



Correlate - Assay

Caspase-6 Fluorometric Assay Kit

Catalog No. 907-021

96 Determination Kit

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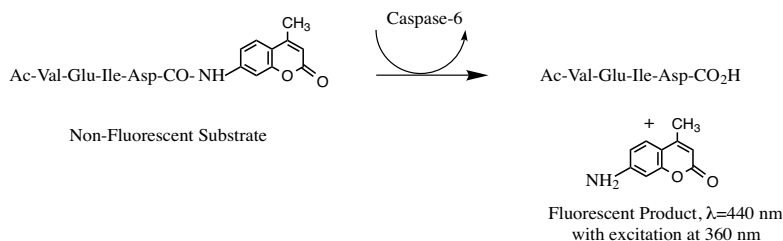
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Description

Assay Designs' Caspase-6 Assay Kit is a complete kit for the quantitative determination of Caspase-6 in buffer and cell lysate samples. Please read the complete kit insert before performing this assay. The kit involves the conversion of a specific fluorogenic substrate for Caspase-6 followed by the fluorometric detection of product of the reaction that emits light at 440 nm with excitation at 360 nm. The conversion of substrate into the product can be measured kinetically or, by use of the stop solution, multiple samples can be read rapidly. The absolute value for Caspase-6 activity can be determined by the conversion of the substrate into the fluorescent product in a system as measured by comparison to the signal given by the AMC product. This kit allows for the determination of Caspase-6 activity in a variety of samples and species.

Caspase Reaction Scheme



Introduction

Caspase-6 is also known as Mch2. It is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the sequence of events associated with apoptosis. Caspase-6 cleaves a variety of cellular molecules that contain the amino acid theme VEID^{1,2}.

Apoptosis was originally reported in 1972 and was described as a mechanism of controlled or programmed cell death³. 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 necrosis⁷.

Precautions

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1. The Substrate Concentrate contains DMSO. Care should be taken in its use.
2. The Stop Solution contains dilute hydrochloric acid. Care should be used in handling this reagent.
3. AMC is a possible toxin/carcinogen. Care should be taken in its use.
4. Do not mix components from different lots of kits.
5. The substrate is light-sensitive and should be protected from direct light and desiccated.
6. Dispose of the contents of the plate with care.

Materials Supplied

1. **White Microtiter Plate, 1 each, Catalog No. 80-0418**
The plate is ready to use.
2. **Desiccated Caspase-6 Enzyme Standard, 2 vials, Catalog No. 80-1034**
2 vials of lyophilized Caspase-6.
3. **Desiccated DTT, 2 Vials, Catalog No. 80-0913**
2 vials of lyophilized dithiothreitol.
4. **AMC Calibrator, 1 mL, Catalog No. 80-0974**
A solution of 7-Amino-4-methyl coumarin at 5 μ M in Caspase Reaction Buffer. The fluorescence of 225 μ L of this solution in a white plate well is equivalent to the FLU's produced by 6.25 Units of fully active Caspase-6 when it reacts with the Caspase-6 Substrate for 3 hours at 30 °C.
5. **Caspase-6 Reaction Buffer Concentrate, 10 mL, Catalog No. 80-1030**
A HEPES based buffer containing detergent and preservatives.
6. **Caspase-6 Fluorometric Substrate Concentrate, 1 vial, Catalog No. 80-1032**
A solution of Caspase-6 Substrate in DMSO.
7. **Stop Solution 2, 11 mL, 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-6 Assay Layout Sheet, 1 each, Catalog No. 30-0184**

Storage

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

Materials Needed but Not Supplied

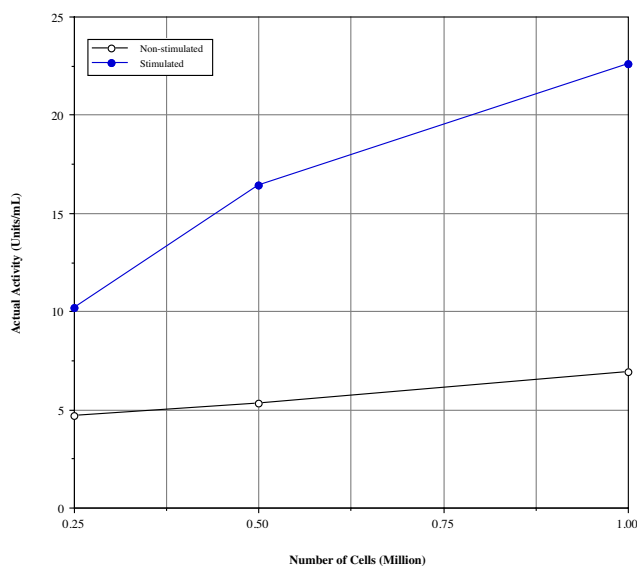
1. Deionized water.
2. Precision pipets for volumes between 25 μL and 1,000 μL .
3. Repeater pipets for dispensing 25 μL and 50 μL .
4. Beakers and cylinders for diluting buffers.
5. Ice bath or refrigerated container capable of maintaining 0 $^{\circ}\text{C}$.
6. A 37 $^{\circ}\text{C}$ Incubator.
7. Fluorescent Microplate reader capable of reading samples at 440 nm, with excitation at 360 nm.
8. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' Caspase-6 assay is compatible with Caspase-6 samples in buffer and cell lysate samples. Samples diluted into Active Caspase-6 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-6 inhibitor, such as the molecule Acetyl-VEID-CHO available from a variety of sources.

Jurkat Cell Stimulation Experiment

Varied numbers of Jurkat cells were incubated for four hours in the presence or absence of 6 μM Camptothecin to induce apoptosis. At the end of the treatment period the cells were collected and washed. Cell pellets were resuspended in 200 μL RIPA buffer (10 mM NaH_2PO_4 , 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 1% Sodium Deoxycholate, 50 mM NaF, 2 mM 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-6 Enzyme Standard** to warm to room temperature for at least 30 minutes before opening. **See Reagent Preparation step 4 on page 5.**
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-6 Reaction Buffer, 1x**
Prepare the Reaction Buffer, 1x by diluting 10 mL of the supplied concentrate with 90 mL of deionized water. This can be stored at room temperature until the expiration date, or for 3 months, whichever is earlier.
2. **Active Caspase-6 Reaction Buffer**
Prepare fresh Caspase-6 Active Reaction Buffer for each assay. Measure out 20 mL of diluted Caspase-6 Reaction Buffer. Add 1 mL of this Buffer to one DTT vial. Vortex and transfer the entire contents of the vial to the remaining 19 mL of Caspase-6 Reaction Buffer. Rinse the same DTT vial by adding 1 mL of this Buffer to the vial, vortex and return the contents to the now Active Caspase-6 Reaction Buffer.
3. **Caspase-6 Fluorometric 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-6 Substrate Concentrate and Active Caspase-6 Reaction Buffer to use for the complete 1x substrate.

A. $(\text{Number of wells} + 1) \times 0.075 \text{ mL} / \text{well} = \text{volume of Active Reaction Buffer needed.}$
Increase the calculated volume to the next whole milliliter.

B. $(\text{Volume from part A}) \times 2 \text{ } \mu\text{L} / \text{mL} = \text{volume of Substrate Concentrate needed.}$

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 3.75 mL. Rounding this volume up to 4 mL, you would remove 18 μL from this volume then add 18 μL of the Substrate Concentrate to the buffer.

4. Caspase-6 Enzyme Standard

NOTE: Keep standards on ice during use.

Reconstitute one vial of Caspase-6 Enzyme Standard with 500 μL of Active Caspase-6 Reaction Buffer and vortex for a concentration of 100 Units/mL. Label five 12 x 75 mm glass tubes #1 through #5. Pipet 250 μL of Active Caspase-6 Reaction Buffer into tubes #1 through #5. Add 250 μL of the reconstituted Caspase-6 Enzyme Standard Stock to tube #1. Vortex thoroughly. Add 250 μL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5. Use within 1 hour of preparation, keeping in an ice bath.

See Caspase-6 Assay Layout Sheet for dilution details.

The concentration of Caspase-6 Enzyme in Standards #1 through #5 is determined by the amount of released AMC. The activity of the Caspase-6 Standards is obtained by correction with the AMC Calibrator.

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 75 μL of Active Caspase-6 Reaction Buffer from step 2 on page 5 into duplicate Blank wells.
4. Pipet 225 μL of AMC Calibrator into duplicate wells.
5. Pipet 75 μL of Standards #1 through #5 into duplicate wells
6. Pipet 75 μL of Samples into duplicate wells.
7. Pipet 150 μL of Caspase Substrate into each well, **except the AMC 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 $^{\circ}\text{C}$.
For **Stopped** reactions add a plate sealer and incubate for 3 hours at 37 $^{\circ}\text{C}$.
10. For **Stopped** reactions, pipet 25 μL of Stop Solution 2 into each well, **including the AMC Calibrator wells.** Read within 1 hour.
11. Blank the plate reader against the blank wells, read the relative fluorescent intensity at 440 nm, with excitation at 360 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean FLU's of the blank wells from all readings.

Calculation of Results

Calculate the concentration of Active Caspase-6 in the samples.

1. Calculate the average net Fluorometric Reading (FLU) for each standard and sample by subtracting the average Blank FLU from the average FLU for each standard and sample.

$$\text{Average Net FLU} = \text{Average FLU} - \text{Average Blank}$$

2. Activity measurements can be quantitated by comparison of the FLU obtained with standards and the AMC calibrator. The FLU of this calibrator is equivalent to the FLU obtained from 6.25 Units of fully active Caspase-6 when reacting with the Caspase-6 Substrate provided at 30 °C for 180 minutes.

$$\text{Conversion Factor (FLU/Unit)} = \frac{\text{Average Net FLU of AMC}}{6.25 \text{ Units}}$$

$$\text{Activity (Unit/mL)} = \frac{\text{Average Net FLU of Standard}}{\text{Conversion Factor}} \div 0.075 \text{ mL}$$

3. Using linear graph paper or graphing software, plot the Average Net FLU for each standard versus Actual Concentration of Active Caspase-6 for the standards. Approximate a straight line through the points. The concentration of Active Caspase-6 in the samples can be determined by interpolation.

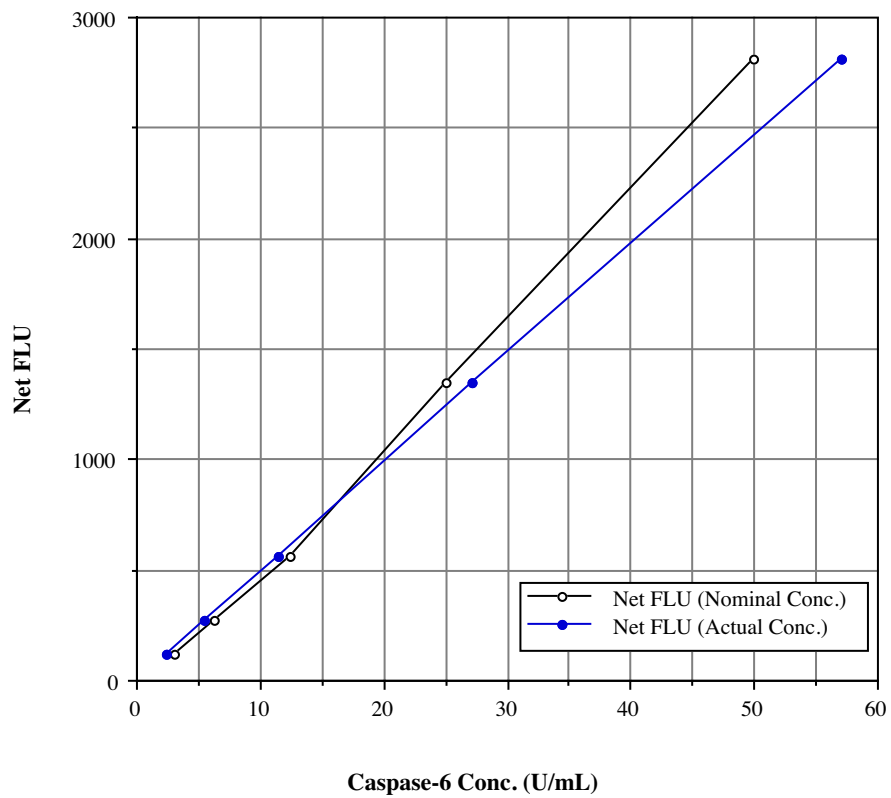
Typical Results

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

<u>Sample</u>	<u>Average FLU</u>	<u>Net FLU</u>	<u>Nominal Conc.</u> <u>(Units / mL)</u>	<u>Actual Conc.</u> <u>(Units / mL)</u>
Blank	195			
S1	3,009	2,814	50	57.1
S2	1,539	1,344	25	27.2
S3	758	563	12.5	11.4
S4	467	272	6.25	5.51
S5	313	118	3.13	2.39
AMC Calibrator	4,305	4,110	---	---

Typical Standard Curve

A typical standard curve is shown below. The curve **must not** be used to calculate Caspase-6 activity; each user must run a standard curve for each assay.



Units of Measure

One unit of Caspase-6 activity is defined as the amount of enzyme needed to convert one picomole of substrate per minute at 30 °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 Protocols⁸.

Sensitivity

Sensitivity was calculated by determining the average FLU for sixteen (16) wells run as Blank, and comparing to the average FLU for sixteen (16) wells run with Standard #5. The detection limit was determined as the concentration of Caspase-6 measured at two (2) standard deviations from the Blank along the standard curve.

$$\begin{aligned} \text{Average FLU's for the Blank} &= 184 \pm 6.4 (3.4\%) \\ \text{Average FLU's for Standard \#5} &= 319 \pm 9.3 (2.9\%) \\ \\ \text{Delta FLU's (3.13 - 0 Units/mL)} &= 319 - 184 = 135 \\ \text{2 SD's of the Blank} &= 2 \times 6.4 = 12.8 \end{aligned}$$

$$\text{Sensitivity} = \frac{12.8}{135} \times 3.13 \text{ Units/mL} = 0.297$$

Linearity

A sample containing 26.8 U/mL Caspase-6 was diluted serially 1:2 three times with Active Caspase-6 Reaction Buffer and measured in the assay. The data was plotted graphically as actual Caspase-6 concentration versus measured Caspase-6 concentration.

The line obtained had a slope of 0.971 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Caspase-6 and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Caspase-6 in multiple assays (n=9).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Caspase-6 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	<u>Caspase-6</u> <u>(U/mL)</u>	<u>Intra-assay</u> <u>%CV</u>	<u>Inter-assay</u> <u>%CV</u>
Low	4.6	6.0	
Medium	10.1	5.0	
High	33.6	2.6	
Low	14.0		7.5
High	37.4		5.7

References

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4. J.C. Ameisen, et al, Curr. Top. Microbiol. Immunol., (1995) 200:195-211.
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7. Z. Darzynkiewicz, et al, Hum. Cell., (1998) 11(1):3-12.
8. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989)
Villanova, PA: NCCLS.

LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

Assay Designs must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if Assay Designs is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

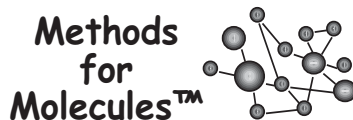


For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

Material Safety Data Sheet (MSDS) available on our website or by fax.

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