Product Manual

Caspase-3 colorimetric detection kit

Catalog # **ADI-907-013**

96 Determination Kit

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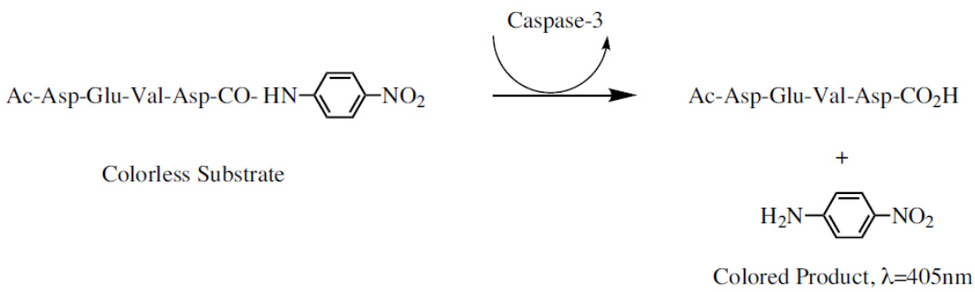
All components, **except the standard**, are stable at 4°C. Store standard at -20°C.

See our website for more product information.

DESCRIPTION

The Caspase-3 colorimetric detection kit is a complete kit for the quantitative determination of Caspase-3 in buffer and cell lysate samples. Please read the complete kit insert before performing this assay. The kit involves the conversion of a specific chromogenic substrate for Caspase-3 followed by the colorimetric detection of the product at 405nm. The conversion of substrate into the colored product can be measured kinetically or at an end point. The absolute value for Caspase-3 activity can be determined by comparison to the signal given by the p-nitroaniline calibrator. This kit allows for the determination of Caspase-3 activity in a variety of samples and species.

Caspase Reaction Scheme



INTRODUCTION

Caspase-3 is also known as Apopain, CPP-32 and Yama. It is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the sequence of events associated with apoptosis. Caspase-3 cleaves a variety of cellular molecules that contain the amino acid theme DEVD such as poly ADP-ribose polymerase (PARP), the 70 kD protein of the U1-ribonucleoprotein and a subunit of the DNA dependent protein kinase[1,2].

Apoptosis was originally reported in 1972 and was described as a mechanism of controlled or programmed cell death[3]. This process is very common in bone marrow and organs with high proliferative activity. It has also been implicated in the progression of a number of diseases, including AIDS, cancer and autoimmune pathologies and has been extensively studied by cellular biologists in fas-mediated cell death[4,5,6].

Apoptosis is characterized by a variety of changes including loss of cellular membrane phospholipid symmetry, chromatin condensation, mitochondrial swelling and eventually leads to damage and fragmentation of DNA. This process results in cell death distinctly different from necrosis. As a result, apoptotic cells avoid the inflammatory response normally associated with necrosis[7].

MATERIALS SUPPLIED

1. **Half-Area Microtiter Plate, 1 Each, Catalog No. 80-2404**
   The plate is ready to use.
2. **Caspase-3 Enzyme Standard, 2 Vials, Catalog No. 80-0930**
   2 vials of lyophilized Caspase-3.
3. **DTT, 2 Vials, Catalog No. 80-0912**
   2 vials of lyophilized dithiothreitol.

Protect substrate from direct light

4. **p-nitroaniline Calibrator, 0.60ml, Catalog No. 80-0934**
   A solution of p-nitroaniline at 50µM in Caspase Reaction Buffer. The optical density of 125µlof this solution in a half-area plate well is equivalent to the optical density produced by 34.72 Units of fully active Caspase-3 when it reacts with the Caspase-3 Substrate for 3 hours at 37°C.

The Substrate Concentrate contains DMSO. Care should be taken in its use.

5. **Caspase-3 Reaction Buffer Concentrate, 10ml, Catalog No. 80-0911**
   A HEPES based buffer containing detergent and preservatives.
6. **Caspase-3 Substrate Concentrate, 0.15ml, Catalog No. 80-0914**
   A solution of Caspase-3 substrate in DMSO.

The Stop Solution contains dilute hydrochloric acid. Care should be used in handling this reagent.

7. **Stop Solution, 11ml, Catalog No. 80-0377**
   A 1N solution of hydrochloric acid. **CAUTION: Acid, wear suitable protective clothing.**
8. **Plate Sealer, 2 Each, Catalog No. 30-0012**
9. **Caspase-3 Assay Layout Sheet, 1 Each, Catalog No. 30-0161**

STORAGE

All components of this kit, **except the standard**, are stable at 4°C until the kit's expiration date. The standard **must** be stored at -20°C.

OTHER MATERIALS NEEDED

1. Deionized water.
2. Precision pipets for volumes between 25µl and 1,000µl.
3. Repeater pipets for dispensing 25µl and 50µl.
4. Beakers and cylinders for diluting buffers.
5. Ice bath or refrigerated container capable of maintaining 0°C.
6. A 37°C Incubator.
7. Microplate reader capable of reading at 405nm, preferably with correction between 570 to 590nm.
8. Graph paper for plotting the standard curve.

SAMPLE HANDLING

Do not mix components from different lots of kits.

Dispose of the contents of the plate with care.

The Caspase-3 colorimetric detection kit is compatible with Caspase-3 in samples in buffer and cell lysate samples. Samples diluted into Active Caspase-3 Reaction Buffer can be read directly from the standard curve. Typically samples will be from cell lysates in a buffer very similar to the Caspase Reaction Buffer, such as a Tris or HEPES buffer containing DTT and detergents. For a suitable buffer please refer to reference 2 on page 11 of this insert. To test for full Caspase activity inhibition, studies should be performed by comparison of the samples with and without Caspase-3 inhibitor, such as the molecule Acetyl-DEVD-CHO available from a variety of sources.

When using cell lysate samples, addition of stop solution should be avoided since some lysis buffers will precipitate.

JURKAT CELL STIMULATION EXPERIMENT

Varied numbers of Jurkat cells were incubated for four hours in the presence or absence of 6µM Camptothecin to induce apoptosis. At the end of the treatment period the cells were collected and washed. Cell pellets were resuspended in 200µl RIPA buffer (10mM NaH_2PO_4, 150mM NaCl, 2mM EDTA, 1% NP-40, 0.1% SDS, 1% Sodium Deoxycholate, 50mM NaF, 2mM Sodium Orthovanadate and Protease Inhibitor Cocktail) and incubated for 3 minutes on ice. The crude lysates were used in the Caspase assay without further modification. The resulting data was kindly provided courtesy of Dr. Jill E. Kolodsick, University of Michigan.

PROCEDURAL NOTES

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents, **except Caspase-3 Enzyme Standard** to warm to room temperature for at least 30 minutes before opening. **See Reagent Preparation step 3 on page 6.**
3. All dilutions should be made in glass tubes.
4. Use deionized water or Caspase Reaction Buffer for dilutions.
5. Pre-rinse the pipet tip, use fresh pipet tips for each sample, standard and reagent.
6. Add the reagent to the inside wall of each well to avoid contamination to other wells.
7. The assay will take approximately one hour to set up once the components and samples have warmed to room temperature.

REAGENT PREPARATION

1. **Caspase-3 Reaction Buffer, 1x**

   Prepare the Reaction Buffer 1x by diluting 10ml of the supplied concentrate with 90ml of deionized water. This can be stored at room temperature until the expiration date, or for 3 months, whichever is earlier.

2. **Active Caspase-3 Reaction Buffer**

   Prepare fresh Active Caspase-3 Reaction Buffer for each assay. Measure out 20ml of diluted Caspase-3 Buffer. Add 1ml of this Buffer to one DTT vial. Vortex and transfer the entire contents of the vial to the remaining 19ml of Caspase-3 Reaction Buffer. Rinse the same DTT vial by adding 1ml of this Buffer to the vial, vortex and return the contents to the now Active Caspase-3 Reaction Buffer.

3. **Caspase-3 Colorimetric Substrate, 1x**

   Prepare fresh substrate for each assay. Count the total number of wells that will receive substrate. Use the following formula to calculate the volume of Caspase-3 Substrate Concentrate and Active Caspase-3 Reaction Buffer to use for the complete 1x substrate.

   A. (Number of wells + 1) x 0.075ml / well = volume of Active Reaction Buffer needed. Increase the calculated volume to the next whole milliliter.

   B. (Volume from part A) x 10µl/ml = volume of Substrate Concentrate needed.

      Pipet the volume of Active Reaction Buffer from part A into a tube. From this volume remove the volume calculated in part B. Add the calculated Substrate Concentrate to the Active Reaction Buffer. Vortex thoroughly and use.

      For example, to run three strips of wells the amount of Active Reaction Buffer needed would be 1.875ml. Rounding this volume up to 2ml, you would remove 20µl from this volume then add 20µl of the Substrate Concentrate to the buffer.

4. **Caspase-3 Enzyme Standard**

   NOTE: Keep standards on ice during use.

   The vial containing the Caspase-3 Enzyme Standard is standard#1. Label four 12 x 75mm glass tubes #2 through #5. Pipet 125µl of Active Caspase-3 Reaction Buffer into tubes #2 through #5. Reconstitute the Caspase-3 Standard with 250µl of Active Caspase-3 Reaction Buffer and vortex.

   Pipet 125µl of the reconstituted Caspase Standard into tube #2 and vortex. Pipet 125µl from tube #2 into tube #3 and vortex. Continue this for tubes #4 and #5. Use within 1 hour of preparation, keeping in an ice bath.

   See Caspase-3 Assay Layout Sheet for dilution details.

   The concentration of Caspase-3 Enzyme in Standards #1 through #5 is determined by the amount of released p-nitroaniline (pNA). The activity of the Caspase-3 Standards is obtained by correction with the pNA Calibrator Optical Density.

ASSAY PROCEDURE

1. Determine the number of wells to be used. Cover unused wells tightly with a plate sealer.

   DO NOT RE-USE WELLS!

2. All standards and samples should be run in duplicate.
3. Pipet 50µl of Active Caspase-3 Reaction Buffer from step 2 on page 5 into duplicate Blank wells.
4. Pipet 125µl of pNA Calibrator into duplicate wells.
5. Pipet 50µl of Standards #1 through #5 into duplicate wells
6. Pipet 50µl of Samples into duplicate wells.
7. Pipet 75µl of Caspase Substrate into each well, except the pNA Calibrator wells.
8. Mix by gently tapping the side of the plate.
9. For Kinetic measurements place the plate into a plate reader capable of reading each well kinetically at 37°C.
10. For Stopped reactions add a plate sealer and incubate for 3 hours at 37°C.
9. For Stopped reactions, pipet 10µl of Stop Solution into each well, including the pNA Calibrator wells. Read within 1 hour.
10. Blank the plate reader against the blank wells, read the optical density at 405nm, preferably with correction at 570 and 590nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

CALCULATION OF RESULTS

Calculate the concentration of Active Caspase-3 in the samples.

1. Calculate the average net Optical Density (OD) for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank

2. Activity measurements can be quantitated by comparison of the optical densities obtained with standards with the p-nitroaniline calibrator. The optical density of this calibrator is equivalent to the optical density obtained from 34.72 units of fully active Caspase-3 when reacting with the Caspase-3 Substrate provided at 37˚C for 180 minutes.

   $$\text{Conversion Factor (OD/Unit)} = \frac{\text{Average Net OD of pNA}}{34.72 \text{ Units}}$$

   $$\text{Activity (Unit/mL)} = \frac{\text{Average Net OD of Standard}}{\text{Conversion Factor}} \div 0.05\text{ml}$$

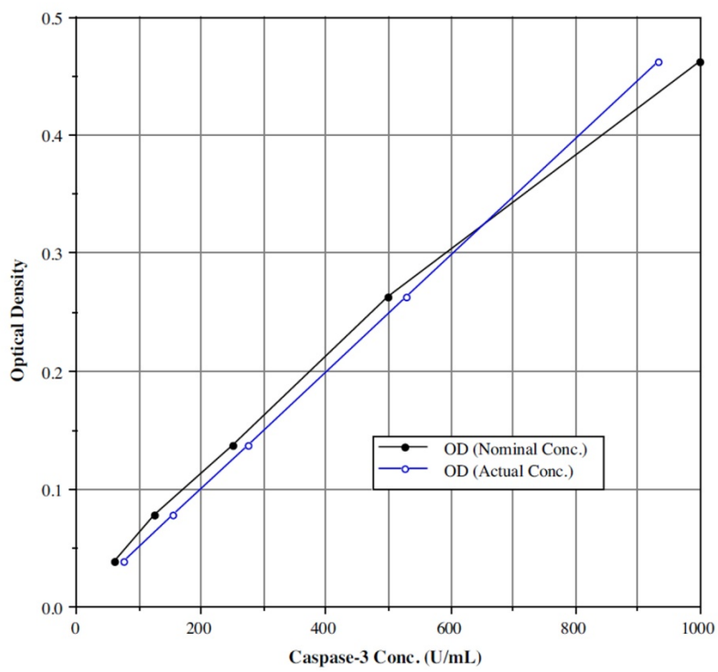
3. Using linear graph paper or graphing software, plot the Average Net OD for each standard versus Actual Concentration of Active Caspase-3 for the standards. Approximate a straight line through the points. The concentration of Active Caspase-3 in the samples can be determined by interpolation.

TYPICAL RESULTS

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Average OD	Net OD	Nominal Conc. (Units /ml)	Actual Conc. (Units /ml)
Blank	(0.054)		**0**	
S1	0.516	0.462	**1,000**	**933**
S2	0.316	0.262	**500**	**529**
S3	0.191	0.136	**250**	**275**
S4	0.131	0.077	**125**	**156**
S5	0.092	0.038	**62.5**	**76**
pNA Calibrator	0.398	0.344	---	---

STANDARD CURVE



Units of Measure

One unit of Caspase-3 activity is defined as the amount of enzyme needed to convert one picomole of substrate per minute at 37°C.

PERFORMANCE CHARACTERISTICS

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[8].

Sensitivity

Sensitivity was calculated by determining the average optical density for sixteen (16) wells run as Blank, and comparing to the average optical density for sixteen (16) wells run with Standard #5. The detection limit was determined as the concentration of Caspase-3 measured at two (2) standard deviations from the Blank along the standard curve.

Average Optical Density for the Blank = 0.052 ± 0.005 (9.5%)

Average Optical Density for Standard #5 = 0.090 ± 0.007 (7.4%)

Delta Optical Density (62.5-0 Units/ml) = 0.038

2 SD's of the Blank = 2 x 0.005 = 0.010

Sensitivity = $\frac{0.010}{0.038}$ x 62.5 Units/ml = **16.4 Units/ml**

Linearity

A sample containing 773 U/mL Caspase-3 was diluted serially 1:2 four times with Active Caspase-3 Reaction Buffer and measured in the assay. The data was plotted graphically as actual Caspase-3 concentration versus measured Caspase-3 concentration.

The line obtained had a slope of 0.965 with a correlation coefficient of 0.992

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Caspase-3 and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring two samples with low and high concentrations of Caspase-3 in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Caspase-3 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Caspase-3 (U/ml)	Intra-assay %CV	Inter-assay %CV
Low	304.0	4.7	
Medium	686.4	3.6	
High	902.9	2.3	
Low	612.1		8.5
High	852.3		3.7

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