

*PRODUCT
INFORMATION*



***Human Cathepsin-L
ELISA***

***IB49651
and
IB49651-10***

Enzyme-linked immunosorbent assay for
detection of human Cathepsin-L.

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

**Human Cathepsin-L
IB49651
IB49651-10**

TABLE OF CONTENTS

1	Intended Use	3
2	Summary	3
3	Principles of the Test	5
4	Reagents Provided	7
5	Storage Instructions – ELISA Kit	9
6	Specimen Collection and Storage Instructions	9
7	Materials Required But Not Provided	10
8	Precautions for Use	11
9	Preparation of Reagents	13
10	Test Protocol	18
11	Calculation of Results	23
12	Limitations	26
13	Performance Characteristics	27
14	Bibliography	32
15	Ordering Information	37
16	Reagent Preparation Summary	39
17	Test Protocol Summary	40

1 Intended Use

The human Cathepsin-L ELISA is an enzyme-linked immunosorbent assay for the detection of human Cathepsin-L. **The human Cathepsin-L ELISA is for research use only. Not for use in diagnostic procedures.**

2 Summary

Cathepsin L is a lysosomal endopeptidase belonging to the papain cysteine protease superfamily. It is synthesized as pre-proenzyme and after posttranslational modifications and its transport to the lysosomes cathepsin L is participating in the bulk lysosomal protein breakdown or in special cellular functions such as antigen pro-processing.

Certain specialized cells like macrophages and osteoclasts secrete the precursor of cathepsin L, procathepsin L, which can easily be processed to some form of active enzyme by acid or surface activation or limited proteolysis and which then is involved in connective tissue degradation and the turnover of extracellular matrix proteins.

Like other lysosomal proteinases procathepsin L was also found to be secreted by many malignantly transformed cells and the level of its mRNA and its protein expression and the extent to which it is secreted seem to be correlated to the malignant potential of such cells. Since cathepsin L is capable of degrading protein constituents of the extracellular matrix it is assumed to play a crucial role in tumor progression and metastasis and a number of other disorders where the destruction of the extracellular matrix is the major cause of disease (e.g. rheumatoid arthritis, neurodegeneration).

Inhibition of the enzyme or the proenzyme by low molecular weight inhibitions or antibodies in in vitro and in vivo test systems led to a suppression of the invasive capabilities of malignant cells or a decline in their ability to form tumors.

Cathepsin L is closely related to the recently described cathepsin V (L2) and the sequence identity of almost 80% between them requires antibodies for the detection of cathepsin L which do not crossreact with cathepsin V. This requirement can only be met by epitope specific monoclonal antibodies as are used in IB49651.

Elevated levels of cathepsin L have been found in primary cell cultures from cancerous prostatic samples. Higher levels of active cathepsin L were found in breast tumor cells that are invasive, compared to those that are not invasive. Enforced expression of the precursor procathepsin L was shown to confer high tumorigenic and metastatic properties to human melanoma cells. The upregulation of cathepsin L and B by *ros* Oncogene has been reported for primary human colorectal carcinomas.

Higher levels of cathepsin L were found in rheumatoid arthritis synovial lining compared with osteoarthritis.

Cathepsin L activity has been described in aortic aneurysm wall and parietal thrombus acting on proteins determining elasticity and mechanical resistance of arteries.

Cathepsin L was found to be necessary for Ii (invariant chain) degradation, a critical step in major histocompatibility complex class II – restricted antigen presentation, in cortical thymic epithelial cells, but not in bone marrow derived antigen – presenting cells.

3 Principles of the Test

An anti-human Cathepsin-L coating antibody is adsorbed onto microwells.

Human Cathepsin-L present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human Cathepsin-L antibody is added and binds to human Cathepsin-L captured by the first antibody.

Following incubation unbound biotin-conjugated anti-human Cathepsin-L antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human Cathepsin-L antibody.

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

Figure 1

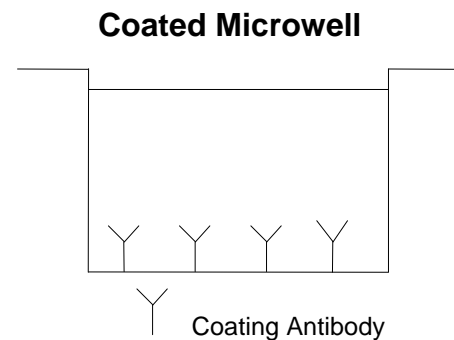


Figure 2

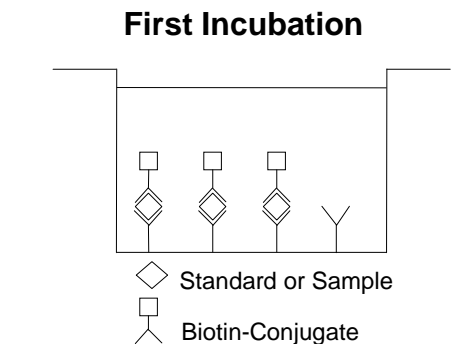


Figure 3

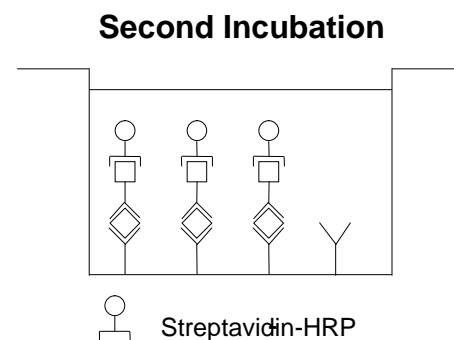
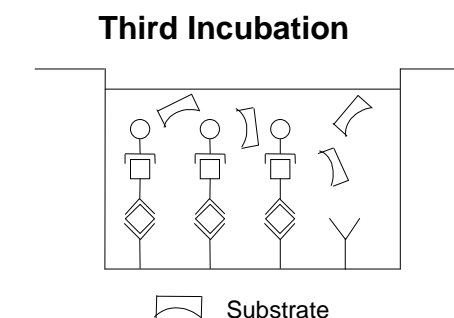
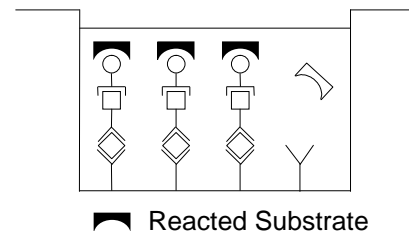


Figure 4



A coloured product is formed in proportion to the amount of human Cathepsin-L present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 5 human Cathepsin-L standard dilutions and human Cathepsin-L sample concentration determined.

Figure 5



4 Reagents Provided

4.1 Reagents for human Cathepsin-L ELISA IB49651 (96 tests)

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human Cathepsin-L
- 1 vial (100 µl) **Biotin-Conjugate** anti-human Cathepsin-L monoclonal antibody
- 1 vial (200 µl) **Streptavidin-HRP**
- 2 vials human Cathepsin-L **Standard** lyophilized, 100 ng/ml upon reconstitution
- 1 vial (12 ml) **Sample Diluent**

Please note: *In some, very rare cases, an insoluble precipitate of stabilizing protein has been seen in the Sample Diluent vial. This precipitate does not interfere in any way with the performance of the test and can thus be ignored.*

- 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 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**

4.2 Reagents for human Cathepsin-L ELISA IB49651-10 (10x96 tests)

- 10 aluminium pouches with a **Microwell Plate coated** with monoclonal antibody to human Cathepsin-L
- 10 vials (100 µl) **Biotin-Conjugate** anti-human Cathepsin-L monoclonal antibody
- 10 vials (200 µl) **Streptavidin-HRP**
- 10 vials human Cathepsin-L **Standard** lyophilized, 100 ng/ml upon reconstitution
- 10 vials (12 ml) **Sample Diluent**
Please note: In some, very rare cases, an insoluble precipitate of stabilizing protein has been seen in the Sample Diluent vial. This precipitate does not interfere in any way with the performance of the test and can thus be ignored.
- 2 vials (5 ml) **Assay Buffer Concentrate 20x** (PBS with 1% Tween 20 and 10% BSA)
- 4 bottles (50 ml) **Wash Buffer Concentrate 20x** (PBS with 1% Tween 20)
- 10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 10 vials (12 ml) **Stop Solution** (1M Phosphoric acid)
- 6 vials (0.4 ml) **Blue-Dye**
- 6 vials (0.4 ml) **Green-Dye**
- 6 vials (0.4 ml) **Red-Dye**
- 20 **Adhesive Films**

5 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.

6 Specimen Collection and Storage Instructions

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.

Pay attention to a possible “**Hook Effect**” due to high sample concentrations (see chapter 11).

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 Cathepsin-L. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).

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

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 μ l to 1000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ l 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

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 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 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 solution 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 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 specimens 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.

9 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.

9.1 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

9.2 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

9.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

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

9.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:200 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

9.5 Human Cathepsin-L Standard

Reconstitute **human Cathepsin-L standard** by addition of distilled water.

Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 100 ng/ml).

After usage remaining standard cannot be stored and has to be discarded.

Standard dilutions can be prepared directly on the microwell plate (see 10.c) or alternatively in tubes (see 9.5.1).

9.5.1 External Standard Dilution

Label 5 tubes, one for each standard point.

S1, S2, S3, S4, S5

Then prepare 1:2 serial dilutions for the standard curve as follows:

Pipette 225 μ l of Sample Diluent into each tube.

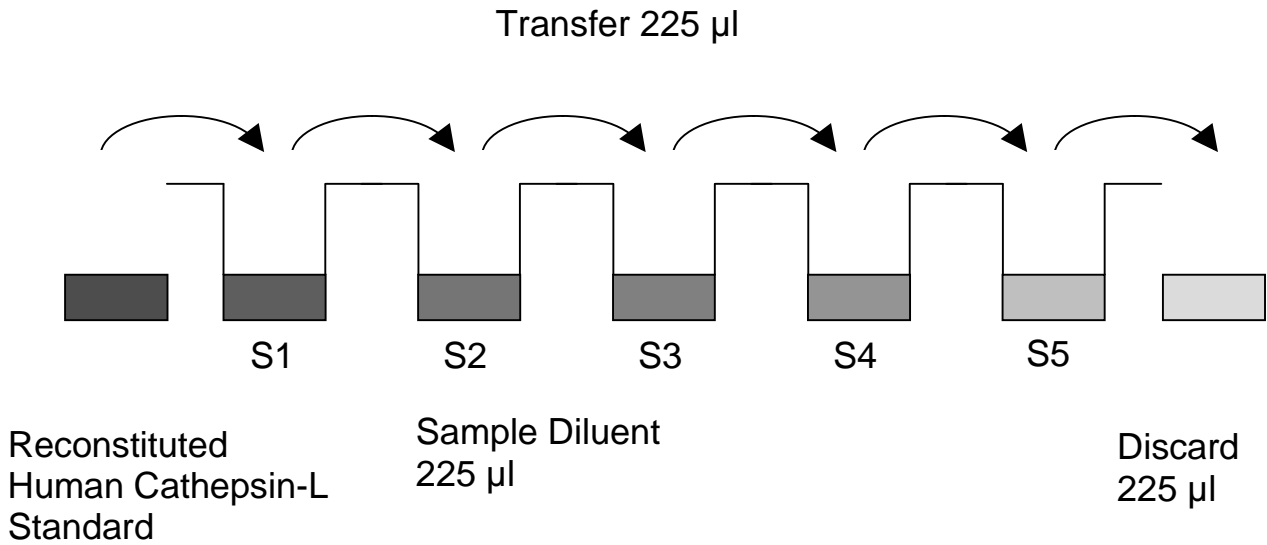
Pipette 225 μ l of reconstituted standard (concentration = 100 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 50 ng/ml).

Pipette 225 μ l of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.

Repeat serial dilutions 3 more times thus creating the points of the standard curve (see Figure 6).

Sample Diluent serves as blank.

Figure 6



9.6 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 standard and 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 Sample Diluent	20 μ l Blue-Dye
12 ml Sample Diluent	48 μ l Blue-Dye
50 ml Sample Diluent	200 μ l Blue-Dye

- 2. Biotin-Conjugate:** Before dilution of the concentrated Biotin-Conjugate, add the **Green-Dye** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) 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 (1x)	30 μ l Green-Dye
6 ml Assay Buffer (1x)	60 μ l Green-Dye

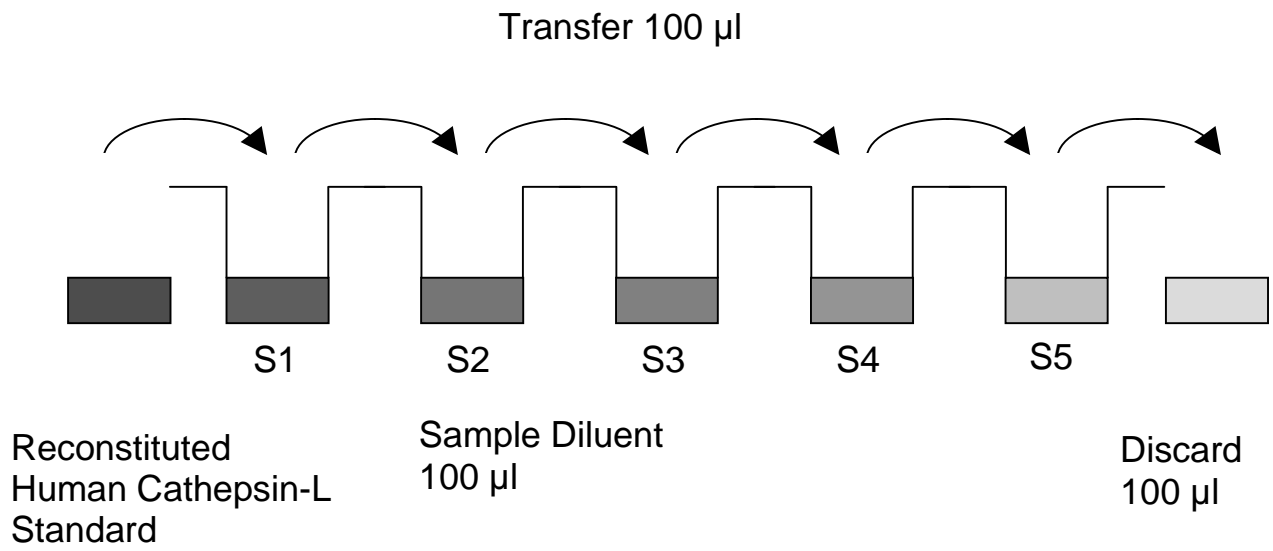
- 3. Streptavidin-HRP:** Before dilution of the concentrated Streptavidin-HRP, add the **Red-Dye** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of **Red-Dye** according to the instruction booklet: Preparation of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

6 ml Assay Buffer (1x)	24 μ l Red-Dye
12 ml Assay Buffer (1x)	48 μ l Red-Dye

10 Test Protocol

- a. 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 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- b. 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. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**
- c. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.5.1):
Add 100 µl of Sample Diluent in duplicate to all **standard wells**.
Pipette 100 µl of prepared **standard** (see Preparation of Standard 9.5, concentration = 100 ng/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 50 ng/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 3 times, creating two rows of human Cathepsin-L standard dilutions ranging from 50.0 to 3.1 ng/ml. Discard 100 µl of the contents from the last microwells (E1, E2) used.

Figure 7



In case of an **external standard dilution** (see 9.5.1), pipette 100 μ l of these standard dilutions (S1 – S5) in the standard wells according to Table 1.

Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (50.0 ng/ml)	Standard 1 (50.0 ng/ml)	Sample 3	Sample 3
B	Standard 2 (25.0 ng/ml)	Standard 2 (25.0 ng/ml)	Sample 4	Sample 4
C	Standard 3 (12.5 ng/ml)	Standard 3 (12.5 ng/ml)	Sample 5	Sample 5
D	Standard 4 (6.3 ng/ml)	Standard 4 (6.3 ng/ml)	Sample 6	Sample 6
E	Standard 5 (3.1 ng/ml)	Standard 5 (3.1 ng/ml)	Sample 7	Sample 7
F	Blank	Blank	Sample 8	Sample 8
G	Sample 1	Sample 1	Sample 9	Sample 9
H	Sample 2	Sample 2	Sample 10	Sample 10

- d. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
- e. Add 50 µl of **Sample Diluent** to the **sample wells**.
- f. Add 50 µl of each **sample** in duplicate to the **sample wells**.
- g. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).
- h. Add 50 µl of **Biotin-Conjugate** to all wells.
- i. 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 100 rpm.
- j. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.4).
- k. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point b. of the test protocol. Proceed immediately to the next step.
- l. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- m. 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 100 rpm.
- n. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point b. of the test protocol. Proceed immediately to the next step.
- o. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- p. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 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 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 Standard 1 has reached an OD of 0.6 – 0.65.

- q. Stop the enzyme reaction by quickly pipetting 100 μ l 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.
- r. 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 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 value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human Cathepsin-L 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 Cathepsin-L 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 human Cathepsin-L concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human Cathepsin-L levels (Hook Effect). Such samples require further external predilution according to expected human Cathepsin-L values with Sample Diluent in order to precisely quantitate the actual human Cathepsin-L level.**
- It is suggested that each testing facility establishes a control sample of known human Cathepsin-L 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.
- A representative standard curve is shown in Figure 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 8

Representative standard curve for human Cathepsin-L ELISA. Human Cathepsin-L was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

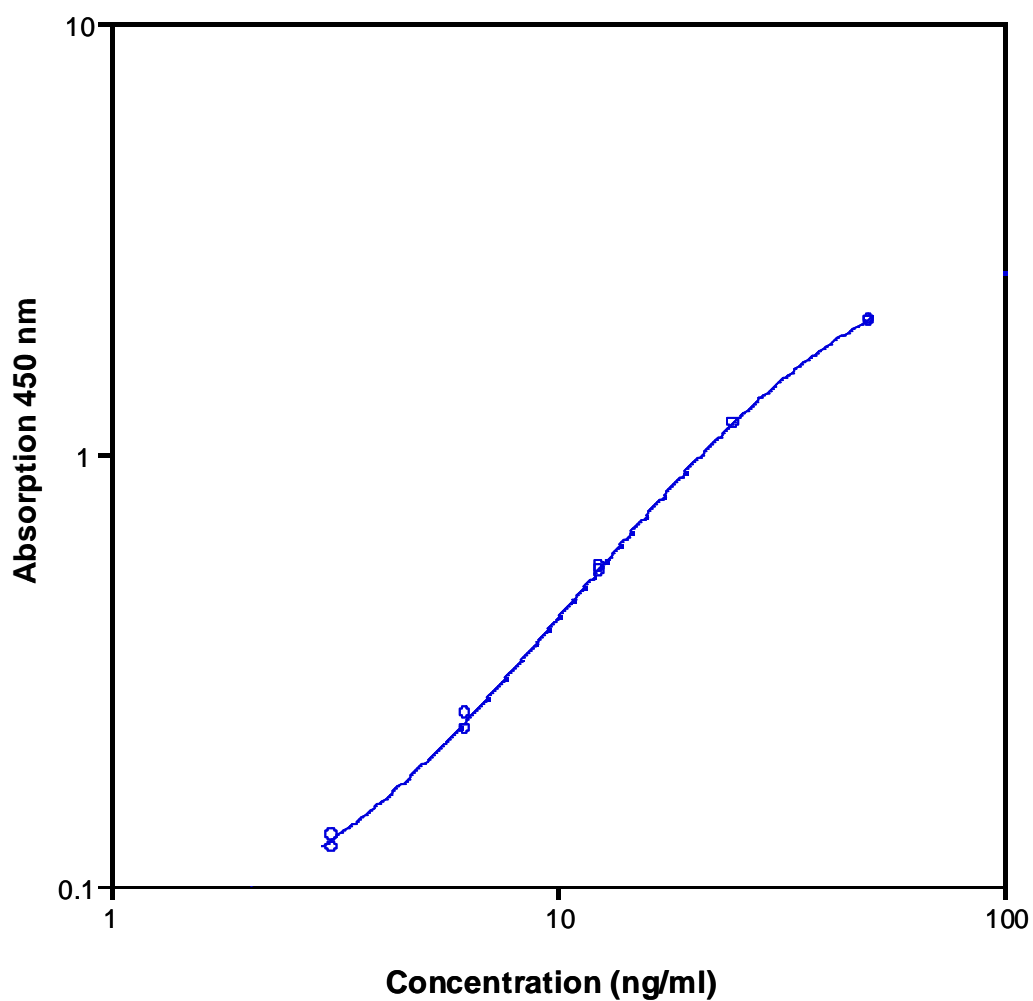


Table 2

Typical data using the human Cathepsin-L ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human Cathepsin-L Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	50.0	2.046	2.04	0.4
	50.0	2.034		
2	25.0	1.182	1.184	0.2
	25.0	1.185		
3	12.5	0.548	0.541	1.8
	12.5	0.534		
4	6.3	0.251	0.24	6.5
	6.3	0.229		
5	3.1	0.130	0.127	3.9
	3.1	0.123		
Blank	0	0.042	0.046	
	0	0.050		

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

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 screen 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. 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 radioimmunotherapy 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 analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

13 Performance Characteristics

13.1 Sensitivity

The limit of detection of human Cathepsin-L 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 1.7 ng/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 7 serum samples containing different concentrations of human Cathepsin-L. 2 standard curves were run on each plate. Data below show the mean human Cathepsin-L concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 6.2%.

Table 3

The mean human Cathepsin-L concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human Cathepsin-L Concentration (ng/ml)	Coefficient of Variation (%)
1	1	15.8	9.0
	2	16.6	6.8
	3	15.6	7.2
2	1	18.8	3.6
	2	17.5	4.3
	3	21.0	4.6
3	1	9.2	3.1
	2	9.1	6.5
	3	9.2	8.8
4	1	13.5	7.1
	2	13.8	11.8
	3	14.9	4.2
5	1	21.0	7.3
	2	20.8	6.8
	3	22.3	5.3
6	1	26.9	2.5
	2	23.1	8.8
	3	25.0	3.6
7	1	7.5	3.9
	2	8.0	15.6
	3	6.0	8.8

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 7 serum samples containing different concentrations of human Cathepsin-L. 2 standard curves were run on each plate. Data below show the mean human Cathepsin-L concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 6.0%.

Table 4

The mean human Cathepsin-L concentration and the coefficient of variation of each sample

Sample	Mean Human Cathepsin-L Concentration (ng/ml)	Coefficient of Variation (%)
1	16.0	3.1
2	19.1	9.2
3	9.2	0.4
4	14.0	5.4
5	21.4	3.6
6	25.0	7.5
7	7.1	14.8

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human Cathepsin-L into different pooled normal human serum samples. Recoveries were determined in 3 independent experiments with 4 replicates each.

The amount of endogenous human Cathepsin-L in unspiked serum was subtracted from the spike values.

The recovery ranged from 78% to 113% with an overall mean recovery of 92%.

13.4 Dilution Parallelism

4 serum samples with different levels of human Cathepsin-L were analysed at serial 2 fold dilutions with 4 replicates each.

The recovery ranged from 94% to 118% with an overall recovery of 106% (see Table 5).

Table 5

Sample	Dilution	Expected Human Cathepsin-L Concentration (ng/ml)	Observed Human Cathepsin-L Concentration (ng/ml)	Recovery of Expected Human Cathepsin-L Concentration (%)
1	1:2	--	51.8	--
	1:4	25.9	26.6	103
	1:8	13.3	13.5	102
	1:16	6.7	6.4	94
2	1:2	--	31.1	--
	1:4	15.6	18.3	118
	1:8	9.1	9.6	105
	1:16	4.8	4.8	101
3	1:2	--	33.8	--
	1:4	16.9	19.4	115
	1:8	9.7	10.7	110
	1:16	5.3	5.5	103
4	1:2	--	36.5	--
	1:4	18.2	21.0	115
	1:8	10.5	12.0	114
	1:16	6.0	5.7	95

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human Cathepsin-L levels determined. There was no significant loss of human Cathepsin-L immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human Cathepsin-L level determined after 24 h. There was no significant loss of human Cathepsin-L immunoreactivity detected during storage under above conditions.

13.6 Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human Cathepsin-L positive serum. There was no crossreactivity detected, namely not with Cathepsin V (80% aa homology).

13.7 Expected Values

A panel of 8 serum samples from randomly selected apparently healthy donors (males and females) was tested for human Cathepsin-L. The detected human Cathepsin-L levels ranged between 2.4 and 14.2 ng/ml with a mean level of 5.0 ng/ml.

14 Bibliography

- 1) Bromme, D., Li, Z., Barnes, M., & Mehler, E. (1999). Human cathepsin V functional expression, tissue distribution, electrostatic surface potential, enzymatic characterization and chromosomal localization. *Biochemistry* 38:2377-2385.
- 2) Chambers, A.F., Colella, R., Denhardt, D.T., & Wilson, S.M. (1992). Increased expression of cathepsin L and B and decreased activity of their inhibitors in metastatic, ras-transformed NIH3T3 cells. *Mol. Carcinog.* 5:238-245.
- 3) Chauhan, S.S., Goldstein, L.J., & Gottesman, M.M. (1991). Expression of cathepsin L in human tumors. *Cancer Res.* 51:1478-1481.
- 4) Cunnane, G., Fitzgerald, O., Hummel, K.M., Gay, R.E., Gay, S., Bresnihan, B. (1999). Collagenase, cathepsin B and cathepsin L gene expression in the synovial membrane of patients with early inflammatory arthritis. *Rheumatology* 38:34-42.
- 5) Denhardt, D.T., Greenberg, A.H., Egon, S.E., Hamilton, R.T., & Wright, J.A. (1987). Cysteine proteinase cathepsin L expression correlates closely with the metastatic potential of H ras-transformed murine fibroblasts. *Oncogene* 2:55.
- 6) Flipo, R.M., Heron, F., Heut, G., Balduyck, M., Degand, P., Duquesnoy, B., and Delcambre, B. (1999). Thiol-proteolytic activity in rheumatoid polyarthritis. Assay by spectrofluorometry. *Mol. Osteoartic.* 58:131-133.
- 7) Frade, R., Rodrigues-Lima, F., Huang, S., Xie, K., Guillaume, N. & Bar-Eli, M. (1998). Procathepsin L, a proteinase that cleaves human C3, the third component of complement, confers high tumorigenic and metastatic properties to human melanoma cells. *Cancer Research* 58:2733-2736.
- 8) Friedrich, B., Jung, K., Lein, M., Turk, I., Rudolph, B., Hampel, G., Schnorr, D., Loening, S.A. (1999). Cathepsins B, H, L and cysteine protease inhibitors in malignant prostate cell lines, primary cultured prostatic cells and prostatic tissue. *Europ. J. of Cancer* 35(1):138-144.

- 9) Gacko, M., Glowinski, S. (1998). Cathepsin D and cathepsin L activities in aortic aneurysm wall and parietal thrombus. *Clin. Chemistry and Laboratory Med.* 36:449-452.
- 10) Gottesman, M.M. (1993). Cathepsin L and cancer, in: *Proteolysis and protein turnover. Portland Press Proceedings: 248-251.*
- 11) Heidtmann, H.-H., Salge, U., Havemann, K., Kirschke, H., & Wiederanders, B. (1994). Secretion of a latent, acid activatable cathepsin L precursor by human non-small cell lung cancer cell line. *Oncology Res.* 5: 441-451.
- 12) Ishido, K. & Kominami, E. (1995). Cathepsin L, gene regulation and extracellular functions in: *Proteases involved Cancer: 27-33* (Eds.:M.Suzuki & T. Hiwasa), Monduzzi Editore, Bologna, Italy
- 13) Ishido, K. & Kominami, E. (1995). Procathepsin L degrades extracellular matrix proteins in the presence of glycosaminoglycans in vitro. *Biochem. Res. Comm.* 217:624-631.
- 14) Jean, D., Bar-Eli, M., Huang, S., Xie, K., Rodrigues-Lima, F., Hermann, J. & Frade, R. (1996). A cysteine proteinase, which cleaves human C3, the third component of complement, is involved in tumorigenicity and metastasis of human melanoma. *Cancer Research* 56:254-258.
- 15) Keyszer, G., Redlich, A., Haupl, T., Zacher, J., Sparmann, M., Ungethuem, U., Gay, S., & Burmester, G.R. (1998). Differential expression of cathepsin B and L compared with matrix metalloproteinases and their respective inhibitors in rheumatoid arthritis and osteoarthritis: A parallel investigation by semiquantitative reverse transcriptase-polymerase chain reaction and immunohistochemistry. *Arthritis and Rheumatism* 41:1378-1387.
- 16) Kim, K., Cai, J.G., Shuja, S., Kuo T., Murnane, M.J. (1998). Presence of activated ras correlates with increased cysteine proteinase activities in human colorectal carcinomas. *Int. J. of Canc.* 79:324-333.

- 17) Kirschke, H. (1998). Cathepsin L in: Handbook of Proteolytic Enzymes, pp.617-621. Eds.: A.J.Barrett, N.D.Rawlings & J.F. Woessner; Academic Press San Diego, London, Boston, New York, Sydney, Tokyo, Toronto.
- 18) Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., and Bohley, P. (1977). Cathepsin L – a new proteinase from rat liver lysosomes. *Eur.J.Biochem.*:74:293-301.
- 19) Kagegawa, H., Nikawaw, T., Tagami, K., Kamioka, H., Sumitani, K., Kawata, T., Drobnic-Kosorok, M., Lenarcic, B., Turk, V., & Katunuma, N. (1993). Participation of cathepsin L in bone resorption. *FEBS Letters* 321:247-250.
- 20) Kane, S.E., & Gottesman, M.M. (1990). The role of cathepsin L in malignant transformation. *Seminars in cancer Biology*1:127-136.
- 21) Kasai, M., Shirasaswa, T., Kitamura, M., Ishido, K., Kominami, E., & Hirokawa, K. (1993). Proenzyme form of cathepsin L produced by thymic epithelial cells promotes proliferation of immature thymocytes in the presence of IL-1, IL-7, and anti-CD3 antibody. *Cell Immunol.* 150:124-136.
- 22) Kolkhorst, V., Stürzebecher, J., & Wiederanders, B. (1998). Inhibition of tumour cell invasion by protease inhibitors: correlation with the protease profile. *Biol. Chem.* 124:598-606.
- 23) Mason, R.W., Johnson, D.A., Barrett, A.J., & Chapman, H.A. (1986). Elastinolytic activity of human cathepsin L. *Biochem. J.* 233:925-927.
- 24) Mason, R.W. & Massey, S.D. (1992). Surface activation of procathepsin L. *Biochem. Biophys. Res. Commun.* 189:1659-1666.
- 25) Mignati, P. & Rifkin, D.B. (1993). Biology and Biochemistry of Proteinases in Tumor Invasion. *Physiological Reviews* 73:161-195.
- 26) Nakagawa, T., Roth, W., Wong, P., Nelson, A., Farr, A., Deussing, J., Villadangos, J.A., Ploegh, H., Peters, C., Rudensky, A.Y. (1998). Cathepsin L: Critical role in li degradation and CD4 T cell selection in the thymus. *Science* 280: 450-453.

- 27) Pennachio, L.A., Lehesjoki, A.-A., Stone, N.E., Willour, V.I., Virtaneva, K., Miao, J., Dámato, E., Ramirez, L., Faham, M., Koskiniemi, M., Warrington, J., Norio, R., de la Chapelle, A., Cox, D.R., Myers, R.M. (1996). Mutations in the gene encoding cystatin B cause progressive myoclonus epilepsy (EPM1). *Science* 271:1731-1734.
- 28) Rozhin, J., Wade, R.L., Hann, K.V., & Sloane, B.F. (1989). Membrane-associated cathepsin L: A role in metastasis in melanomas. *Biochem. Biophys. Res. Comm.* 164:556-561.
- 29) Santamaria, I., Velasco, G., Cazorla, M., Fueyo, A., Campo, E., Lopez-Utin, C. (1998). Cathepsin L2, a novel human cysteine proteinase produced by breast and colorectal carcinomas. *Cancer Res.* 58: 1624-1630.
- 30) Santamaria, I., Velasco, G., Pendas, A.M., Paz, A., Lopez-Utin, C. (1999). Molecular cloning and structural and functional characterization of human cathepsin L , a new cysteine proteinase of the papain family with a long propeptide domain. *Journal of Biol. Chem.* 274:13800-13809.
- 31) Sivaparvathi, M., Sawaya, R., Wang, S.W., Rayfor, A., Yamamoto, M., Liotta, L.A., Nicolson, G.L., and Rao, J.S. (1996). Expression and immunohistochemical localization of cathepsin L during the progression of human gliomas. *Clin. Experim. Metastasis* 14:27-34.
- 32) Schröter, C.J., Beck, H., Deeg, M., Weber, E., Schmid, H., & Kalbacher, H. (1998). The immunodominant epitope is the most protease sensitive region of myelin basic protein: Implications for antigen processing. *Proceedings of the 10th International Congress of Immunology*, pp. 379-382. New Delhi, India. Eds.: G.P Talwar, I. Nath, N.K. Ganguly & K.V.S. Rao, Monduzzi Editore, Bologna, Italy.
- 33) Tagami, K., Kakegawa, H., Kamioka, H., Sumitani, K., Kawata, T., Lenarcic, B., Turk, V., & Katunuma N. (1994). The mechanisms and regulation of procathepsin L secretion from osteoclasts in bone resorption. *FEBS Letters* 342:308-312.

- 34) Taniguchi, S., Nishimura, Y., Takahashi, T., Baba, T., & Kato, K. (1990). Augmented excretion of procathepsin L of a ras-transformed highly metastatic rat cell line. *Biochem. Biophys. Res. Comm.* 168:520-526.
- 35) Thomssen, C., Oppelt, P., Janicke, F., Ulm, K., Harbeck, N., Hofler, H., Kuhn, W., Graeff, H., Schmitt, W. (1998). Identification of low-risk node-negative breast cancer patients by tumor biological factors PAI-1 and cathepsin L. *Anticancer Res.* 18:2173-2180.
- 36) Trabandt, A., Aicher, W.K., Gay, R.E., Sukhatme, V.P., Hamilton, M.N., Hamilton, R.T., McGhee, J.R., Fassbender, H.G., and Gay, S. (1990). Expression of the collagenolytic and Ras-induced cysteine proteinase cathepsin L and proliferation-associated oncogenes in synovial cells of MRL/l mice and patients with rheumatoid arthritis. *Matrix* 10:349-361
- 37) Wang, B., Shi, G.P., Yao, P.M., Li, Z., Chapman, H.A., Bromme, D. (1998). Human cathepsin F – Molecular cloning, functional expression, tissue localization, and enzymatic characterization. *Journal of Biol. Chem.* 273:32000-32008.
- 38) Weber, E., Günther, D., Laube, F., Wiederanders, B. & Kirschke, H. (1994). Hybridoma cells producing antibodies to cathepsin L have greatly reduced potential for tumour growth. *J. Canc. Res. Clin. Oncol.* 120:564-567.
- 39) Weber, E., Günther, D., Laube, F., Wiederanders, B. & Kirschke, H. (1995). Suppression of malignancy of myeloma cells by their production of antibodies to cathepsin L. *Proteases Involved in Cancer*. Eds.: M. Suzuki & T. Hiwasa, pp. 75-79. Monduzzi Editore, Bologna, Italy.
- 40) Weber, E., Bahn, H. & Günther, G. (1997). Monoclonal antibodies against cathepsin L and procathepsin L of different species. *Hybridoma* 16:159-166.
- 41) Wex, T., Levy, B., Smeekens, S.P., Ansorge, S., Desnick, R.J., Bromme, D. (1998). Genomic structure, chromosomal localization, and expression of human cathepsin W. *Biochem. and Biophys. Res. Comm.* 248:255-261.

- 42) Wurl, P., Taubert, H., Meye, A., Berger, D., Lautenschlager, C., Schmidt, H., Hinze, R., Rath, F.W., Schönfelder, M. (1998). Expression of vascular endothelial growth factor and its receptor flk-1 in soft tissue sarcomas: Correlation to cathepsin expression and prognosis. *Acta Histochem. et Cytochem.* 31:55-63.
- 43) Xing, R.Y., Addington, A.K., Mason R.W. (1998). Quantification of cathepsins B and L in cells. *Biochem. Journal* 332 part 2: 499-505.
- 44) Yagel, S., Warner, A.H., Nellam, H.N., Lala, P.K., Waghorne, C. & Denhardt, D.T. (1989). Suppression by cathepsin L inhibitors of the invasion of amnion membranes by murine cancer cells. *Cancer Res.* 49:3533-3557.x

For literature update refer to **www.ibl-america.com**

15 Ordering Information

For orders please contact:

USA

IBL-America, Inc.

8201 Central Ave NE. Suite P

Minneapolis, MN 55432, USA

toll-free phone: +1 (888) 523 1246

phone: +1 (763) 780 2955

fax: +1 (763) 780 2988

For technical information please contact:

e-mail: ibl@ibl-america.com

www.IBL-America.com

Cat.No. IB49651 human Cathepsin-L ELISA

Cat.No. IB49651-10 human Cathepsin-L ELISA

16 Reagent Preparation Summary

16.1 Wash Buffer (1x)

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

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

16.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 ml) to 95 ml distilled water.

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

16.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Assay Buffer (1x):

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

16.4 Streptavidin-HRP

Make a 1:200 dilution of **Streptavidin-HRP** in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

16.5 Human Cathepsin-L Standard

Reconstitute lyophilized **human Cathepsin-L standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

17 Test Protocol Summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
Alternatively external standard dilution in tubes (see 9.5.1): Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
5. Add 50 µl Sample Diluent to sample wells.
6. Add 50 µl sample in duplicate, to designated sample wells.
7. Prepare Biotin-Conjugate.
8. Add 50 µl Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C).
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 3 times with Wash Buffer.
12. Add 100 µl diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
14. Empty and wash microwell strips 3 times with Wash Buffer.
15. Add 100 µl of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
17. Add 100 µl Stop Solution to all wells.
18. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (x 2).