conducting a kinetic determination, immediately proceed to Step 3. If not, shield the plate from ambient light. Cover the plate with a plate sealer if incubating for extended periods (>4 hours).

   Note: To perform this assay in a 384-well plate, simply maintain the 1:1 volume ratio of Apo-ONE® Caspase-3/7 Reagent to sample. Adjust the total reaction volume such that the bottom of each well is covered but liquid does not splash from the wells during the assay.

2. Gently mix contents of wells using a plate shaker at 300–500rpm from 30 seconds up to read time. Incubate at room temperature for 30 minutes to 18 hours depending upon the level of the caspase-3 or -7 activity in the wells analyzed. The optimal incubation period should be determined empirically.
3. Measure the fluorescence of each well. The optimal excitation wavelength for detection is 499nm with emission maximum at a wavelength of 521nm (5). This protocol was developed using a spectrofluorometer configured to detect caspase-3/7 activity at an excitation wavelength range of 485 ± 20nm and an emission wavelength range of 530 ± 25nm. Kinetic readings will require several measurements/well and will be limited by spectrofluorometer instrumentation. Typically, total time frames for kinetic analysis range from 1 minute to 1 hour.

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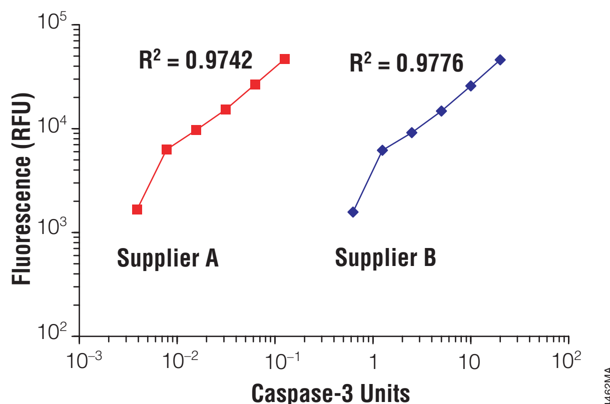

Figure 6. Comparison of caspase-3 enzymes from two different suppliers. The enzymes were compared based on the units listed on the labels. Both enzymes were serially diluted using phosphate-buffered saline containing 0.1% BSA in a single 96-well plate. Apo-ONE® Homogeneous Caspase-3/7 Reagent (100µl) was added to 100µl of diluted enzyme and incubated as described in Section 5.B for 10 minutes and the fluorescence was measured. The data were plotted using a $\log_{10}$ scale due to the broad range of units used.

6. Positive and Negative Cell Culture Controls

Apoptosis can be induced in experimental systems by a variety of methods that lead to caspase activation. These include:

- Treatment of FAS or TNF receptor-bearing cells by cross-linking with agonistic antibodies (8,9).
- Treatment of cells with DNA topoisomerase inhibitors, e.g., etoposide (10), with the protein kinase inhibitor staurosporine (11) or with microtubule damaging agents such as paclitaxel (12).
- Exposure of cells to genotoxic damage induced by ionizing radiation (13,14).

Example: Analysis of Caspase Activity in Jurkat Cells Treated with Anti-Fas Antibody (Figure 7)

Materials to Be Supplied by the User

- anti-Fas receptor antibody (MBL International, Cat.# SY001)
- human Jurkat T-cells
- FBS
- RPMI 1640
- penicillin and streptomycin
- CO_2 incubator

1. Grow Jurkat cells to a density of 5×10^5 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum, 2mM glutamine and 1% penicillin-streptomycin in a humidified, 5% CO_2 incubator at 37°C.
2. Dilute cells in culture medium to the desired density. We recommend starting with 2×10^5 cells/ml in a volume of 50µl. Dispense the cells into the wells of a 96-well plate. Leave at least 3 wells empty for blanks.
3. Add anti-Fas mAb diluted in 50µl of RPMI 1640 to assay wells. Make sure that the final concentration of the mAb in each well is 100ng/ml. For untreated samples (negative control), add 50µl of RPMI only.
4. Incubate for 5 hours at 37°C in a humidified, 5% CO_2 atmosphere.
5. Measure caspase-3/7 activity of anti-Fas antibody-treated (induced, positive control) and uninduced cells (negative control) as described in Section 4.B (standard assay).

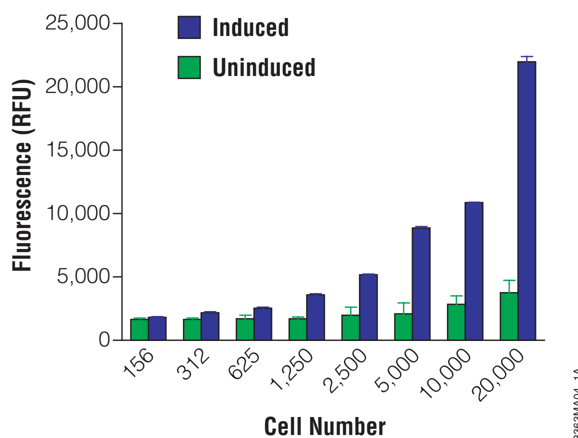

Figure 7. Measurement of caspase-3/7 activity in anti-Fas antibody-treated human Jurkat T-cells. Twofold serial dilutions of Jurkat T-cells were treated with 100ng/ml of anti-Fas antibody in RPMI 1640 or RPMI 1640 only (control) for 5 hours at 37°C. Individual wells were assayed for caspase-3/7 activity according to the conditions described in Section 4. Measurements were taken using a CytoFluor® II. Typically background measurements (no cells) are approximately 400RFU.

7. Purified Enzyme Analysis

Example: Determination of IC_{50} for Caspase-3 with Ac-DEVD-CHO (Figure 8)

Materials to Be Supplied by the User

- caspase inhibitor Ac-DEVD-CHO (MilliporeSigma, Cat.# 235420)
- RPMI 1640
- recombinant, active caspase-3 (MilliporeSigma, Cat.# CC119)
- PBS buffer
- 0.1% BSA

Serial dilutions of the caspase inhibitor Ac-DEVD-CHO are mixed with caspase-3, allowed to reach equilibrium and then assayed for remaining activity.

1. Serially dilute Ac-DEVD-CHO from 200nM to 0.194nM in PBS buffer (pH 7.5) with 0.1% BSA. Add 50µl per well to a black or white multiwell plate for each experimental well. Add PBS buffer (pH 7.5), 0.1% BSA without Ac-DEVD-CHO to a separate well for a maximal enzyme rate control.
2. Add 50µl of active, recombinant human caspase-3 at 2.0u/ml in PBS buffer (pH 7.5), 0.1% BSA to each well.
3. Incubate the plate on a plate shaker for 1 hour with shaking (300–500rpm).
4. Add 100µl of Apo-ONE® Caspase-3/7 Reagent to each well.
5. Measure remaining caspase activity rates at each dilution for kinetic measurements as described in Section 5.B, Step 3 (standard assay).

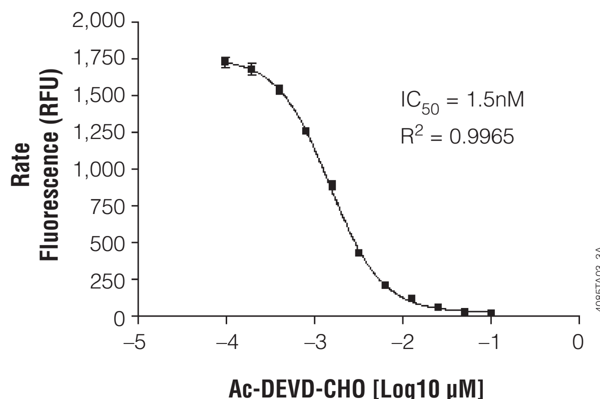

Figure 8. IC_{50} determination for Ac-DEVD-CHO. The experiment was performed as described in Section 7 except that RPMI was used in place of PBS buffer with 0.1% BSA. Serial dilutions of the caspase inhibitor Ac-DEVD-CHO were mixed with caspase-3 and allowed to reach equilibrium. Apo-ONE® Homogeneous Caspase-3/7 Reagent was added to the system, and activity rates were measured. To generate this plot, we performed nonlinear regression analysis on the data using GraphPad Prism® software.

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8. Calculation of Caspase-3/7 Activity

Caspase-3/7 activity is indicated by net fluorescence:

assay RFU – blank RFU

or

assay RFU – negative control RFU

9. References

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10. Related Products

Product	Size	Cat.#
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011
Caspase-Glo® 3/7 Assay	10ml	G8091
Caspase-Glo® 8 Assay	10ml	G8201
Caspase-Glo® 9 Assay	10ml	G8211
Caspase Inhibitor Z-VAD-FMK	50µl	G7231
Anti-PARP p85 Fragment pAb	50µl	G7341
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl	G7461
CaspACE™ Assay System, Colorimetric	100 assays	G7220
DeadEnd™ Colorimetric TUNEL System	40 reactions	G7130
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
rhTNF-α	10µg	G5241
Terminal Deoxynucleotidyl Transferase, Recombinant (TdT)	300u	M1871
CellTiter-Glo® Luminescent Cell Viability Assay	10 × 10ml	G7571
CellTiter-Glo® 2.0 Assay	10ml	G9241
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
CellTiter-Blue® Cell Viability Assay	100ml	G8081
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	1,000–4,000 assays	G7891
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP	1,000–4,000 assays	G7892
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
CellTox™ Green Cytotoxicity Assay	10ml	G8741
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
RealTime-Glo™ MT Cell Viability Assay	100 reactions	G9711
GloMax® Discover Multimode Microplate Reader	1 each	GM3000

11. Summary of Changes

The following changes were made to the 12/18 revision of this document:

1. Updated document format.
2. New products added to Related Products.
3. Discontinued products were removed.

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