ab119508 – Caspase-9 Human ELISA Kit

Instructions for Use

For the quantitative measurement of Human Caspase-9 concentrations in cell lysate, cell culture supernatant and serum

This product is for research use only and is not intended for diagnostic use.

Version 6 Last Updated 11 January 2019

Table of Contents

1. BACKGROUND

Abcam's Caspase-9 Human *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for accurate quantitative measurement of Human Caspase-9 concentrations in cell lysate, cell culture supernatant and serum.

Caspase-9 specific antibodies have been precoated onto 96-well plates. Standards and test samples are added to the wells and along with a Caspase-9 detection antibody and the microplate is then incubated at room temperature. Following washing a HRP conjugated anti-rabbit-IgG antibody is added to each well, incubated at room temperature then again washed. TMB is added and then catalyzed by HRP to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the Caspase-9 amount of sample captured in plate.

Caspases are the executioners of apoptosis. This cysteine protease family consists of more than 10 related members characterized by almost absolute specificity for aspartic acid in the P1 position. Caspases are synthesized as inactive proenzymes comprising an N-terminal peptide together with one large and one small subunit. Activation of caspases during apoptosis results in the cleavage of critical cellular substrates so precipitating the dramatic morphological changes of apoptosis.

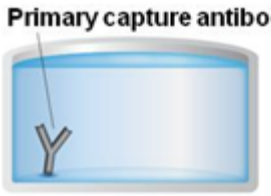
Caspase-9, formerly called Apaf-3, binds to Apaf-1 in the presence of cytochrome c and dATP, which leads to caspase-9 activation. Activated caspase-9 in turn cleaves and activates caspase-3; caspase-9 thus is the most upstream member of the apoptotic protease cascade that is triggered by cytochrome c and dATP.

Caspase-9 is essentially localized in mitochondria, the disruption of the outer mitochondrial membrane occurring early during apoptosis is critical for its sub-cellular redistribution and activation.

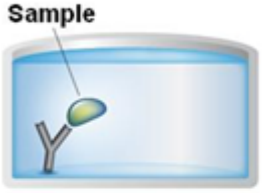
Apart from cleavage of caspase-3, the downstream enzymes caspase-6 and –7 are targets of caspase-9 activity. Caspase-9 forms dimers in the course of activation. Cytochrome c/Apaf-1/caspase-9 forms a so-called apoptosome, amplifying the caspase cascade. Feedback cleavage of caspase-9 by caspase-3 again significantly increases the activity of the apoptosome.

Caspase-9 plays a crucial role in all pathologies involving apoptotic processes. Specifically the involvement of caspase-9 has been described in Human gastric cancer, ovarian carcinoma, neuroblastoma, gliomas as well as in Alzheimer´s disease, Huntington´s disease and osteoarthritis.

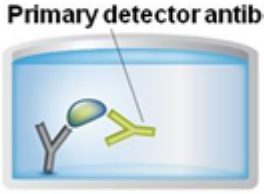
2. ASSAY SUMMARY



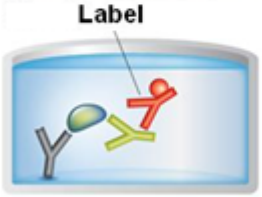
Prepare all reagents, samples and standards as instructed.



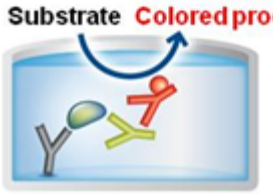
Add standard or sample to each well used.



Add prepared detection antibody to each well. Incubate at room temperature.



Wash and add prepared anti-rabbit-IgG HRP Conjugated antibody. Incubate at room temperature.



Add TMB Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8ºC immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9 Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Microplate coated with monoclonal antibody to Human Caspase-9 (12 x 8 wells)	96 wells	2-8 ºC
Polyclonal anti-Human Caspase-9 Detection Antibody (rabbit)	70 µL	2-8 ºC
Anti-rabbit-IgG HRP conjugated antibody	10 µL	2-8 ºC
Human Caspase-9 Standard lyophilized	2 Vials	2-8 ºC
Sample Diluent	12 mL	2-8 ºC
20X Assay Buffer Concentrate	5 mL	2-8 ºC
20X Wash Buffer Concentrate	50 mL	2-8 ºC
10X Lysis Buffer	15 mL	2-8 ºC
TMB Substrate Solution	15 mL	2-8 ºC
Stop Solution (1M Phosphoric acid)	15 mL	2-8 ºC
Adhesive Films	4 units	2-8 ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if expiration date printed on the label has been exceeded
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers
- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps.
- As exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radio immunotherapy has significantly increased the number of patients with Human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.
- **This kit is sold based on number of tests.** A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use.

9.1 1X Wash Buffer

Prepare 1X Wash Buffer by diluting the 20X Wash Buffer Concentrate with distilled or deionized water. To make 500 mL 1X Wash Buffer, combine 25 mL 20X Wash Buffer Concentrate with 475 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Wash Buffer should be stored at 2-8 ºC and is stable for 30 days.

9.2 1X Assay Buffer

Prepare 1X Assay Buffer by diluting the 20X Assay Buffer Concentrate with distilled or deionized water. To make 50 mL 1X Assay Buffer, combine 2.5 mL 20X Assay Buffer Concentrate with 47.5 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Assay Buffer should be stored at 2-8 ºC and is stable for 30 days.

9.3 1X Lysis Buffer

Prepare 1X Lysis Buffer by diluting the 10X Lysis Buffer Concentrate with distilled or deionized water. To make 150 mL 1X Lysis Buffer, combine 15 mL 10X Lysis Buffer Concentrate with 135 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Lysis Buffer should be stored at 2-8 ºC and is stable for 30 days.

9.4 1X Detection Antibody

To prepare the Detection Antibody, dilute the Polyclonal anti-Human Caspase-9 Detection Antibody 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Detection Antibody as needed by adding the required volume (µL) of the Detection Antibody to the required volume (mL) of 1X Assay Buffer. Mix gently and thoroughly.

Number of strips	Volume of Detection Antibody (µL)	1X Assay Buffer (mL)
1 - 6	30	2.97
7 - 12	60	5.94

Note: The 1X Detection Antibody should be used within 30 minutes after dilution.

9.5 1X Anti-rabbit-IgG-HRP Conjugate

To prepare the Anti-rabbit-IgG-HRP Conjugate, dilute the Anti-rabbit-IgG-HRP Conjugate 2000-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Antibody-HRP Conjugate as needed by adding the required volume (µL) of the Anti-rabbit-IgG-HRP Conjugate to the required volume (mL) of 1X Assay Buffer. Mix gently and thoroughly

Number of strips	Volume of Anti-rabbit-IgG-HRP Conjugate (µL)	1X Assay Buffer (mL)
1 - 6	3	6
7 - 12	6	12

Note: The 1X Anti-rabbit-IgG-HRP Conjugate should be used within 30 minutes after dilution.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.

10.1 Prepare a 200 ng/mL **Stock Standard** by reconstituting one vial of the Human Caspase-9 standard with the volume of distilled water stated on the label. Hold at room temperature for 10-30 minutes. The 200 ng/mL **Stock Standard** cannot be stored for later use.

10.2 Label eight tubes with numbers 1 - 8.

10.3 Add 225 µL of sample diluent to all tubes.

10.4 Prepare a 100 ng/mL **Standard 1** by adding 225 µL of the 200 ng/mL Stock Standard to tube 1. Mix thoroughly and gently.

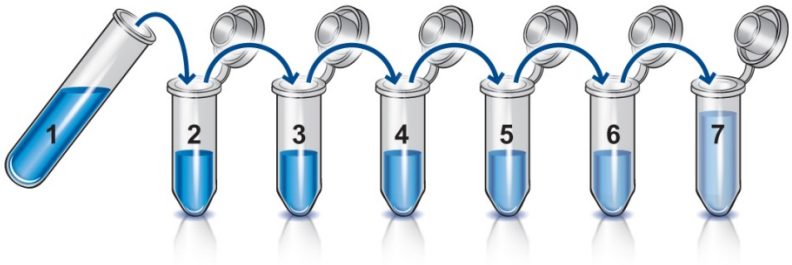
10.5 Prepare **Standard 2** by transferring 225 µL from standard 1 to tube 2. Mix thoroughly and gently.

10.6 Prepare **Standard 3** by transferring 225 µL from standard 2 to tube 3. Mix thoroughly and gently.

10.7 Using the table below as a guide, repeat for tubes number 4 through to 7.

10.8 **Standard 8** contains no protein and is the Blank control

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock	225	225	200	100
2	Standard 1	225	225	100	50
3	Standard 2	225	225	50	25
4	Standard 3	225	225	25	12.5
5	Standard 4	225	225	12.5	6.3
6	Standard 5	225	225	6.3	3.1
7	Standard 6	225	225	3.1	1.6
8	None	-	225	-	0



11. SAMPLE COLLECTION AND STORAGE

- Cell lysate, cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting

- Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens

- Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive Human Caspase-9. If samples are to be run within 24 hours, they may be stored at 2° to 8°C

- Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently

- Aliquots of samples were stored at -20°C and thawed 3 times, and the Human Caspase-9 levels determined. A significant decrease of Human Caspase-9 immunoreactivity was detected (40% at 3 freeze/thaw cycles). Therefore samples should be stored in aliquots at -20°C and thawed only once

- Aliquots of samples were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the Human Caspase-9 level determined after 48 h. There was no significant loss of Human Caspase-9 immunoreactivity detected during storage at -20°C and 2-8°C. A significant loss of Human Caspase-9 immunoreactivity was detected during storage at RT (50%) and 37°C (80%) after 48 h

12. SAMPLE PREPARATION

Cell Lysate Protocol

- Numerous extraction protocols can be used. The following protocol is provided as an example of a suitable extraction procedure, but should not be construed as necessarily being the method of choice. Users may wish to experiment with extraction procedures to find the optimal method

- **For suspension cells:** Pellet by centrifugation, remove supernatant and proceed to Addition of Lysis Buffer

- **For attached cells:** Remove supernatant from cells, wash cells once with PBS, harvest cells by scraping and gentle centrifugation, aspirate PBS, leaving an intact cell pellet (at this point the cell pellet can be frozen at -80°C and lysed at a later date) and proceed to Addition of Lysis Buffer

- **Addition of Lysis Buffer:** Resuspend the pellet in 1X Lysis Buffer (we recommend a concentration of 5 x10^6cells/mL.), incubate 60 minutes at room temperature with gentle shaking and transfer extracts to microcentrifuge tubes and centrifuge at 1000 x g for 15 minutes. Aliquot the cleared lysate to clean microfuge tubes and continue the test procedure (Alternatively lysates can be stored at -80°C and assayed at a later time. Divide lysates into small aliquots to avoid multiple freeze/thaw cycles.)

13. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use
- Unused well strips should be returned to the plate packet and stored at 2-8°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay

14. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

13.1. Prepare all reagents, working standards, and samples as directed in the previous sections. Determine the number of microplate strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards.

13.2. Wash the microplate twice with approximately 400 µL 1X Wash Buffer per well with thorough aspiration of microplate contents between washes. Allow the 1X Wash Buffer to remain in the wells for about 10 - 15 seconds before aspiration. Take care not to scratch the surface of the microplate.

13.3. After the last wash step, empty wells and tap microplate on absorbent pad or paper towel to remove excess 1X Wash Buffer. Use the microplate strips immediately after washing. Alternatively the microplate strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

13.4. Add 100 µL of prepared standards (including the standard blank control) to the appropriate wells.

13.5. Add 50 µL of the Sample Diluent to all sample wells.

13.6. Add 50 µL of samples to appropriate wells.

13.7. Add 50 µL of 1X Detection Antibody to all wells.

13.8. Cover with adhesive film and incubate at room temperature (18° to 25°C) for 2 hours (microplate can be incubated on a shaker set at 400 rpm).

13.9. Remove adhesive film and empty wells. Wash microplate strips 3 times according to step 13.2. Proceed immediately to step 13.19.

13.10. Add 100 µL of 1X anti-rabbit-IgG-HRP to all wells, including the blank wells.

13.11. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour (microplate can be incubated on a shaker set at 400 rpm).

13.12. Remove adhesive film and empty wells. Wash microplate strips 3 times according to step 13.2. Proceed immediately to the next step.

13.13. Pipette 100 µL of TMB Substrate Solution to all wells.

13.14. Incubate the microplate strips at room temperature (18 to 25°C) for 10 minutes. Avoid direct exposure to intense light.

Note: The color development on the plate should be monitored and the substrate reaction stopped (see step 13.14) before the signal in the positive wells becomes saturated. Determination of the ideal time period for color development should to be done individually for each assay. It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 - 0.95.

13.15. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well.

Note: It is important that the Stop Solution is mixed quickly and uniformly throughout the microplate to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microplate strips are stored at 2 - 8°C in the dark.

13.16. Read absorbance of each microplate on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader

according to the manufacturer's instructions by using the blank wells (standard 8). Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

15. CALCULATIONS

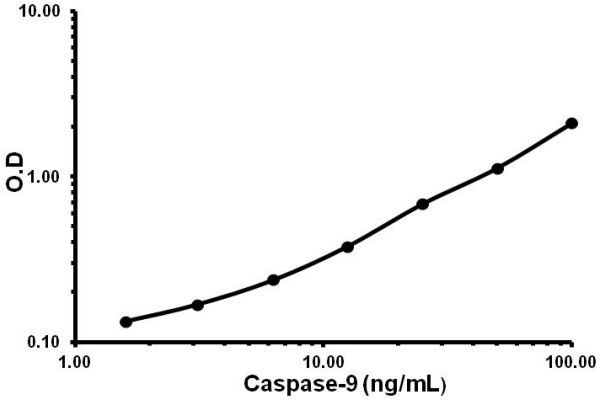
Average the duplicate readings for each standard, sample and control blank. Subtract the no protein control blank from all mean readings. Plot the mean standard readings against their concentrations and draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4-parameter logistic). Extrapolate protein concentrations for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

If samples have been diluted 1:2, as stated in step 13.5, the concentration obtained from the standard curve must be multiplied by the dilution factor (x 2) to obtain an accurate value, in addition to any initial sample dilution factor.

Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human Caspase-9 levels. Such samples require further external predilution according to expected human Caspase-9 values with Sample in order to precisely quantitate the actual human Caspase-9 level.

16. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc.	**O.D. 450 nm**		**Mean**
(ng/mL)	**1**	**2**	**O.D.**
0	0.087	0.089	0.088
1.6	0.135	0.130	0.133
3.2	0.176	0.156	0.168
6.3	0.245	0.229	0.237
12.5	0.388	0.367	0.378
25	0.668	0.695	0.682
50	1.142	1.106	1.124
100	2.056	2.153	2.105

Figure 1. Example of Human Caspase-9 protein standard curve.

17. TYPICAL SAMPLE VALUES

SERUM/PLASMA –

A panel of 40 serum samples from randomly selected donors (males and females) was tested for Human Caspase-9. The detected Human Caspase-9 levels ranged between n.d. (not detectable) and 11.8 ng/mL.

SENSITIVITY -

The limit of detection of Human Caspase-9 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.4 ng/mL (mean of 6 independent assays).

RECOVERY –

The spike recovery was evaluated by spiking 4 levels of Human Caspase-9 into different cell lysates. Recoveries were determined in 3 independent experiments with 4 replicates each. The amount of endogenous Human Caspase-9 in unspiked lysates was subtracted from the spike values. The overall mean recovery was 103%.

PRECISION –

Intra- and Inter-assay reproducibility was determined by measuring samples containing different concentrations of Capase-9.

	Intra-Assay	Inter-Assay
n=	8	8
%CV	6.6	9.0

LINEARITY OF DILUTION –

4 samples with different levels of Human Caspase-9 were analyzed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 89% to 114% with an overall recovery of 103%.

Sample	Dilution	Expected Human Caspase-9 Concentration. (ng/mL)	Observed Human Caspase-9 Concentration (ng/mL)	Recovery of Expected Human Caspase-9 Concentration (%)
1	1:2	-	47.2	-
	1:4	23.6	25.7	108.9
	1:8	12.9	14.1	109.9
	1:16	7.1	7.2	101.5
2	1:2	-	54.6	-
	1:4	27.3	24.3	89.0
	1:8	12.2	11.8	96.7
	1:16	5.9	6.2	104.9
3	1:2	-	47.0	-
	1:4	23.5	26.7	113.7
	1:8	13.4	15.3	114.2
	1:16	7.6	8.0	105.2
4	1:2	-	54.2	-
	1:4	27.1	24.1	88.8
	1:8	12.0	11.5	95.3
	1:16	5.7	6.1	105.9

18. ASSAY SPECIFICITY

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a Human Caspase-9 positive sample. There was no cross reactivity detected.

19. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

20. NOTES

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