Caspase-4 Inhibitor Screening Assay Kit

Item No. 701820

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
701841	Caspase Assay Buffer (5X)	1 vial/5 ml	-20°C
700416	DTT (1 M) Assay Reagent	1 vial/1 ml	-20°C
701821	Caspase-4 Substrate (Ac-LEVD-AFC)	1 vial/50 µl	-20°C
701822	Caspase-4 Enzyme (human, recombinant)	1 vial/50 µl	-80°C
701823	Caspase-4 Inhibitor (Ac-LEVD-CHO)	1 vial/25 µl	-20°C
400093	384-Well Solid Plate (low volume; black)	1 plate	RT
400023	Foil Plate Cover	1 ea	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3640

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure fluorescence with excitation and emission wavelengths of 400 and 505 nm, respectively
2. Adjustable pipettes and a multichannel pipette
3. A source of pure water; glass-distilled water or deionized water is acceptable *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*

INTRODUCTION

Background

Caspase-4 is a cysteinyl aspartic protease and a putative human ortholog to murine caspase-11.[1] It is constitutively expressed in human cells and acts as a sensor for cytosolic Gram-negative bacteria that have escaped extracellular detection by toll-like receptor 4 (TLR4).[2] Procaspase-4, the inactive form of the enzyme, binds to LPS on the outer membrane of Gram-negative bacteria *via* its caspase activation and recruitment domain (CARD), which leads to caspase-4 oligomerization and activation.[2,3] Activated caspase-4 induces non-canonical inflammasome activation by cleaving the pore effector protein gasdermin D at the aspartate in position 276 (Asp^{276}) to release the N-terminal domain that activates the NLRP3 inflammasome and forms pores in the cell membrane to induce pyroptosis, a lytic, inflammatory form of cell death.[4,5] Inhibition of caspase-4 activity by oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-PC (oxPAPC), which competes for LPS binding, or the *Brucella* TIR domain-containing protein TcpB, which promotes degradation of caspase-4, inhibits non-canonical inflammasome activation and LPS-induced pyroptosis in macrophages *in vitro*.[6,7] Caspase-4 has non-inflammasome-related activity as well.[8] It cleaves TAR DNA-binding protein 43 (TDP-43) *in vitro* leading to the accumulation of TDP-43 fragments in the cytosol, which is a pathological hallmark of amyotrophic lateral sclerosis (ALS).[8] In addition, protein levels of caspase-4 are increased in postmortem brain tissue from patients with ALS. The role of caspase-4 in sensing and responding to LPS without activation of TLR4 indicates the potential for caspase-4 inhibition as a therapeutic target for sepsis. Caspase-4 inhibitors would also be useful in characterizing the role of caspase-4 in neurological diseases such as ALS.

About This Assay

Cayman's Caspase-4 Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of human caspase-4, a major upstream effector of non-canonical inflammasome activation. The assay uses a caspase-4-specific fluorogenic substrate, Ac-LEVD-AFC. Caspase-4 cleaves this substrate generating free AFC, which can be easily quantified using a fluorescence plate reader at excitation and emission wavelengths of 400 and 505 nm, respectively. The potent and reversible caspase-4 inhibitor AC-LEVD-CHO is included as a positive control.

PRE-ASSAY PREPARATION

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in diluted Caspase Assay Buffer at a concentration 4X the desired final assay concentration (*e.g.*, for 1 µM final assay concentration, a 4 µM stock should be made). This solution may contain up to 16% DMSO, DMF, or short-chain alcohols (*e.g.*, MeOH, EtOH). The final concentration of organic solvents in the assay will then be ≤4% (see 'Effects of Solvents' on page 17).

Reagent Preparation

1. **Caspase Assay Buffer (5X)**

   Mix 2 ml of Caspase Assay Buffer (5X) (Item No. 701841) with 7.9 ml of water and 100 µl of the supplied DTT (1 M) Assay Reagent (Item No. 700416) to make 10 ml of Caspase Assay Buffer (1X). The Caspase Assay Buffer (1X) should be discarded if not used within the same day. Once thawed, the Caspase Assay Buffer (5X) may be stored at 4°C for at least one month.

2. **Caspase-4 Substrate (Ac-LEVD-AFC)**

   This vial contains Caspase-4 Substrate (Ac-LEVD-AFC) (Item No. 701821) in DMSO. Mix 20 µl of Caspase-4 Substrate (Ac-LEVD-AFC) with 3.98 ml Caspase Assay Buffer (1X). The diluted substrate will be stable at room temperature for four hours. If all of the Caspase-4 Substrate (Ac-LEVD-AFC) will not be used at one time, aliquot the undiluted substrate and store at -20°C where it will be stable for at least one month.

3. **Caspase-4 Enzyme (human, recombinant)**

   Caspase-4 Enzyme (human, recombinant) (Item No. 701822) should be thawed on ice and mixed prior to dilution. To dilute the enzyme, mix 40 µl of Caspase-4 Enzyme (human, recombinant) with 1.96 ml Caspase Assay Buffer (1X). It is recommended that the enzyme be diluted immediately prior to performing the assay. The diluted enzyme loses 30% of its activity when stored on ice for four hours. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

4. **Caspase-4 Inhibitor (Ac-LEVD-CHO)**

   This vial contains Caspase-4 Inhibitor (Ac-LEVD-CHO) (Item No. 701823) in DMSO, which can be used as a positive control. Mix 5 µl of Caspase-4 Inhibitor with 45 µl of Caspase Assay Buffer (1X) to make a 100 µM stock solution. Then mix 4 µl of the 100 µM Caspase-4 Inhibitor stock solution with 96 µl Caspase Assay Buffer (1X) to make a 4 µM working solution. If all of the Caspase-4 Inhibitor will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

ASSAY PROTOCOL

Plate Set Up

The 384-well plate(s) included with this kit is supplied ready to use. There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background. It is suggested that each inhibitor (including the positive control Caspase-4 Inhibitor) be assayed in triplicate.

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 20 µl in all the wells.
- Use the diluted assay buffer in the assay.
- All reagents should be prepared as described above and kept at room temperature before beginning the assay, except Caspase-4 Enzyme (human, recombinant).
- It is not necessary to use all the wells on the plate at one time.
- If the appropriate inhibitor concentration is not known, it may be necessary to assay at several concentrations.
- It is recommended to assay the samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Performing the Assay

1. **Background Wells**: add 10 µl Caspase Assay Buffer (1X) to three wells.
2. **100% Initial Activity Wells**: add 5 µl of the diluted Caspase-4 Enzyme (human, recombinant) and 5 µl of solvent to three wells. Use the same solvent concentration used for the unknown inhibitor and the positive control, Caspase-4 Inhibitor (Ac-LEVD-CHO).
3. **Inhibitor/Positive Control Wells**: add 5 µl of Caspase-4 Enzyme (human, recombinant) and 5 µl of unknown inhibitor or the 4 µM positive control (Caspase-4 Inhibitor) working solution to three wells. If inhibitors in different solvents are to be assayed at the same time, separate sets of 100% initial activity wells should be run for each solvent. *NOTE: To determine an IC_{50} value for an inhibitor, multiple concentrations of the inhibitor should be tested in the assay.*
4. Incubate for 10 minutes at room temperature.
5. Initiate the reactions by adding 10 µl of Caspase-4 Substrate (Ac-LEVD-AFC) to all the wells being used. Mixing the contents is not necessary.
6. Cover the plate with the Foil Plate Cover (Item No. 400023) and incubate for 2.5 hours at room temperature.
7. Remove the plate cover and read the plate with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence (AF) of each sample.
2. Subtract the AF of the background wells from the AF of the 100% initial activity and inhibitor wells. These are the corrected values.
3. Determine the percent inhibition or percent activity for each inhibitor using one of the following equations:

$$\text{\% Inhibition} = \left[\frac{\text{(corrected 100\% initial activity - corrected inhibitor activity)}}{\text{corrected 100\% initial activity}} \right] \times 100$$

$$\text{\% Activity} = \left[\frac{\text{(corrected inhibitor activity)}}{\text{corrected 100\% initial activity}} \right] \times 100$$

4. Graph the percent inhibition or percent activity as a function of inhibitor concentration to determine the IC_{50} value (the concentration at which there is 50% inhibition) of the inhibitor. Inhibition of recombinant human caspase-4 by Caspase-4 Inhibitor (Ac-LEVD-CHO) is shown in figure 1 (see page 15).

Performance Characteristics

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[9]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z´ factor is 1.0. A robust assay has a Z´ factor >0.5. The Z´ factor for Cayman's Caspase-4 Inhibitor Screening Assay Kit was determined to be 0.85.

Sample Data:

The data shown here is an example of the data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to directly compare to your samples. Your results could differ substantially.

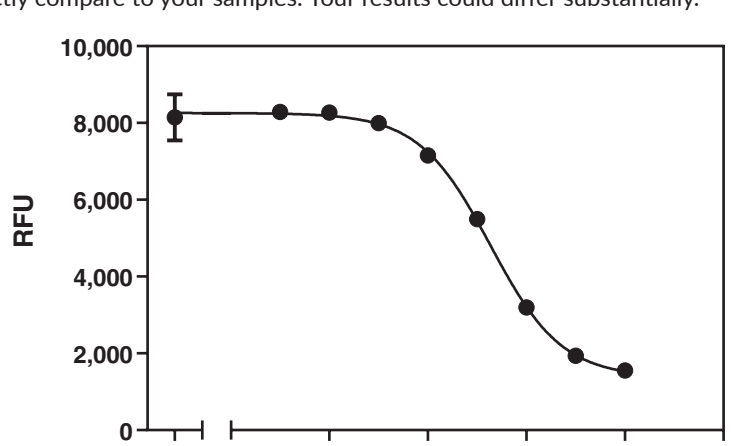

Figure 1. Inhibition of recombinant human caspase-4 by Caspase-4 Inhibitor (Ac-LEVD-CHO). Data are plotted as the mean of quadruplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% initial activity.

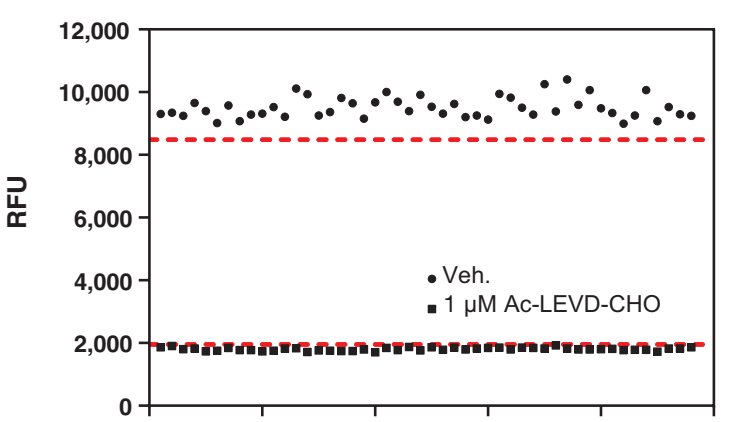

Figure 2. Typical Z′ data for the Caspase-4 Inhibitor Screening Assay Kit. Data are shown from 48 replicates each for vehicle control (Veh.) and 1 µM Caspase-4 Inhibitor (Ac-LEVD-CHO) prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.85. The red lines correspond to three standard deviations from the mean for each control value.

Effects of Solvents:

Compounds may be prepared in organic solvents such as DMSO, DMF, or short-chain alcohols (*e.g.* MeOH, EtOH), as long as the final concentration of organic solvents in the assay is ≤4%. A titration of organic solvents showed that the signal increases slightly with increasing solvent concentration so the proper vehicle control should be included in the assay.

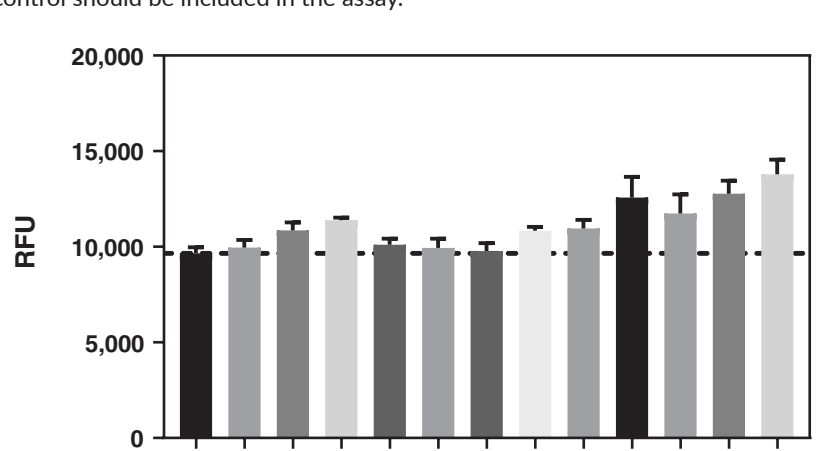

Figure 3. The effect of solvent on the readout of caspase-4 activity. The data are shown as the mean ± standard deviation for triplicate reactions containing the indicated concentration of solvents.

Precision:

Intra-assay precision was determined by analyzing 32 measurements of the background, vehicle, and 1 µM inihibitor Ac-LEVD-CHO on the same day. The intra-assay coefficients of variation were 6.7, 3.8, and 4.4%, respectively.

Inter-assay precision was determined by analyzing inhibition with Ac-LEVD-CHO in separate assays on three different days. The inter-assay coefficient of variance for the IC_{50} value was 13%.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in the inhibitor wells	A. Enzyme or substrate was not added to the well(s) B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all the components to the well(s) B. Reduce the inhibitor concentration and re-assay
The fluorometer exhibited 'MAX' values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the compound concentration and re-assay

References

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3. Yang, J., Zhao, Y., and Shao, F. Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity. *Curr. Opin. Immunol.* **32**, 78-83 (2015).
4. Shi, J., Zhao, Y., Wang, K., *et al.* Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **526(7575)**, 660-665 (2015).
5. Man, S.M. and Kanneganti, T.-D. Gasdermin D: The long-awaited exectioner of pyroptosis. *Cell Res.* **25(11)**, 1183-1184 (2015).
6. Chu, L.H., Indramohan, M., Ratsimandresey, R.A., *et al.* The oxidized phospholipid oxPAPC protects from septic shock by targeting the non-canonical inflammasome in macrophages. *Nat. Commun.* **9(1)**, 996 (2018).
7. Jakka, P., Namani, S., Murugan, S., *et al.* The *Brucella* effector protein TcpB induces degradation of inflammatory caspases and thereby subverts non-canonical inflammasome activation in macrophages. *J. Biol. Chem.* **292(50)**, 20613-20627 (2017).
8. Yin, P., Guo, X., Yang, W., *et al.* Caspase-4 mediates cytoplasmic accumulation of TDP-43 in the primate brains. *Acta Neuropathol.* **137(6)**, 919-937 (2019).
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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.