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MANUAL

Caspase-1 (mouse) ELISA Kit

[Interleukin-1 β -converting Enzyme (mouse) ELISA Kit]

For research use only. Not for diagnostic use

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1. Intended Use

The caspase-1 (mouse) ELISA Kit is to be used for the *in vitro* quantitative determination of mouse caspase-1 in cell culture supernatants, serum and plasma. This ELISA Kit is for research use only.

2. Introduction

Caspase-1 is the best-described inflammatory caspase (1). It processes the cytokines interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) and induces pyroptotic cell death. Caspase-1 is synthesized as inactive pro-Caspase-1, which is activated and processed by multiprotein complexes called inflammasomes in response to numerous stimuli that are detected through distinct inflammasomes (2). NLRP4 responds to cytosolic flagellin (3), murine NLRP1b responds to anthrax lethal toxin (4), AIM2 responds to cytosolic DNA (5) and NLRP3/NALP3 responds to a variety of agonists including crystals (6). Pro-Caspase-1, once activated by an inflammasome complex is processed into p20 and p10 subunits that assemble to form a p20-p20 /p10-p10 active complex that is secreted by a nonconventional (non-ER-dependent) mechanism (7).

How to measure inflammasome activation using this ELISA kit

Cleavage and secretion of Caspase-1 are important events in inflammasome activation, especially since cleavage is currently thought to be a prerequisite for secretion (8). The Caspase-1 (mouse) ELISA Kit can detect both proform or cleaved mouse Caspase-1. Therefore, it cannot distinguish between the specific, inflammasome-dependent release of the activated cleaved form of Caspase-1 and the accidental release of the proform (e.g. due to cell death). In order to specifically demonstrate inflammasome activation, a western blot should be performed to show that cleaved Caspase-1 is present in the cellular supernatants (9). To this end the amount of the biotinylated detection antibody (DET) included in the ELISA kit is present in excess and 10 μ l of DET could be used (dilution 1/1000) for western blotting to confirm the cleavage of Caspase-1 and the activation of inflammasome on positive samples obtained using the Caspase-1 (mouse) ELISA Kit. The isotype of the antibody DET is mouse IgG1 and the antibody recognizes the p20 fragment of mouse Caspase-1 (see also www.adipogen.com: Caspase-1 (mouse), mAb).

3. General References

- (1) Inflammatory caspases in innate immunity and inflammation: A.S. Yazdi, et al.; *J. Innate Immun.* **2**, 228 (2010)
- (2) The inflammasomes: K. Schroder & J. Tschopp; *Cell* **140**, 821 (2010)
- (3) The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus: Y. Zhao, et al.; *Nature* **477**, 596 (2011)
- (4) Anthrax lethal factor cleavage of Nlrp1 is required for activation of the inflammasome: J.L. Levinsohn, et al.; *PLoS Pathog.* **8**, e1002638 (2012)
- (5) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC: V. Hornung, et al.; *Nature* **458**, 514 (2009)
- (6) NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? J. Tschopp & K. Schroder; *Nat. Rev. Immunol.* **10**, 210 (2010)
- (7) Mechanisms of regulated unconventional protein secretion: W. Nickel & C. Rabouille; *Nat. Rev. Mol. Cell Biol.* **10**, 148 (2009)
- (8) Active caspase-1 is a regulator of unconventional protein secretion: M. Keller, et al.; *Cell* **132**, 818 (2008)
- (9) Measuring the inflammasome: O. Gross; *Methods Mol. Biol.* **844**, 199 (2012)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of mouse Caspase-1 (Caspase-1) in cell culture supernatants, serum and plasma. A monoclonal antibody specific for Caspase-1 has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, Caspase-1 is recognized by the addition of a biotinylated monoclonal antibody specific for Caspase-1 (DET). After removal of excess biotinylated antibody, streptavidine-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of Caspase-1 in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

- 1 vial mouse Caspase-1 Standard (lyophilized) (1 µg) (STD)
- 1 vial Detection Antibody (40 µl) (DET)
- 1 vial HRP Labeled Streptavidin (lyophilized) (2 µg) (STREP-HRP)
- 2 bottles Wash Buffer 10X (2 x 30 ml) (Wash Buffer 10X)
- 2 bottles ELISA Buffer 10X (2 x 30 ml) (ELISA Buffer 10X)
- 1 bottle TMB Substrate Solution (12 ml) (TMB)
- 1 bottle Stop Solution (12 ml) (STOP)
- 1 plate coated with mCaspase-1 Antibody (6 x 16-well strips)
- 2 plate Covers (plastic film)
- 2 silica Gel Minibags

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: Prepare just the appropriate amount of the buffers necessary for the assay.

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain Wash Buffer 1X.
- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 10 ml ELISA Buffer 10X + 90 ml water) to obtain ELISA Buffer 1X.
- **Detection Antibody (DET)** has to be diluted to 1:500 in ELISA Buffer 1X (4 µl DET + 2 ml ELISA Buffer 1X).

NOTE: The diluted Detection Antibody is not stable and cannot be stored!

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
 - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles.**
 - Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

NOTE: The diluted STREP-HRP is not stable and cannot be stored!

- **Mouse Caspase-1 Standard (STD)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
 - This reconstitution produces a stock solution of 10 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

NOTE: The reconstituted standard is aliquoted and stored at -20°C!

- Dilute the standard protein concentrate (STD) (**10 µg/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.
- Suggested standard points are:
10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0 ng/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
100 ng/ml	10 µl of Caspase-1 (STD) (10 µg/ml)	990 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
10 ng/ml	100 µl of Caspase-1 (100 ng/ml)	900 µl of ELISA Buffer 1X
5 ng/ml	300 µl of Caspase-1 (10 ng/ml)	300 µl of ELISA Buffer 1X
2.5 ng/ml	300 µl of Caspase-1 (5 ng/ml)	300 µl of ELISA Buffer 1X
1.25 ng/ml	300 µl of Caspase-1 (2.5 ng/ml)	300 µl of ELISA Buffer 1X
0.625 ng/ml	300 µl of Caspase-1 (1.25 ng/ml)	300 µl of ELISA Buffer 1X
0.312 ng/ml	300 µl of Caspase-1 (0.625 ng/ml)	300 µl of ELISA Buffer 1X
0.156 ng/ml	300 µl of Caspase-1 (0.312 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample collection, storage and dilution

Cell Culture Supernatants, serum and plasma have to be diluted in ELISA Buffer 1X. Starting dilutions of 1/2 to 1/20 are recommended.

8.3. Assay Procedure (Checklist)

<input type="checkbox"/>	<p>1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C.</p> <p>NOTE: Remaining 16-well strips coated with Caspase-1 antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.</p>
<input type="checkbox"/>	<p>2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted serum, plasma or cell culture supernatant samples in duplicate to the wells (see 8.1. Preparation and Storage of Reagents and 8.2 Preparation of Samples).</p>
<input type="checkbox"/>	<p>3. Cover the plate with plastic film and incubate for 2 h at room temperature (RT).</p>
<input type="checkbox"/>	<p>4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.</p>
<input type="checkbox"/>	<p>5. Add 100 µl to each well of the diluted Detection Antibody (DET) (see 8.1 Preparation and Storage of Reagents).</p>
<input type="checkbox"/>	<p>6. Cover the plate with plastic film and incubate for 1 h at RT.</p>
<input type="checkbox"/>	<p>7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.</p>
<input type="checkbox"/>	<p>8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin (STREP-HRP) (see 8.1. Preparation and Storage of Reagents).</p>
<input type="checkbox"/>	<p>9. Cover the plate with plastic film and incubate for 30 min at RT.</p>
<input type="checkbox"/>	<p>10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.</p>
<input type="checkbox"/>	<p>11. Add 100 µl to each well of TMB substrate solution (TMB).</p>
<input type="checkbox"/>	<p>12. Allow the color reaction to develop at RT in the dark for 10-20 minutes. Do not cover the plate.</p>
<input type="checkbox"/>	<p>13. Stop the reaction by adding 50 µl of Stop Solution (STOP). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (STOP) is added.</p>
<input type="checkbox"/>	<p>! CAUTION: CORROSIVE SOLUTION !</p>
<input type="checkbox"/>	<p>14. Measure the OD at 450 nm in an ELISA reader.</p>

9. Calculation of Results

- Average the duplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding Caspase-1 concentration (pg/ml) on the vertical axis (see **10. TYPICAL DATA**).
- Calculate the Caspase-1 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of mouse Caspase-1 in the sample.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

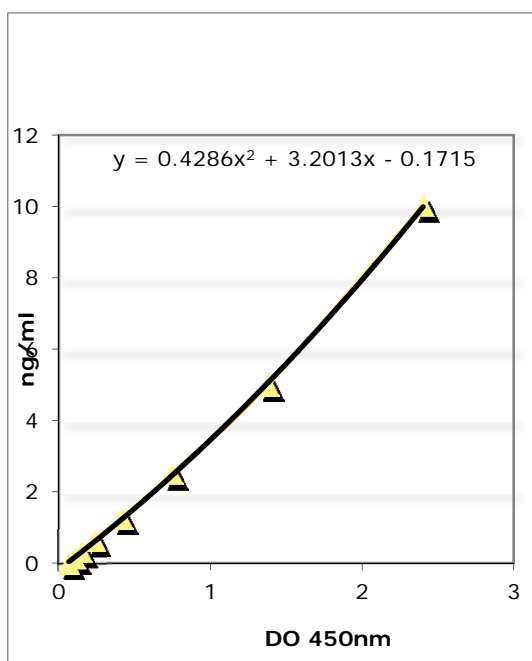


Figure: Standard curve

Standard Caspase-1 (ng/ml)	Optical Density (mean)
10	2.401
5	1.377
2.5	0.747
1.25	0.415
0.625	0.235
0.3125	0.147
0.156	0.103
0	0.064

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of Caspase-1 that can be detected by this assay is 33 pg/ml. **NOTE:** *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.156 ng/ml – 10 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant mouse Caspase-1 (proform and p20 fragment). It does not cross-react with human Caspase-1.

D. Intra-assay precision:

Four samples of known concentrations of mouse Caspase-1 were assayed in replicates 7 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A1	9.49	0.129	1.35	7
A2	0.30	0.003	0.87	7
A3	1.20	0.016	1.30	7
A4	2.40	0.015	1.30	7

E. Inter-assay precision:

Four samples of known concentrations of mouse Caspase-1 were assayed in 3 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
B1	2.5	0.10	4.10	3
B2	7.95	0.209	2.71	3
B3	1.38	0.016	1.20	3
B4	0.412	0.024	5.81	3

F. Linearity:

Different samples containing mouse Caspase-1 were diluted several fold (1/2 to 1/20) and the measured recoveries ranged from 98% to 102%.

G. Expected values:

Mouse Caspase-1 levels range in cell culture supernatant of bone marrow-derived dendritic cells (BMDCs) activated by Nigericin from **5 to >10ng /ml**.

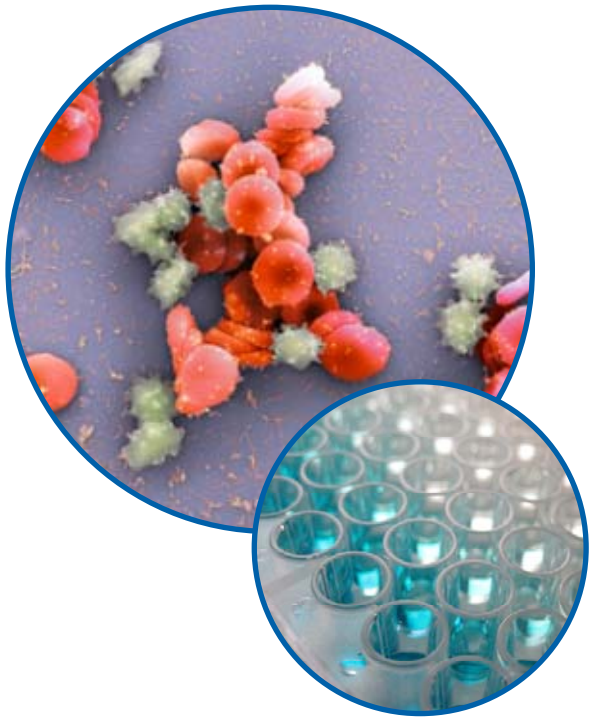
12. Technical Hints and Limitations

- It is recommended that all standards, controls and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes



Product Specific References:

1. D. Miyazaki, et al.; Am. J. Pathol. **183**, 96 (2013)

For more References please visit www.adipogen.com!

Adipogen Life Sciences
Schützenstrasse 12
CH-1410 Liestal
Switzerland
TEL: +41-61-926-60-40
FAX: +41-61-926-60-49
Email: info@adipogen.com

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