PRODUCT INFORMATION

Human Caspase-8 ELISA

Enzyme-linked immunosorbent assay for

detection of human Caspase-8.

For research use only, not for use in diagnostic procedures.

REF ***IB49679***

Manufactured for:
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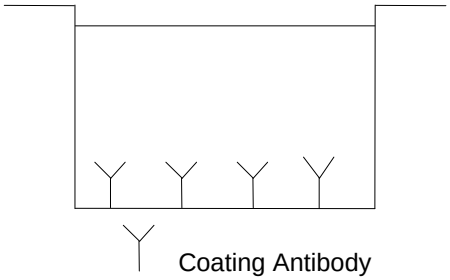
1. Intended Use

The human Caspase-8 ELISA is an enzyme-linked immunosorbent assay for the detection of human Caspase-8. For research use only, not for use in diagnostic procedures.

2. Principle of the Test

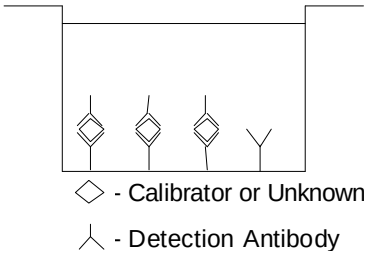
An anti-human Caspase-8 coating antibody is adsorbed onto microwells.

Figure 1 **Coated Microwell**



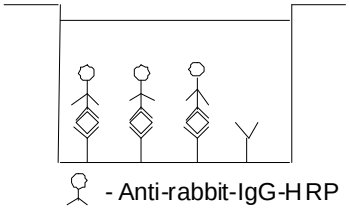
Human Caspase-8 present in the unknown or calibrator binds to antibodies adsorbed to the microwells. The detection antibody binds to human Caspase-8 captured by the first antibody.

Figure 2 **First Incubation**



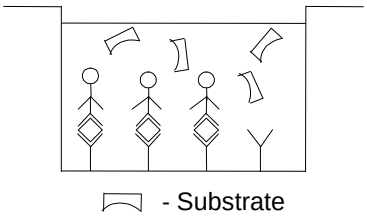
Following incubation unbound detection antibody is removed during a wash step. Anti-rabbit-IgG-HRP is added and binds to the Detection Antibody.

Figure 3 **Second Incubation**



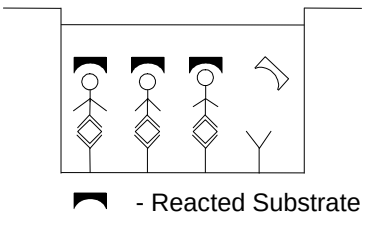
Following incubation unbound anti-rabbit-IgG-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 4 **Third Incubation**



A coloured product is formed in proportion to the amount of human Caspase-8 present in the unknown or calibrator. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibrator curve is prepared from 7 human Caspase-8 calibrator dilutions and human Caspase-8 concentration determined.

Figure 5



3. Reagents Provided

1 aluminum pouch with a **Microwell Plate coated** with monoclonal antibody to human Caspase-8

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

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

2 vials human Caspase-8 **Calibrator** lyophilized, 20.00 ng/ml upon reconstitution

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

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

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

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

1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)

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

1 vial (0.4 ml) **Blue-Dye**

1 vial (0.4 ml) **Green-Dye**

1 vial (0.4 ml) **Red-Dye**

4 **Adhesive Films**

4. Storage Instructions – ELISA Kit

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. 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, this reagent is not contaminated by the first handling.

5. Collection and Handling of Unknowns

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

Pay attention to a possible **“Hook Effect”** due to high concentrations of unknown (see chapter 10).

Unknown containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic unknowns. Unknowns should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Caspase-8. If unknowns 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 unknown should be brought to room temperature slowly and mixed gently.

6. Materials Required But Not Provided

- 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)
- 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 regression analysis

7. 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 statement(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 unknowns are handled.
- Avoid contact of skin or mucous membranes with kit reagents or unknowns.
- Rubber or disposable latex gloves should be worn while handling kit reagents or unknowns.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or unknowns which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.

- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose unknowns and all potentially contaminated materials as 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.

8. Preparation of Reagents

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

8.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 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 Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

8.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

8.3 Lysis Buffer

Pour the entire contents (15 ml) of the **Lysis Buffer Concentrate** (10x) into a clean 150 ml graduated cylinder. Bring to final volume of 150 ml with distilled or deionized water and mix gently. Store at room temperature. Please note that the Lysis Buffer is stable for 30 days.

8.4 Detection Antibody

Please note that the Detection Antibody should be used within 30 minutes after dilution. Make a 1:100 dilution of the concentrated **Detection Antibody** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

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

8.5 Anti-rabbit-IgG-HRP

Please note that the anti-rabbit-IgG-HRP should be used within 30 minutes after dilution. Make a 1:2000 dilution of the concentrated **anti-rabbit-IgG-HRP** solution as needed according to the following table:

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

8.6 Human Caspase-8 Calibrator

Reconstitute **human Caspase-8 calibrator** by addition of distilled water. Reconstitution volume is stated on the label of the calibrator vial. Swirl or mix gently to insure complete and homogeneous solubilisation (concentration of reconstituted calibrator = 20.00 ng/ml). After usage remaining calibrator cannot be stored and has to be discarded.

8.6.1 Calibrator Dilution

Label 7 tubes, one for each calibrator point.

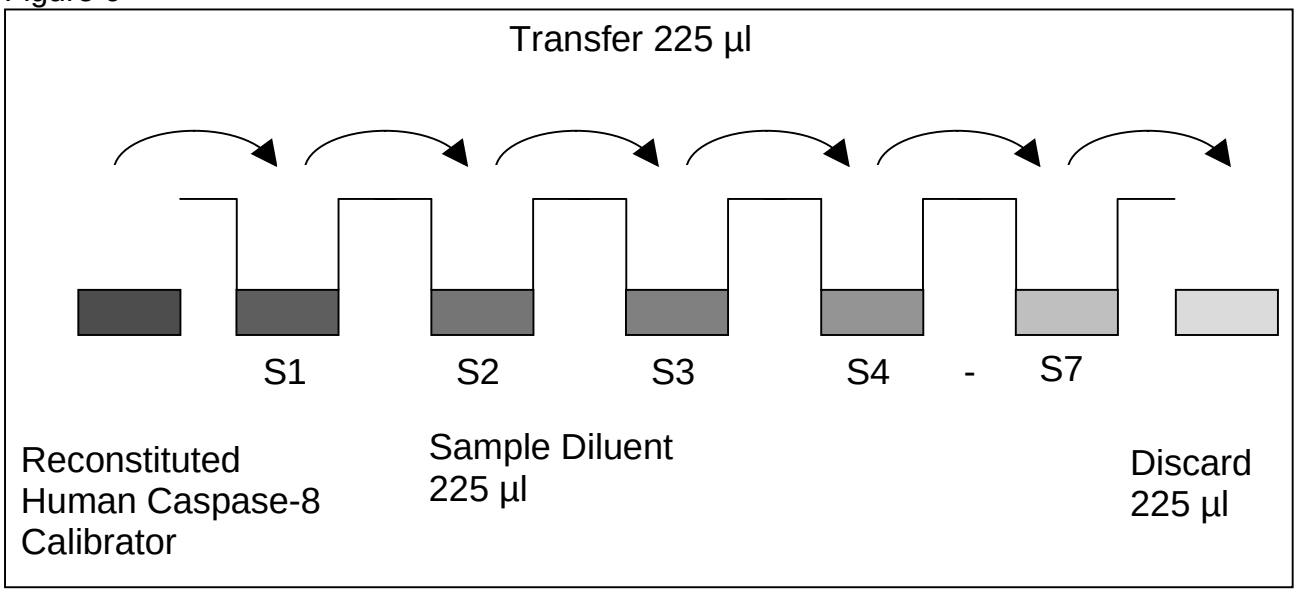
S1, S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the calibrator curve as follows:
Pipette 225 µl of Sample Diluent into each tube.

Pipette 225 µl of reconstituted calibrator (concentration of calibrator = 20.00 ng/ml) into the first tube, labelled S1, and mix (concentration of calibrator 1 = 10.00 ng/ml).
Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.
Repeat serial dilutions 5 more times thus creating the points of the calibrator curve (see Figure 6).

Sample Diluent serves as blank.

Figure 6



8.7 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-America ELISAs, IBL-America offers a 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 calibrator and unknown 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 Sample Diluent	20 µl ***Blue-Dye***
12 ml Sample Diluent	48 µl ***Blue-Dye***
50 ml Sample Diluent	200 µl ***Blue-Dye***

2. Detection Antibody: Before dilution of the concentrated Detection antibody, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final antibody dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet: Preparation of Detection Antibody.

3 ml Assay Buffer (1x)	30 µl ***Green-Dye***
6 ml Assay Buffer (1x)	60 µl ***Green-Dye***
12 ml Assay Buffer (1x)	120 µl ***Green-Dye***

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

6 ml Assay Buffer (1x)	24 µl ***Red-Dye***
12 ml Assay Buffer (1x)	48 µl ***Red-Dye***

9. Test Protocol

1. For cell lysis follow the cell lysate protocol:

   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.

   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 Lysis Buffer (1x) (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.).

2. Unknowns expected to contain more than 20.0 ng/ml Caspase-8 must be diluted with Sample Diluent, according to expected human Caspase-8 values.

3. Determine the number of microwell strips required to test the desired number of unknowns plus appropriate number of wells needed for running blanks and calibrators. Each unknown, calibrator, blank and optional control should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

4. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. **Do not allow wells to dry**.

5. Pipette 100 µl of calibrator dilutions (see section 8.5.1) in the calibrator wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, calibrators and unknowns in the microwell strips:

	1	2	3	4
A	Calibrator 1 (10.00 ng/ml)	Calibrator 1 (10.00 ng/ml)	Unknown 1	Unknown 1
B	Calibrator 2 (5.00 ng/ml)	Calibrator 2 (5.00 ng/ml)	Unknown 2	Unknown 2
C	Calibrator 3 (2.50 ng/ml)	Calibrator 3 (2.50 ng/ml)	Unknown 3	Unknown 3
D	Calibrator 4 (1.25 ng/ml)	Calibrator 4 (1.25 ng/ml)	Unknown 4	Unknown 4
E	Calibrator 5 (0.63 ng/ml)	Calibrator 5 (0.63 ng/ml)	Unknown 5	Unknown 5
F	Calibrator 6 (0.31 ng/ml)	Calibrator 6 (0.31 ng/ml)	Unknown 6	Unknown 6
G	Calibrator 7 (0.16 ng/ml)	Calibrator 7 (0.16 ng/ml)	Unknown 7	Unknown 7
H	Blank	Blank	Unknown 8	Unknown 8

6. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
7. Add 50 µl of **Sample Diluent** to the wells designated for unknowns.
8. Add 50 µl of each **unknown** in duplicate to the appropriately assigned wells.
9. Prepare **Detection Antibody** (see Preparation of Detection Antibody 8.4).
10. Add 50 µl of **Detection Antibody** to all wells.
11. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 200 rpm.
12. Prepare **anti-rabbit-IgG-HRP** (refer to Preparation of Anti-rabbit-IgG-HRP 8.5).
13. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point 4. of the test protocol. Proceed immediately to the next step.
14. Add 100 µl of diluted **anti-rabbit-IgG-HRP** to all wells, including the blank wells.
15. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 200 rpm.

16. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point 4. of the test protocol. Proceed immediately to the next step.

17. Pipette 100 µl of **TMB Substrate Solution** to all wells.

18. Incubate the microwell strips at room temperature (18° to 25°C) for about 15 min. Avoid direct exposure to intense light.

    The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

    It is recommended to add the stop solution when the highest calibrator 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 Calibrator 1 has reached an OD of 0.6 – 0.65.

19. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. 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.

20. Read absorbance of each microwell 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. Determine the absorbance of both the unknowns and the unknowns.

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

10. Results

- Calculate the average absorbance values for each set of duplicate calibrators and unknowns. Duplicates should be within 20 percent of the mean value.
- Create a calibrator curve by plotting the mean absorbance for each calibrator concentration on the ordinate against the human Caspase-8 concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human Caspase-8 for each unknown, first find the mean absorbance value on the ordinate and extend a horizontal line to the calibrator curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human Caspase-8 concentration.
- **If instructions in this protocol have been followed unknowns have been diluted 1:2 (50 µl unknown + 50 µl Sample Diluent), the concentration read from the calibrator curve must be multiplied by the dilution factor (x 2).**

- **Calculation of unknowns with a concentration exceeding calibrator 1 may result in incorrect, low human Caspase-8 levels (Hook Effect). Such unknowns require further external predilution according to expected human Caspase-8 values with Sample Diluent in order to precisely determine the actual human Caspase-8 level.**
- It is suggested that each testing facility establishes a control of known human Caspase-8 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

11. Limitations

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every run.
- Bacterial or fungal contamination of either screen unknowns 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 may result in inaccurate 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.
- Human anti-mouse IgG antibodies (HAMA) may interfere with assays utilizing murine monoclonal antibodies leading to inaccurate results. Serum unknowns containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the unknown.

12. Performance Characteristics

12.1 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human Caspase-8 into serum. Recoveries were determined in 3 independent experiments with 4 replicates each. The unspiked serum was used as blank in these experiments. The overall mean recovery was 89.5%.

12.2 Dilution Parallelism

Serums with different levels of human Caspase-8 were analyzed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 85% to 111% with an overall recovery of 100% (see Table 2).

Table 2

Serum	Dilution	Expected Human Caspase-8 Concentration (ng/ml)	Observed Human Caspase-8 Concentration (ng/ml)	Recovery of Expected Human Caspase-8 Concentration (%)
1	1:2	--	5.96	--
	1:4	2.98	3.27	110
	1:8	1.63	1.73	106
	1:16	0.86	0.87	101
2	1:2	--	9.42	--
	1:4	4.71	4.19	89
	1:8	2.10	2.02	97
	1:16	1.01	1.05	104
3	1:2	--	2.94	--
	1:4	1.47	1.63	111
	1:8	0.81	0.84	104
	1:16	0.42	0.44	104
4	1:2	--	5.02	--
	1:4	2.51	2.12	85
	1:8	1.06	0.99	94
	1:16	0.50	0.46	93

12.3 Stability of Unknowns

12.3.1 Freeze-Thaw Stability

Aliquots of serum were stored at -20°C and thawed 5 times, and the human Caspase-8 levels determined. There was no significant loss of human Caspase-8 immunoreactivity detected by freezing and thawing.

12.3.2 Storage Stability

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

12.4 Specificity

The assay detects both natural and recombinant human Caspase-8. The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a Caspase-8 positive serum. There was no cross-reactivity detected.

13. Ordering Information

This kit is manufactured for Immuno-Biological Laboratories, Inc. (IBL-America). For ordering information, please contact:

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