Instructions for Use

Caspase-8 ELISA

Enzyme immunassay for the quantitative determination of human Caspase-8 in serum, plasma, cell culture supernatants and other body fluids.

REF **BE52081**

Σ **96**

2-8 °C

EU: *For research use only.*

U.S.: *For research use only.*
Not for use in diagnostic procedures.

I B L I N T E R N A T I O N A L G M B H
Flughafenstrasse 52a
D-22335 Hamburg, Germany
Phone: +49 (0)40-53 28 91-0
Fax: +49 (0)40-53 28 91-11
IBL@IBL-International.com
www.IBL-International.com

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1. INTENDED USE

The Caspase-8 ELISA is an enzyme-linked immunosorbent assay for quantitative detection of human Caspase-8 in cell lysates, cell culture supernatants, human serum, plasma or other body fluids. **The Caspase-8 ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

2. SUMMARY

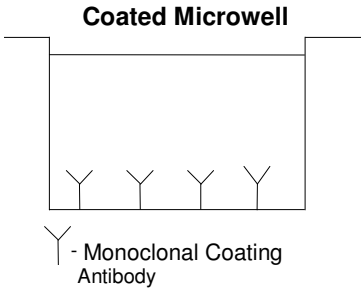
Caspases are the executioners of apoptosis. These cysteine protease family consists of more than 10 related members characterized by almost absolute specificity for aspartic acid in the P1 position. Caspases are synthesized as inactive proenzymes comprising an N-terminal peptide together with one large and one small subunit (8,23). Activation of caspases during apoptosis results in the cleavage of critical cellular substrates so precipitating the dramatic morphological changes of apoptosis.
Apoptosis induced by CD95 (Fas/APO-1) and tumor necrosis factor activates caspase-8 (MACH/FLICE/Mch5) so providing a direct link between cell death receptors and the caspases, caspase-8 being at the apex of the apoptotic cascade (3,13,16,24;27). Caspase-8 is a 55 kDa protein binding the death effector domain of FADD (20). A total of eight different iso forms of FLICE have been described, only two of them being predominantly expressed (25). The CASP8 gene contains at least 11 exons spanning approximately 30 Kb on human chromosome band 2q33-34 (2,7).
The protein encoded shows a complex testiary structure (2,7). Apart from being activated by CD95 cleavage of caspase-8 by granzymeB during T-lymphocyte induced apoptosis has been shown (17).
Further digomerization at the membrane turned out to be sufficient for caspase-8 autoactivation (15). The apoptosis induction by caspase-8 is then amplified through the mitochondrial release of cytochrome c (14).

FLIP was shown to be a regulatory protein of lymphocyte proliferation and death (10) and germinal center B cell apoptosis (9), its expression inhibits T-cell activation (26). On the other hand FLIP(L), the long form of the protein, activates caspase-8 by forming heterodimeric structures (1).

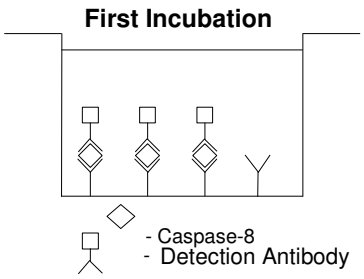
Caspase-8 plays an important role in all physiological disorders where apoptosis is involoved primarily in the development (and treatment) of tumors (5,6,11,12,18,19,22,28,29,30,31) and cardiac diseases (4,21).

3. PRINCIPLES OF THE TEST

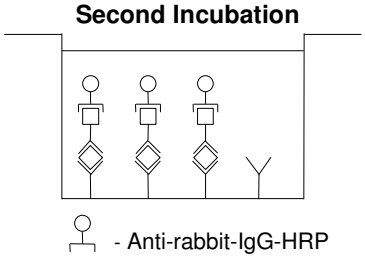
An anti-Caspase-8 monoclonal coating antibody is adsorbed onto microwells.



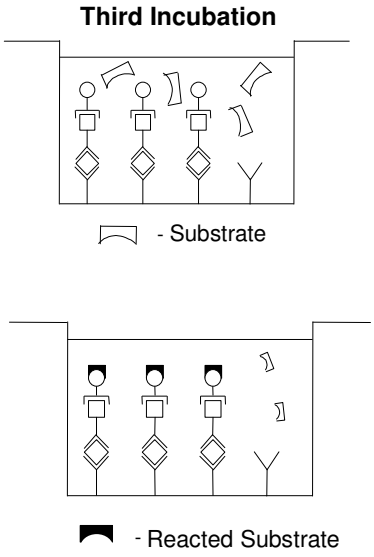
Caspase-8 present in the sample or standard binds to antibodies adsorbed to the microwells; a polyclonal anti-Caspase-8 detection antibody (rabbit) is added and binds to Caspase-8 captured by the first antibody.



Following incubation unbound anti-Caspase-8 detection antibody is removed during a wash step. Anti-rabbit-IgG-HRP is added and binds to the anti-Caspase-8 detection antibody. Following incubation unbound anti-rabbit-IgG-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.



A coloured product is formed in proportion to the amount of Caspase-8 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven Caspase-8 standard dilutions and Caspase-8 sample concentration determined.



4. REAGENTS PROVIDED

1 aluminium pouch with **a Microwell Plate coated with Monoclonal Antibody** (murine) to human Caspase-8

1 vial (100 µl) anti-Caspase-8 polyclonal **Detection Antibody** (rabbit)

1 vial (10 µl) **anti-rabbit-IgG-HRP**

2 vials **Caspase-8 Standard**, lyophilized, 20 ng/ml upon reconstitution

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10 % BSA)

1 bottle (12 ml) **Sample Diluent**

1 bottle (15 ml) **Lysis Buffer** 10x

1 vial (7 ml) **Substrate Solution I** (tetramethyl-benzidine)

1 vial (7 ml) **Substrate Solution II** (0.02 % buffered hydrogen peroxide)

1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 ml each) **Blue-Dye**, **Green-Dye**, **Red-Dye**

4 adhesive **Plate Covers**

Reagent Labels

5. STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.
The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

6. SPECIMEN COLLECTION

Cell extracts (see cell lysate protocol below), cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

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

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive Caspase-8. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

For stability and suitability of samples refer to respective chapter.

<u>Cell Lysate Protocol:</u>

Prepare cell extracts after induction of apoptsis. 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 that work best in their hands.

1. For suspension cells, pellet by centrifugation, remove supernatant and proceed to step # 3. For attached cells, remove supernatant from cells.

2. Wash cells once with PBS, harvest cells by scraping and gentle centrifugation.

3. 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). We recommend for every 5 X 10^6 cells, resuspend the pellet in 1 ml of Lysis Buffer.

4. Incubate 60 minutes at room temperature with gentle shaking.

5. Transfer extracts to microcentrifuge tubes and centrifuge at 1000 x g for 15 minutes.

6. Aliquot cleared lysate to clean microfuge tubes. These samples are now ready for analysis according to the instructions provided in **Test Protocol**. Lysates can be frozen at -80°C and assayed at a later time. The sample should be divided into small aliquots to avoid multiple freeze/thaw cycles.

Note: Samples found to contain greater than 20 ng/ml Caspase-8 (i.e., outside the range of the standard curve) must be diluted with Sample Diluent (provided), so that the Caspase-8 concentration falls within the range spanned by the standard curve, and assayed again.

7. MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes

- 10 µl to 1,000 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)

- Microwell 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 linear regression analysis.

8. PRECAUTIONS FOR USE

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements(s) for specific advice.

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.

- Do not mix or substitute reagents with those from other lots or other sources.

- Do not use kit reagents beyond expiration date on label.

- Do not expose kit reagents to strong light during storage or incubation.

- Do not pipette by mouth.

- Do not eat or smoke in areas where kit reagents or samples are handled.

- Avoid contact of skin or mucous membranes with kit reagents or specimens.

- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.

- Avoid contact of substrate solutions with oxidizing agents and metal.

- Avoid splashing or generation of aerosols.

- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.

- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.

- Exposure to acids will inactivate the conjugate.

- Glass-distilled water or deionized water must be used for reagent preparation.

- Substrate solutions must be at room temperature prior to use.

- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.

- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9. PREPARATION OF REAGENTS

A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

B. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer** Concentrate (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2º to 8ºC. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Preparation of Detection Antibody

Make a 1:100 dilution of the concentrated **Detection Antibody** solution with **Assay Buffer** (reagent B) in a clean plastic tube as needed according to the following table:

Number of Strips	Detection Antibody (ml)	Assay Buffer (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

D. Preparation of Caspase-8 Standard

Reconstitute Caspase-8 **Standard** by addition of distilled water. Reconstitution volume is stated on the label. Mix gently to ensure complete solubilization.

E. Preparation of anti-rabbit-IgG-HRP

Make a 1:2000 dilution of the concentrated **anti-rabbit-IgG-HRP** solution in a clean plastic tube with **Assay Buffer** as needed according to the following table:

Number of Strips	anit-rabbit-IgG- HRP (ml)	Assay Buffer (ml)
1 - 6	0.003	6.000
1 - 12	0.006	12.000

F. TMB Substrate Solution

Using clean pipettes and containers known to be metal free, dispense an equal volume of **Substrate Solution I** into **Substrate Solution II** and swirl gently to mix. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect product performance. A blue colour present in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. The TMB Substrate Solution must be used within a few minutes after mixing. Warm to room temperature before use. Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation.

Substrate preparation according to assay size:

Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

G. Addition of colour-giving reagents: Blue-Dye, Green-Dye, Red-Dye

In order to help our customers to avoid any mistakes in pipetting the IBL-International ELISAs, IBL-International now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye, Red-Dye***) can be added to the reagents according to the following guidelines:

1. Diluent: Before sample dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml Diluent	20 µl ***Blue-Dye***
12 ml Diluent	48 µl ***Blue-Dye***

2. Biotin-Conjugate: Before dilution of the concentrated conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet, preparation of Biotin-conjugate.

3 ml Assay Buffer	30 µl ***Green-Dye***
6 ml Assay Buffer	60 µl ***Green-Dye***
12 ml Assay Buffer	120 µl ***Green-Dye***

3. Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP; add the ***Red-Dye*** at a dilution of 1:250 (see table below) to the Assay Buffer used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet, preparation of Streptavidin-HRP.

6 ml Assay Buffer	24 µl ***Red-Dye***
12 ml Assay Buffer	48 µl ***Red-Dye***

10. TEST PROTOCOL

a. Mix all reagents thoroughly without foaming before use.

b. Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with Monoclonal Antibody** to human Caspase-8 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

c. Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

   After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

d. Add 100 µl of **Sample Diluent** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of reconstituted (refer to preparation of reagents, 9.D.) **Caspase-8 Standard**, in duplicate, into wells A1 and A2. Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of Caspase-8 standard dilutions ranging from 10 to 0.16 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of Caspase-8 standard dilutions:

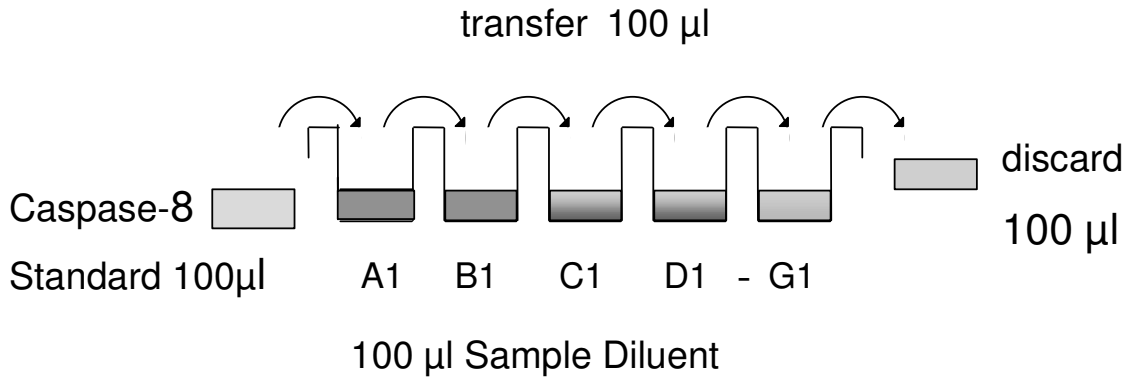


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**	**3**	**4**
A	Standard 1 (10 ng/ml)	Standard 1 (10 ng/ml)	Sample 1	Sample 1
B	Standard 2 (5 ng/ml)	Standard 2 (5 ng/ml)	Sample 2	Sample 2
C	Standard 3 (2.5 ng/ml)	Standard 3 (2.5 ng/ml)	Sample 3	Sample 3
D	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Sample 4	Sample 4
E	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.31 ng/ml)	Standard 6 (0.31 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.16 ng/ml)	Standard 7 (0.16 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

e. Add 100 µl of **Sample Diluent** in duplicate to the blank wells.

f. Add 50 µl of **Sample Diluent**, in duplicate, to the sample wells.

g. Add 50 µl of each **Sample**, in duplicate, to the designated wells.

h. Prepare **Detection Antibody** (refer to preparation of reagents, 9.C).

i. Add 50 µl of diluted **Detection Antibody** to all wells, including the blank wells.

j. Cover with a **Plate Cover** and incubate at room temperature **(18° to 25°C)** for 2 hours, if available on a microplate shaker set at 200 rpm.

k. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

l. Prepare **anti-rabbit-IgG-HRP** (refer to preparation of reagents 9.E).

m. Add 100 µl of diluted **anti-rabbit-IgG-HRP** to all wells, including the blank wells.

n. Cover with a **Plate Cover** and incubate at room temperature **(18° to 25°C)** for 1 hour, if available on a microplate shaker set at 200 rpm.

o. Prepare **TMB Substrate Solution** a few minutes prior to use (refer to preparation of reagents 9.F).

p. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

q. Pipette 100 µl of mixed **TMB Substrate Solution** to all wells, including the blank wells.

r. Incubate the microwell strips at room temperature **(18° to 25°C)** for about **20 minutes,** if available on a microplate shaker set at 200 rpm. Avoid direct exposure to intense light.
The colour development on the plate should be monitored and the substrate reaction stopped (see point s. of this protocol) before positive wells are no longer properly recordable.
It is recommended to add the Stop Solution when the highest standard has developed a dark blue colour.
Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

s. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

t. Read absorbance of each microwell on a spectro-photometer 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. Determine the absorbance of both, the samples and the Caspase-8 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.

11. CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the Caspase-8 concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating Caspase-8 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding Caspase-8 concentration.

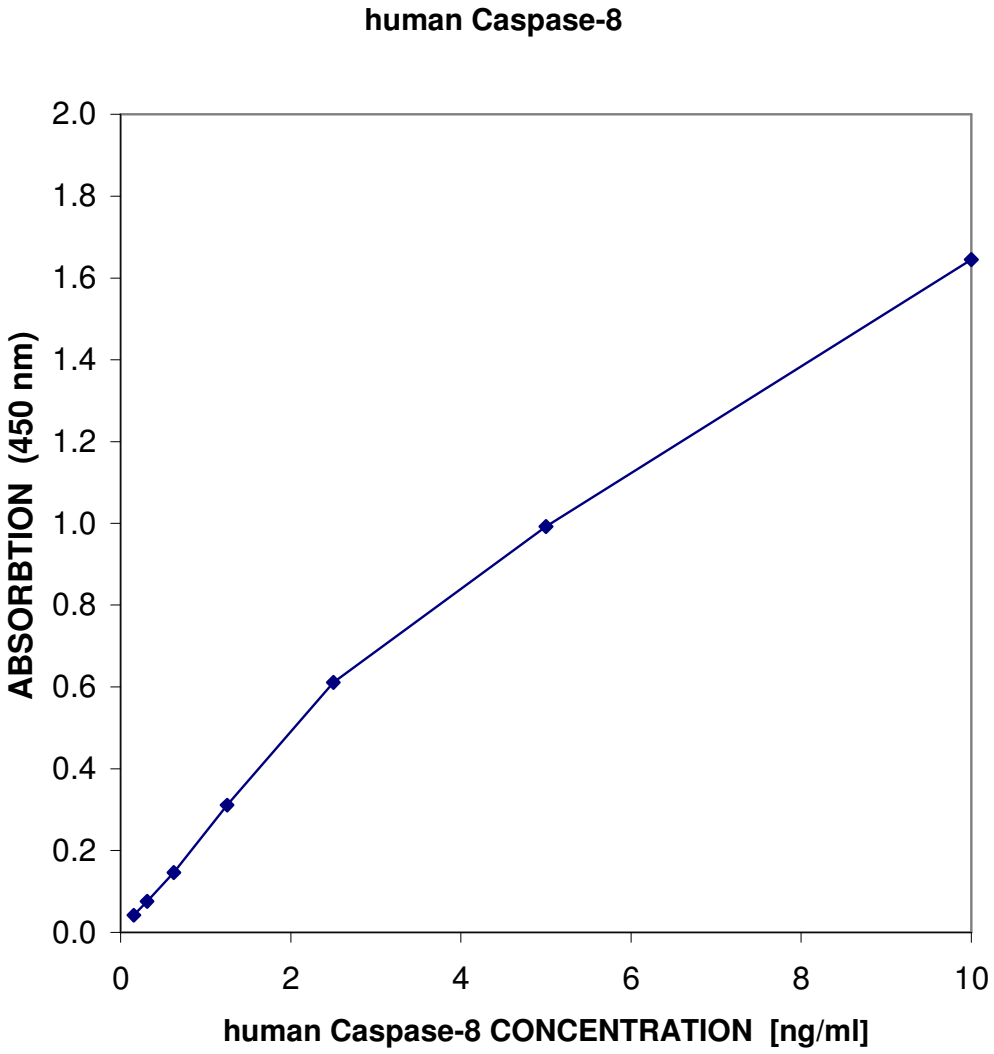
  For samples which have been diluted according to the instructions given in this manual 1:2, the concentration read from the standard curve must be multiplied by the dilution factor (x2).

  Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low Caspase-8 levels. Such samples should be re-analyzed at higher dilution rate in order to precisely quantitate the actual Caspase-8 level.

  It is suggested that each testing facility establishes a control sample of known Caspase-8 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for Caspase-8 ELISA. Caspase-8 was diluted in serial two-fold steps in Sample Diluent, symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Caspase-8

Typical data using the Caspase-8 ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Caspase-8 Concentration (ng/ml)	O.D. (450 nm)	O.D. Mean	CV %
1	10	1.776	1.738	3.1
	10	1.700		
2	5	1.139	1.086	6.9
	5	1.033		
3	2.5	0.714	0.705	1.9
	2.5	0.695		
4	1.25	0.405	0.405	0.3
	1.25	0.404		
5	0.63	0.239	0.240	0.6
	0.63	0.241		
6	0.31	0.169	0.170	0.4
	0.31	0.170		
7	0.16	0.134	0.136	1.6
	0.16	0.137		
Blank	0	0.097	0.094	5.3
	0	0.090		

12. LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.

- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.

- 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. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

13. PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of Caspase-8 defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be less than 0,10 ng/ml (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of Caspase-8. Two standard curves were run on each plate. Data below show the mean Caspase-8 concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 6.7%.

Positive Sample	Experiment	Caspase-8 Concentration (ng/ml)	Coefficient of Variation (%)
1	1	5.93	7
	2	6.22	9
	3	5.75	8
2	1	4.49	10
	2	4.21	10
	3	4.91	8
3	1	3.15	4
	2	3.51	8
	3	3.90	5
4	1	2.94	6
	2	3.17	6
	3	3.18	4
5	1	8.00	2
	2	7.68	4
	3	8.80	4
6	1	3.63	6
	2	3.58	4
	3	4.40	6
7	1	1.87	5
	2	1.77	9
	3	2.17	7
8	1	0.93	8
	2	0.91	10
	3	1.12	9

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by three technicians. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of Caspase-8. Two standard curves were run on each plate. Data below show the mean Caspase-8 concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 8.5%.

Sample	Caspase-8 Concentration (ng/ml)	Coefficient of Variation (%)
1	5.97	4.0
2	4.54	7.8
3	3.52	10.7
4	3.10	4.4
5	8.16	7.1
6	3.87	11.9
7	1.94	10.7
8	0.99	11.5

C. Spike Recovery

The spike recovery was evaluated by spiking four levels of Caspase-8 into 4 different pooled normal human sera. The amount of endogenous Caspase-8 in unspiked serum was substracted from the spike values. Mean recovery was 89.5%.

D. Dilution Parallelism

Four samples with different levels of Caspase-8 were assayed at four serial two-fold dilutions (1:2-1:16) with 4 replicates each. In the table below the per cent recovery of expected values is listed. Recoveries ranged from 84.7 – 110.6 % with an overall mean recovery of 99.6%.

Sample	Dilution	Caspase-8 Concentration (ng/ml) Expected Value	Observed Value	% Recovery of Exp. Value
1	1:2	--	5.96	--
	1:4	2.98	3.27	109.7 %
	1:8	1.63	1.73	105.6 %
	1:16	0.86	0.87	101.2 %
2	1:2	--	9.42	--
	1:4	4.71	4.19	89.0 %
	1:8	2.10	2.02	96.5 %
	1:16	1.01	1.05	103.6 %
3	1:2	--	2.94	--
	1:4	1.47	1.63	110.6 %
	1:8	0.81	0.84	103.6 %
	1:16	0.42	0.44	104.2 %
4	1:2	--	5.02	--
	1:4	2.51	2.12	84.7 %
	1:8	1.06	0.99	93.7 %
	1:16	0.50	0.46	92.6 %

E. Sample Stability

a. Freeze-Thaw Stability

Aliquots of samples were stored frozen at -20℃ and thawed up to 5 times, and Caspase-8 levels determined. There was no significant loss of Caspase-8 by freezing and thawing up to 5 cycles of freezing and thawing.

b. Storage Stability

Aliquots of samples were stored at -20℃, 2-8℃, room temperature (RT), and 37℃ and the Caspase-8 level determined after 72 h. There was no significant loss of Caspase-8 immunoreactivity during above storage conditions.

F. Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a Caspase-8 positive serum. There was no detectable cross reactivity.

G. Expected Serum Values

A panel of 40 sera was tested for Caspase-8. The detected Caspase-8 levels ranged between n.d. and 1.20 ng/ml.

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15. ORDERING INFORMATION

For orders please contact:

IBL-International GmbH
Flughafenstr. 52A
D-22335 Hamburg
Germany
phone: +49 40 5328 91-0
fax: +49 40 5328 91-40
e-mail: IBL@IBL-International.com

For technical information please contact:

e-mail: IBL@IBL-International.com
www.IBL-International.com

Cat.No. BE52081 Caspase-8 ELISA

16. PREPARATION SUMMARY

A. Wash Buffer

Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

B. Assay Buffer

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Detection Antibody

Make a 1:100 dilution according to the table.

Number of Strips	Detection Antibody (ml)	Assay Buffer (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

D. Standard

Add distilled water to each vial of lyophilized **Caspase-8 Standard** (volume is stated on the label) as needed.

E. anti-rabbit-IgG-HRP

Make a 1:2000 dilution according to the table.

Number of Strips	anti-rabbit-IgG-HRP (ml)	Assay Buffer (ml)
1 - 6	0.003	6.000
1 - 12	0.006	12.000

F. TMB Substrate Solution

Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

17. TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Sample Diluent**, in duplicate, to all standard wells
- Pipette 100 µl reconstituted **Caspase-8 Standard** into the first wells and create standard dilutions ranging from 10 to 0.16 ng/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells
- Add 100 µl **Sample Diluent**, in duplicate, to the blank wells
- Add 50 µl **Sample Diluent** to the sample wells
- Add 50 µl **Sample**, in duplicate, to designated wells
- Prepare **Detection Antibody**
- Add 50 µl of diluted **Detection Antibody** to all wells
- Cover microwell strips and incubate 2 hours at room temperature **(18° to 25°C)** on microplate shaker
- Prepare **anit-rabbit-IgG-HRP**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of diluted **anti-rabbit-IgG-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature **(18° to 25°C)** on a microplate shaker
- Prepare **TMB Substrate Solution** few minutes prior to use
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of mixed **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 20 minutes at room temperature **(18°to 25°C)** on a microplate shaker
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low Caspase-8 levels. Such samples require further dilution with Sample Diluent in order to precisely quantitate the actual Caspase-8 level.

Symbols / Symbole / Symbôles / Símbolos / Símbolos / Σύμβολα

REF	Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.–Cat.: / Αριθμός-Κατ.:
LOT	Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός -Παραγωγή:
	Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro: / Χρησιμοποιείται από:
	No. of Tests: / Kitgröße: / Nb. de Tests: / No. de Determ.: / N.º de Testes: / Quantità dei tests: / Αριθμός εξετάσεων:
CONC	Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
LYO	Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λυοφιλιασμένο
IVD	In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Dispositivo Médico para Diagnóstico In Vitro. / Equipamento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostico In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
	Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluació. / Kit de avaliação. / Kit di evaluazione. / Κιτ Αξιολόγησης.
	Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Ler as instruções antes de usar. / Leggere le istruzioni prima dell'uso. / Διαβάστε τις οδηγίες πριν την χρήση.
	Keep away from heat or direct sun light. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l'abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Manter longe do calor ou luz solar directa. / Non esporre ai raggi solari. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.
	Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazenar a: / Conservare a: / Αποθήκευση στους:
	Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabbricante: / Παραγωγός:
	Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!

Symbols of the kit components see MATERIALS SUPPLIED.
Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben.
Voir MATERIEL FOURNI pour les symbôles des composants du kit.
Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS.
Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS.
Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT.
Για τα σύμβολα των συστατικών του κιτ συμβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.

IBL AFFILIATES WORLDWIDE

IBL International GmbH Flughafenstr. 52A, 22335 Hamburg, Germany	Tel.: E-MAIL: WEB:	+ 49 (0) 40 532891 -0 Fax: -11 IBL@IBL-International.com http://www.IBL-International.com
IBL International B.V. Zutphenseweg 55, 7418 AH Deventer, The Netherlands	Tel.: E-MAIL: WEB:	+ 49 (0) 40 532891 -0 Fax: -11 IBL@IBL-International.com http://www.IBL-International.com
IBL International Corp. 194 Wildcat Road, Toronto, Ontario M3J 2N5, Canada	Tel.: E-MAIL: WEB:	+1 (416) 645 -1703 Fax: -1704 Sales@IBL-International.com http://www.IBL-International.com

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