# PRODUCT INFORMATION & MANUAL

# Human IL-1β
# Instant ELISA

### *BMS224INST*

Enzyme-linked immunosorbent assay for
quantitative detection of human IL-1β.
For research use only.
Not for diagnostic or therapeutic procedures.
128 Tests

**eBioscience®**

An Affymetrix Company

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Instant ELISA*

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## 1    Intended Use

The human IL-1$\beta$ Instant ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human IL-1$\beta$. **The human IL-1$\beta$ Instant ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

## 2    Summary

Interleukin-1 (IL-1), originally described in 1972 as lymphocyte activating factor (LAF) for its effects on thymocytes, is a polypeptide cytokine with two molecular forms. Both forms appear to mediate identical ranges of biological activity which include synthesis of the acute phase proteins by hepatocytes, chemotaxis of polymorphonucleocytes, and release of polymorphonucleocytes from blood and bone marrow. These effects coined the acronym leukocyte endogenous mediator (LEM). Early researchers also called IL-1$\beta$ endogenous pyrogen, and it has been shown to induce fever and is thought to contribute to wasting of muscles (PIF, proteolysis inducing factor). Other activities associated with IL-1 are the induction of Prostaglandin $E_2$ by synovial cells and release of collagenase with resulting destruction of cartilage and bone resorption (catabolin, osteoclast activation factor). In addition, IL-1 has multiple immunological functions including enhancement of IL-2 production by T cells and activation of B-cells (BAF) and thymocytes. A true pleiotrope, IL-1 may have tumoricidal activity via its release of IL-2 and interferon gamma and be indirectly antiviral by stimulating fibroblasts to release interferon beta. In its role as mediator of sepsis, IL-1 has most recently been described as enhancing the growth of virulent *E.coli*.

The two distinct molecular forms of IL-1 are thought to be derived from two genes. After transcription, as 31 kD precursor polypeptide is cleaved to give rise to mostly cell membrane associated IL-1$\alpha$ and secreted IL-1$\beta$. Both have the same molecular weight of 15 kD but have different isoelectric points of 5 and 7, respectively.

Despite sequence homology of only 20 %, both forms are thought to bind to the same receptor. IL-1 inhibitors that vary only in their degree of glycosylation have been described to bind to the IL-1 receptor. These inhibitors are structurally related to IL-1$\beta$ and may be important in regulation of IL-1$\beta$ action.

Low levels of IL-1$\beta$ have been reported in normal serum. It is thought that IL-1 genes are induced to respond to tissue damage or in infection. Elevated levels have been reported in a number of infectious disease conditions and in noninfectious inflammatory conditions such as Crohn's disease. In addition to elevated serum levels, IL-1 has been found in synovial fluids of patients with rheumatoid arthritis and in cerebrospinal fluid after neurological inflammation or insult. At the other end of the spectrum, low levels of IL-1 have been found in malnutrition and advanced neoplasia suggesting perhaps a complex immunological and physiological regulatory role for this cytokine.

For literature update refer to **www.eBioscience.com**

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## 3   Principles of the Test

An anti-human IL-1$\beta$ coating antibody is adsorbed onto microwells. Human IL-1$\beta$ present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-human IL-1$\beta$ antibody binds to human IL-1$\beta$ captured by the first antibody. Streptavidin-HRP binds to the biotin conjugated anti-human IL-1$\beta$.

Figure 1

First-Incubation

- Streptavidin Conjugate
- Coating Antibody
- Biotin Conjugate
- Standard or Sample

Following incubation unbound biotin conjugated anti human IL-1$\beta$ and Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 2

Second Incubation

- Substrate

A colored product is formed in proportion to the amount of soluble human IL-1$\beta$ 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 7 human IL-1$\beta$ standard dilutions and human IL-1$\beta$ sample concentration determined.
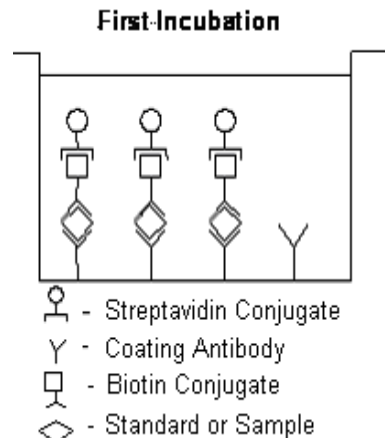
Figure 3

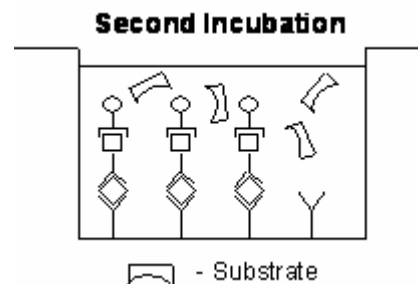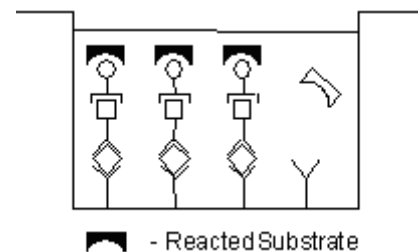- Reacted Substrate

## 4    Reagents Provided

1    aluminium pouch with **a Microwell Plate coated with Monoclonal Antibody** (murine) to human IL-1$\beta$, **Biotin-Conjugate** (anti-IL-1$\beta$ monoclonal antibody), **Streptavidin-HRP** and Sample Diluent, lyophilized

2    aluminium pouches with a human IL-1$\beta$ **Standard curve** (coloured)

1    bottle (25 ml) **Wash Buffer Concentrate** 20x (phosphate-buffered saline with 1% Tween 20)

1    vial (12 ml) **Sample Diluent** (Use when an external predilution of the samples is needed)

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

1    vial (15 ml) **Stop Solution** (1M Phosphoric acid)

2    **Adhesive Films**

## 5    Storage Instructions

Store ELISA plate and standard curves or whole kit at -20°C. The plate and standard curves can also be removed, stored at -20°C, remaining kit reagents can be stored between 2° and 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 culture supernatant, serum and plasma (citrate, heparin) were tested with this assay. Other body fluids might be suitable for use in the assay.
Remove serum from the clot as soon as possible after clotting.

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

Samples must be stored frozen at -20°C to avoid loss of bioactive human IL-1$\beta$. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13).
Avoid repeated freeze-thaw cycles. Prior to assay, frozen serum or plasma should be brought to room temperature slowly and mixed gently.

## 7    Materials Required But Not Provided

− 5 ml and 10 ml graduated pipettes

− 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips

− adjustable multichannel micropipettes (for volumes between 50 µl and 500 µl) 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 reagents 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 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 substrate reagent.

– 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 and Samples

**Buffer concentrate** should be brought to room temperature and diluted before starting the test procedure. If crystals have formed in the **buffer concentrate**, warm it gently until crytals have completely dissolved.

### 9.1    Wash Buffer (1x)

Pour entire contents (25 ml) of the Wash Buffer Concentrate (20x) into a clean 500 ml graduated cylinder. Bring to final volume to 500 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

## 10  Test Protocol

- **Use plate immediately after removal from -20°C!**
- **Do not wait until pellets have completely dissolved before applying samples - the binding reaction in the standard strips starts immediately after addition of water!**
- **Do not try to dissolve pellets by pipetting up and down in the wells - some parts of the pellet could stick to the tip creating high variation of results.**
- **Perform the washing step with at least 400 µl of washing buffer as stated in the manual or fill the wells completely - otherwise any pellet residues sticking to the rim of the well will not be removed and create high variation of results.**
- **Allow the washing buffer to sit in the wells for a few seconds before aspiration.**
- **Remove covers of the standard strips carefully so that all the lyophilized pellets remain in the wells.**

a. Determine the number of microwell strips required to test the desired number of samples plus microwell strips for blanks and standards (coloured). Each sample, standard and blank should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at -20°C sealed tightly. Place microwell strips containing the standard curve in position A1/A2 to H1/H2 (see Table 1).

b. Add **distilled water** to all **standard and blank wells** as indicated on the label of the standard strips (A1, A2 to H1, H2).

c. Add 100 µl of **distilled water** to the **sample wells**.

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 (500.0 pg/ml) | Standard 1 (500.0 pg/ml) | Sample 1 | Sample 1 |
| **B** | Standard 2 (250.0 pg/ml) | Standard 2 (250.0 pg/ml) | Sample 2 | Sample 2 |
| **C** | Standard 3 (125.0 pg/ml) | Standard 3 (125.0 pg/ml) | Sample 3 | Sample 3 |
| **D** | Standard 4 (62.5 pg/ml) | Standard 4 (62.5 pg/ml) | Sample 4 | Sample 4 |
| **E** | Standard 5 (31.3 pg/ml) | Standard 5 (31.3 pg/ml) | Sample 5 | Sample 5 |
| **F** | Standard 6 (15.6 pg/ml) | Standard 6 (15.6 pg/ml) | Sample 6 | Sample 6 |
| **G** | Standard 7 (7.8 pg/ml) | Standard 7 (7.8 pg/ml) | Sample 7 | Sample 7 |
| **H** | Blank | Blank | Sample 8 | Sample 8 |

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d. Add 50 µl of each **sample**, in duplicate, to the **designated wells** and mix the contents.

e. Cover with an adhesive film and incubate at room temperature (18ºC to 25ºC) for 3 hours, if available on a microplate shaker at 400 rpm.

f. Remove adhesive film and empty wells. **Wash** the microwell strips 6 times 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, 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 no longer than 15 minutes. Do not allow wells to dry.

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

h. 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 0.9 – 0.95.

i. 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.

j.  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 human IL-1$\beta$ 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 human IL-1β 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 IL-1β 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 IL-1β concentration.

− **\*Samples have been diluted 1:2, thus 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 IL-1β levels. Such samples require further external predilution according to expected human IL-1β values with Sample Diluent in order to precisely quantitate the actual human IL-1β level.**

− It is suggested that each testing facility establishes a control sample of known human IL-1β 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 4. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

* N.B: There is a common dilution factor for samples due to the conjugate which must then be included in the calculation. The samples contribute 100 µl to the final volume per well. These 100 µl are composed of 50 µl of Sample Diluent plus 50 µl of the sample. This is a 1:2 dilution.

The remaining 50 µl to give 150 µl are due to the addition of 50 µl conjugate to all wells.

50 µl Sample Diluent and 50 µl conjugate results in 100 µl reconstitution volume, addition of 50 µl sample (50 µl + 50 µl = 1:2 dilution)

Figure 4
Representative standard curve for human IL-1β Instant ELISA. human
IL-1β was diluted in serial 2-fold steps in Sample Diluent. Each symbol
represents the mean of 3 parallel titrations.
Do not use this standard curve to derive test results. A standard curve
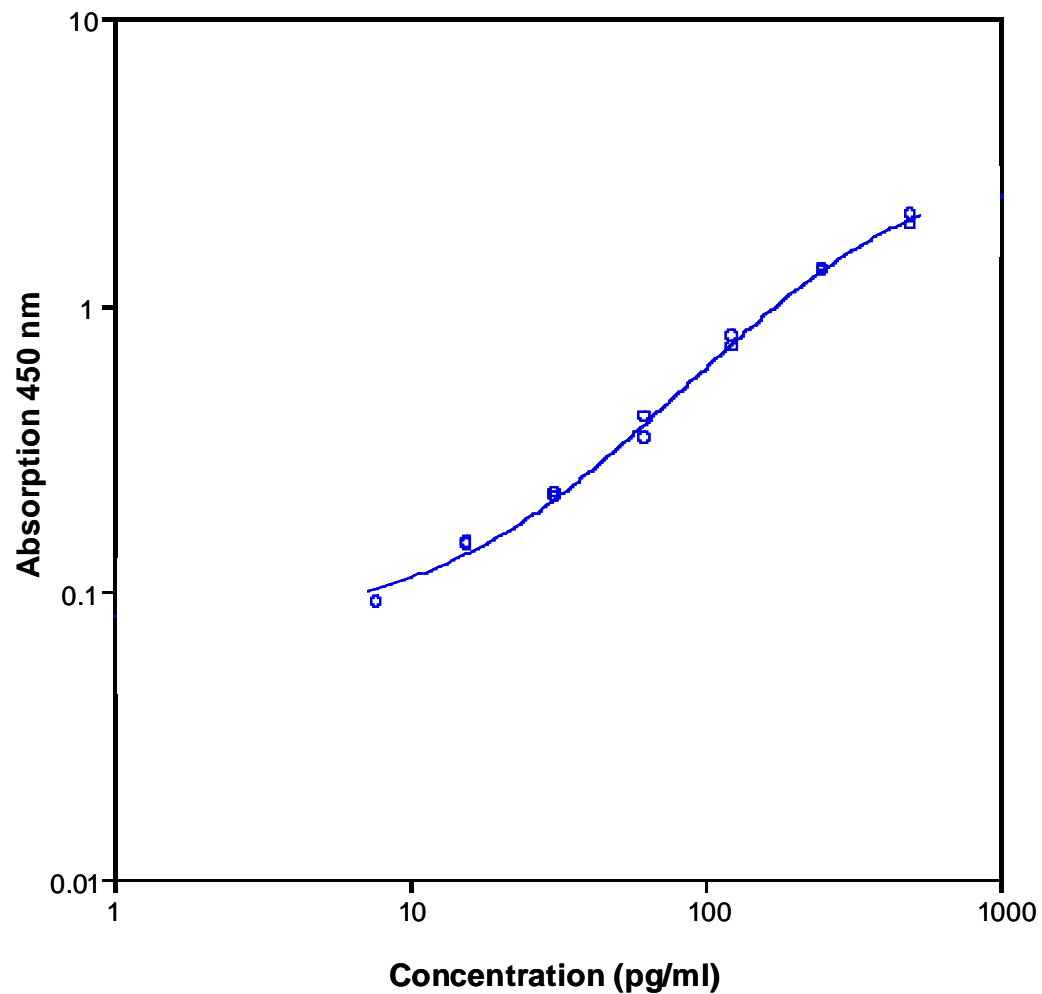must be run for each group of microwell strips assayed.

Table 2
Typical data using the human IL-1β INSTANT ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

| Standard | Human IL-1β Concentration (pg/ml) | O.D. (450 nm) | O.D. Mean | C.V. (%) |
|---|---|---|---|---|
| 1 | 500.0 | 2.045<br>1.902 | 1.974 | 3.6 |
| 2 | 250.0 | 1.325<br>1.304 | 1.310 | 0.8 |
| 3 | 125.0 | 0.765<br>0.710 | 0.737 | 3.7 |
| 4 | 62.5 | 0.339<br>0.404 | 0.370 | 8.7 |
| 5 | 31.3 | 0.221<br>0.212 | 0.218 | 2.1 |
| 6 | 15.6 | 0.147<br>0.145 | 0.146 | 0.7 |
| 7 | 7.8 | 0.092<br>0.091 | 0.092 | 0.5 |
| Blank | 0.0 | 0.023<br>0.020 | 0.022 | 9.9 |

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.

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## 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 IL-1$\beta$ defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.7 pg/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 8 serum samples containing different concentrations of human IL-1$\beta$. 2 standard curves were run on each plate. Data below show the mean human IL-1$\beta$ concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 7.6%.

Table 3
The mean human IL-1β concentration and the coefficient of variation for each sample.

| Positive Sample | Experiment | Mean Human IL-1β Concentration (pg/ml) | Coefficient of Variation (%) |
|---|---|---|---|
| 1 | 1 | 497 | 4 |
|  | 2 | 438 | 8 |
|  | 3 | 530 | 7 |
| 2 | 1 | 194 | 11 |
|  | 2 | 183 | 10 |
|  | 3 | 235 | 6 |
| 3 | 1 | 96 | 4 |
|  | 2 | 98 | 6 |
|  | 3 | 111 | 9 |
| 4 | 1 | 113 | 8 |
|  | 2 | 110 | 4 |
|  | 3 | 95 | 10 |
| 5 | 1 | 304 | 6 |
|  | 2 | 263 | 8 |
|  | 3 | 306 | 11 |
| 6 | 1 | 99 | 7 |
|  | 2 | 91 | 5 |
|  | 3 | 110 | 9 |
| 7 | 1 | 4.3 | 9 |
|  | 2 | 3.8 | 10 |
|  | 3 | 3.3 | 9 |
| 8 | 1 | 73 | 8 |
|  | 2 | 72 | 6 |
|  | 3 | 68 | 7 |

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### 13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments by 3 technicians. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human IL-1$\beta$. 2 standard curves were run on each plate. Data below (see Table 4) show the mean human IL-1$\beta$ concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall coefficient of variation was 10.3%.

Table 4

The mean human IL-1$\beta$ concentration and the coefficient of variation of each sample

| Sample | Mean Human IL-1$\beta$ Concentration (pg/ml) | Coefficient of Variation (%) |
|:------:|:--------------------------------------------:|:----------------------------:|
| 1 | 488 | 9.6 |
| 2 | 204 | 13.3 |
| 3 | 102 | 8.0 |
| 4 | 106 | 9.4 |
| 5 | 291 | 8.2 |
| 6 | 100 | 9.6 |
| 7 | 3.8 | 12.3 |
| 8 | 68 | 12.2 |

## 13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human IL-1β into pooled normal human serum. Recoveries were determined in 3 independent experiments with 6 replicates each. The unspiked serum was used as blank in these experiments. The overall mean recovery has been determined to be 86%.

## 13.4 Dilution Parallelism

4 serum samples with different levels of human IL-1β were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 83% and 113% with an overall mean recovery of 96% (see Table 5).

Table 5

| Sample | Dilution | Expected Human IL-1β Concentration (pg/ml) | Observed Human IL-1β Concentration (pg/ml) | Recovery of Expected Human IL-1β Concentration (%) |
|---|---|---|---|---|
| 1 | 1:2 | -- | 451 | -- |
|   | 1:4 | 1845 | 198 | 88 |
|   | 1:8 | 923 | 107 | 108 |
| 2 | 1:2 | -- | 484 | -- |
|   | 1:4 | 2063 | 262 | 108 |
|   | 1:8 | 1032 | 116 | 88 |
| 3 | 1:2 | -- | 540 | -- |
|   | 1:4 | 1934 | 225 | 83 |
|   | 1:8 | 967 | 109 | 96 |
| 4 | 1:2 | -- | 351 | -- |
|   | 1:4 | 1580 | 150 | 86 |
|   | 1:8 | 790 | 85 | 113 |

### 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 IL-1$\beta$ levels determined. There was no significant loss of human IL-1$\beta$ immunoreactivity 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 IL-1$\beta$ level determined after 24 h. There was no significant loss of human IL-1$\beta$ immunoreactivity during storage under above conditions.

## 13.6  Specificity

The interference of circulating factors of the immune syteme was evaluated by spiking these proteins at physiologically relevant concentrations into a human IL-1$\beta$ positive serum.
There was no cross reactivity detected.

## 13.7  Expected Values

Panels of 40 serum as well as plasma (citrate, heparin) samples from randomly selected apparently healthy donors (males and females) was tested for human IL-1$\beta$.
For detected human IL-1$\beta$ levels see Table 6:

Table 6

| Sample Matrix | Number of Samples Evaluated | Range (pg/ml) | % Detectable | Mean of Detectable (pg/ml) |
|---|---|---|---|---|
| Serum | 40 | n.d. | 0 | -- |
| Plasma (Citrate) | 40 | n.d. – 8.7 | 2.5 | 8.7 |
| Plasma (Heparin) | 40 | n.d. | 0 | -- |

* n.d. = non-detectable, samples measured below the lowest standard point are considered to be non-detectable.

## 13.8  Calibration

The immunoassay is calibrated with highly purified recombinant human IL-1$\beta$ which has been evaluated against the W.H.O. First International Standard for Interleukin-1$\beta$ (human) 86/552 and has been shown to be equivalent.
W.H.O. First International Standard for Interleukin-1$\beta$ (human) 86/552 is quantitated in WHO units, 1 WHO unit corresponding to 10 pg human IL-1$\beta$.

## 14  Ordering Information

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our website at www.eBioscience.com/distributors.

## 15   Reagent Preparation Summary

### 15.1  Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20 x (25 ml) to 475 ml distilled water

## 16  Test Protocol Summary

− Place standard strips in position A1/A2 to H1/H2.
− Add distilled water, in duplicate, to all standard and blank wells as indicated on the label of the standard strips.
− Add 100 µl distilled water to sample wells.
− Add 50 µl sample to designated wells.
− Cover microwell strips and incubate 3 hours at room temperature (18° to 25°C) if available on a microplate shaker at 400 rpm.
− Empty and wash microwell strips 6 times with 400 µl Wash Buffer.
− Add 100 µl of TMB Substrate Solution to all wells including blank wells.
− Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
− Add 100 µl Stop Solution to all wells including blank wells.
− Blank microwell reader and measure colour intensity at 450 nm.

**Note: Samples have been diluted 1:2, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**