

# PRODUCT INFORMATION & MANUAL

## Human IL-8/NAP-1 Instant ELISA

***BMS204/3INST***

Enzyme-linked immunosorbent assay for  
quantitative detection of human IL-8/NAP-1.

For research use only.

Not for diagnostic or therapeutic procedures.

128 Tests



### *Human IL-8/NAP-1 Instant ELISA*

#### **North America**

Technical Support:

Research Products:

888.810.6168

858.642.2058

tech@eBioscience.com

Clinical Products:

877.726.8559

858.642.2058

tech@eBioscience.com

Customer Service:

888.999.1371

858.642.2058

info@eBioscience.com

Fax:

858.642.2046

#### **Europe/International\***

Technical Support:

+43 1 796 40 40-120

tech@eBioscience.com

Customer Service:

+43 1 796 40 40-304

europa@eBioscience.com

Fax:

+43 1 796 40 40-400



Bender MedSystems GmbH

Campus Vienna Biocenter 2

1030 Vienna, Austria

www.eBioscience.com

\* Customers outside North America and Europe may contact their eBioscience distributor listed on our website at [www.eBioscience.com/distributors](http://www.eBioscience.com/distributors).

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## 1 Intended Use

The human IL-8/NAP-1 Instant ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human IL-8/NAP-1. **The human IL-8/NAP-1 Instant ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

## 2 Summary

Interleukin-8/Neutrophil-Activating Peptide-1 selectively stimulates the ability of neutrophils and T-lymphocytes to invade injured or inflamed tissue. IL-8 purified from peripheral blood monocytes is a 10 kDa protein showing at least 4 variant forms (77, 72, 70, and 69 amino acids, respectively) which differ in the length of the N-terminal and readily form dimers in solution. Two cystin-bridges are essential for the biological activity of IL-8. Exogenous stimuli like LPS, but also IL-1, TNF $\alpha$  and TNF $\beta$  induce the secretion of IL-8 in a variety of different cell types including monocytes, endothelial and epithelial cells, peripheral blood mononuclear cells, dermal fibroblasts, keratinocytes, neutrophils, hepatocytes, synovial cells, and T-lymphocytes. When IL-8 was subcutaneously injected into rats, both lymphocytes and neutrophils migrated to the site of injection within 3 hours. At lower dosages, only lymphocytes migrated towards the site of injection, while at higher dosages primarily neutrophils were attracted. It was found that T-lymphocytes are 10 times more sensitive to IL-8 than neutrophils. IL-8 exerts its effects via specific cell membrane receptors (chemotactic agonist receptor-family) with homogeneous high-affinity activity and two binding sites for its ligand. The receptor density is determined by the cell type and ranges from 300 on T-lymphocytes up to 20.000 on neutrophils. After binding of IL-8, the receptor expression is downregulated >90 % within 10 minutes at 37°C, together with the internalization of the ligand. IL-8 is proteolytically degraded in the cytoplasm and released into the culture medium as soluble fragments. The IL-8 receptors are probably recycled. Besides its chemotactic influence, IL-8 exerts other distinct characteristics. In neutrophils it triggers the secretion of superoxide anions and lysosomal enzymes, thereby indirectly augmenting the permeability of blood vessels, and IL-8 enhances the fungicidal activity against *Candida albicans*. Neutrophils are more readily liberated from the bone marrow reservoir under the influence of this cytokine. In vitro, IL-8 stimulates a rapid Mac-1 as well as CR 1, p150,95 and LFA-1 expression on neutrophils which enables

the adherence to activated vascular endothelial cells expressing e.g. ICAM-1. This may account for the accumulation of neutrophils at IL-8 injection sites. Other findings suggest that endothelial-derived IL-8 may function to attenuate inflammatory events at the interface between vessel wall and blood, via inhibiting neutrophil adhesion to cytokine-activated endothelial monolayers. Therefore these cells seem to be protected against neutrophil-mediated damage. In basophils, besides its chemotactic effects, IL-8 stimulates the histamin liberation in atopic as well as healthy persons.

The property of IL-8 to stimulate movement of neutrophils across endothelial monolayers in vitro supports the concept of a central role for this molecule in the accumulation of neutrophils at inflammatory lesions in vivo. Data indicate that IL-8 may participate in the pathogenesis of rheumatoid arthritis via the induction of neutrophil-mediated cartilage damage, and psoriasis. A causative involvement of IL-8 is found within a broad range of clinico-pathological conditions: adult respiratory distress syndrome, asthma, bacterial infections, bladder cancer, blood incompatability, contact dermatitis, empyema, graft rejection, glomerulonephritis, gout, hemolytic uremic syndrome, immune vasculitis, inflammatory bowel disease, influenza virus infection, Jarisch-Herxheimer reaction, malignancies of the central nervous system, myocardial infarction, placental infection, sepsis, uveitis, among others.

Due to the nowadays known biological properties of IL-8, this cytokine, especially in combinations with other neutrophil activating agents, may prove helpful in the treatment of patients suffering from granulocytopenia, severe infections against which antibiotics are not effective, and immunodeficiency caused by HI-virus.

For literature update refer to **[www.eBioscience.com](http://www.eBioscience.com)**

### 3 Principles of the Test

An anti-human IL-8/NAP-1 coating antibody is adsorbed onto microwells. Human IL-8/NAP-1 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated anti-human IL-8/NAP-1 antibody binds to human IL-8/NAP-1 captured by the first antibody. Streptavidin-HRP binds to the biotin conjugated anti-human IL-8/NAP-1.

Following incubation unbound biotin conjugated antihuman IL-8/NAP-1 and Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of soluble human IL-8/NAP-1 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-8/NAP-1 standard dilutions and human IL-8/NAP-1 sample concentration determined.

Figure 1

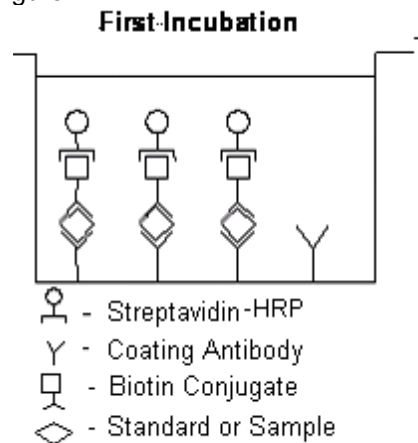


Figure 2

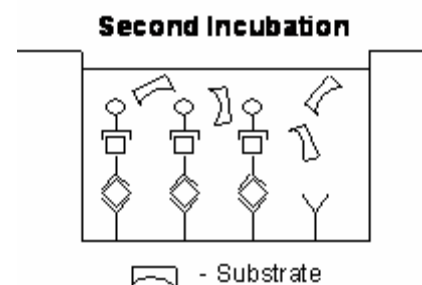
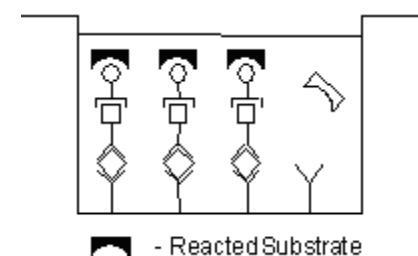


Figure 3



#### 4 Reagents Provided

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human IL-8/NAP-1, **Biotin-Conjugate** (anti-human IL-8/NAP-1 polyclonal antibody), **Streptavidin-HRP** and Sample Diluent, lyophilized
- 2 aluminium pouches with a human IL-8/NAP-1 **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 the 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, plasma (EDTA and heparin) and amniotic fluid were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

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

Samples must be stored frozen at -20°C to avoid loss of bioactive human IL-8/NAP-1. 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, the frozen sample should be brought to room temperature slowly and mixed gently.

\* Elevated blank signal was observed when using DMEM cell culture medium. No elevated blank signal was observed when using RPMI cell culture medium.

## **7 Materials Required But Not Provided**

- 5 ml and 10 ml graduated pipettes
- 5  $\mu$ l to 1000  $\mu$ l adjustable single channel micropipettes with disposable tips
- adjustable multichannel micropipettes (for volumes between 50  $\mu$ l and 500  $\mu$ l) 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 crystals 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 sample 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:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Standard 1 (1000.0 pg/ml)	Standard 1 (1000.0 pg/ml)	Sample 1	Sample 1
<b>B</b>	Standard 2 (500.0 pg/ml)	Standard 2 (500.0 pg/ml)	Sample 2	Sample 2
<b>C</b>	Standard 3 (250.0 pg/ml)	Standard 3 (250.0 pg/ml)	Sample 3	Sample 3
<b>D</b>	Standard 4 (125.0 pg/ml)	Standard 4 (125.0 pg/ml)	Sample 4	Sample 4
<b>E</b>	Standard 5 (62.5 pg/ml)	Standard 5 (62.5 pg/ml)	Sample 5	Sample 5
<b>F</b>	Standard 6 (31.3 pg/ml)	Standard 6 (31.3 pg/ml)	Sample 6	Sample 6
<b>G</b>	Standard 7 (15.6 pg/ml)	Standard 7 (15.6 pg/ml)	Sample 7	Sample 7
<b>H</b>	Blank	Blank	Sample 8	Sample 8

- 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 (18°C to 25°C) for 3 hours, if available on a microplate shaker at 100 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-8/NAP-1 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-8/NAP-1 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-8/NAP-1 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-8/NAP-1 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-8/NAP-1 levels. Such samples require further external predilution according to expected human IL-8/NAP-1 values with Sample Diluent in order to precisely quantitate the actual human IL-8/NAP-1 level.**
- It is suggested that each testing facility establishes a control sample of known human IL-8/NAP-1 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  $\mu\text{l}$  to the final volume per well. These 100  $\mu\text{l}$  are composed of 50  $\mu\text{l}$  of Sample Diluent plus 50  $\mu\text{l}$  of the sample. This is a 1:2 dilution.

The remaining 50  $\mu\text{l}$  to give 150  $\mu\text{l}$  are due to the addition of 50  $\mu\text{l}$  conjugate to all wells.

50  $\mu\text{l}$  Sample Diluent and 50  $\mu\text{l}$  conjugate results in 100  $\mu\text{l}$  reconstitution volume, addition of 50  $\mu\text{l}$  sample (50  $\mu\text{l}$  + 50  $\mu\text{l}$  = 1:2 dilution)

Figure 4

Representative standard curve for human IL-8/NAP-1 Instant ELISA. Human IL-8/NAP-1 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.

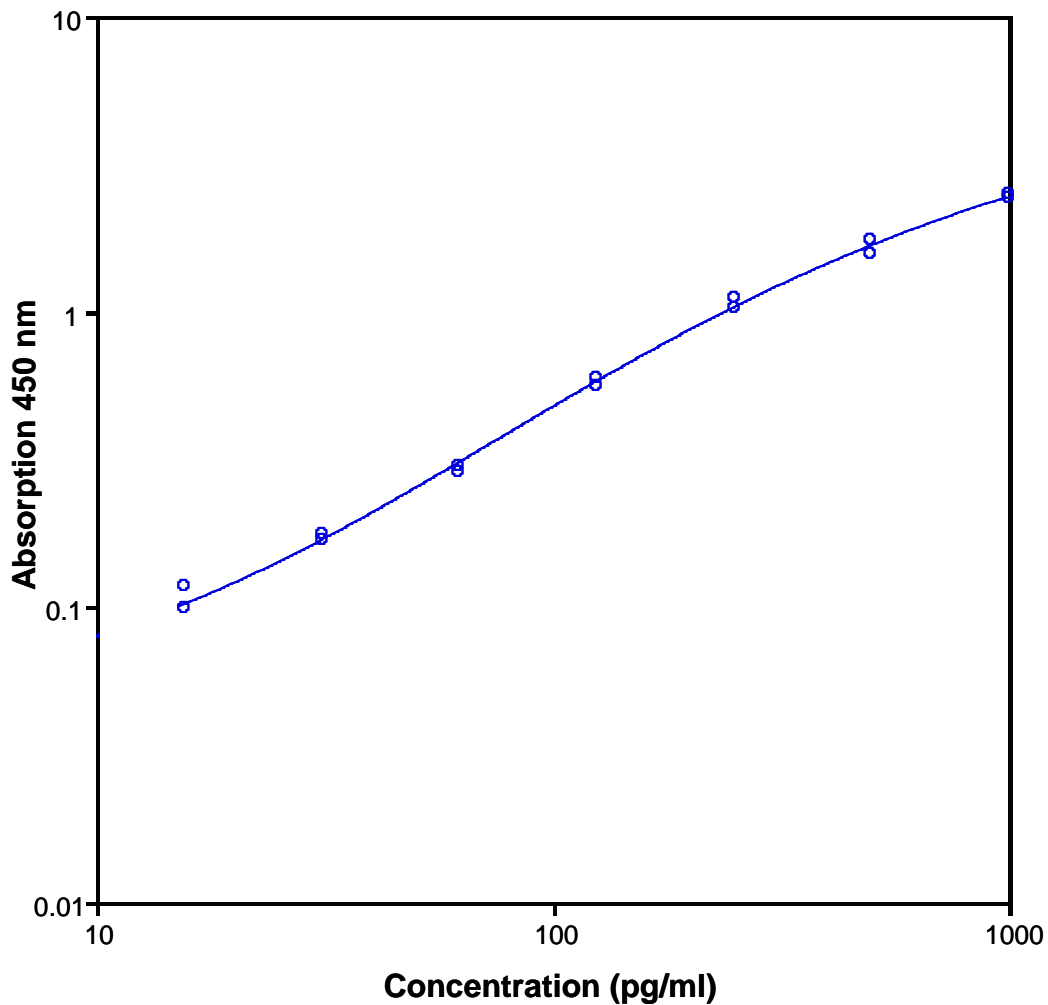


Table 2

Typical data using the human IL-8/NAP-1 INSTANT ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human IL-8/NAP-1 Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	1000.0	2.402 2.508	2.455	2.2
2	500.0	1.731 1.556	1.644	5.3
3	250.0	1.020 1.118	1.069	4.6
4	125.0	0.559 0.591	0.575	2.8
5	62.5	0.286 0.299	0.293	2.2
6	31.3	0.167 0.177	0.172	2.9
7	15.6	0.099 0.118	0.109	8.8
Blank	0.0	0.026 0.027	0.027	1.9

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.

## 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-8/NAP-1 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 1.3 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-8/NAP-1. 2 standard curves were run on each plate. Data below show the mean human IL-8/NAP-1 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 8.1%.

Table 3

The mean human IL-8/NAP-1 concentration and the coefficient of variation for each sample.

Positive Sample	Experiment	Human IL-8/NAP-1 Concentration (pg/ml)	Coefficient of Variation (%)
1	1	606.9	15
	2	543.7	11
	3	527.1	9
2	1	344.1	2
	2	306.2	11
	3	295.4	10
3	1	187.6	4
	2	144.1	6
	3	140.5	9
4	1	65.2	4
	2	57.0	7
	3	57.8	10
5	1	56.2	7
	2	47.4	10
	3	47.4	5
6	1	554.0	7
	2	506.4	11
	3	390.6	5
7	1	296.3	6
	2	254.8	9
	3	207.3	3
8	1	200.0	13
	2	196.5	12
	3	202.2	10

### 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 8 serum samples containing different concentrations of human IL-8/NAP-1. 2 standard curves were run on each plate. Data below show the mean human IL-8/NAP-1 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 10.8%.

Table 4

The mean human IL-8/NAP-1 concentration and the coefficient of variation of each sample

Sample	Mean Human IL-8/NAP-1 Concentration (pg/ml)	Coefficient of Variation (%)
1	559.2	7.5
2	315.2	8.1
3	157.4	16.7
4	60.0	7.6
5	50.3	10.0
6	483.7	17.4
7	252.8	17.6
8	199.6	1.4

### 13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human IL-8/NAP-1 into serum. Recoveries were determined in 3 independent experiments with 6 replicates each. The unspiked serum was used as blank in these experiments.

Average recovery ranged from 72% to 118% with an overall mean recovery of 88%.

### 13.4 Dilution Parallelism

4 serum samples with different levels of human IL-8/NAP-1 were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 108% and 120% with an overall recovery of 115% (see Table 5).

Table 5

Sample	Dilution	Mean Human IL-8/NAP-1 Concentration (pg/ml)		% Recovery of Exp. Val.
		Expected Value	Observed Value	
1	---	---	598.1	---
	1:2	299.0	349.7	116.9
	1:4	174.8	195.7	111.9
	1:8	97.8	108.9	111.3
2	---	---	322.4	---
	1:2	161.2	174.7	108.4
	1:4	87.4	101.3	116.0
	1:8	50.7	59.6	117.6
3	---	---	56.1	---
	1:2	28.0	33.4	119.1
	1:4	16.7	19.2	114.9
	1:8	9.6	10.6	110.0
4	---	---	220.3	---
	1:2	110.1	122.2	111.0
	1:4	61.1	73.1	119.6



## 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-8/NAP-1 levels determined. There was no significant loss of human IL-8/NAP-1 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 IL-8/NAP-1 level determined after 24 h.

There was no significant loss of human IL-8/NAP-1 immunoreactivity detected during storage under above conditions.

## 13.6 Specificity

The assay detects both natural and recombinant human IL-8/NAP-1. To define the specificity of this ELISA several peptides with high homology to human IL-8/NAP-1 were tested for cross reactivity. At concentrations up to 1000 pg/ml, connective tissue activating peptide 3, platelet factor 4, and neutrophil activating peptide 2 as well as IL-6, IL-2R, rhTNF $\beta$  and CD8 did not show any cross reactivity.

## 13.7 Expected Values

A panel of 40 serum samples from randomly selected apparently healthy donors (males and females) was tested for human IL-8/NAP-1.

For detected human IL-8/NAP-1 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. - 666	22.5	114

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

## 14 Ordering Information

### North America

#### Technical Support:

Research Products:  
888.810.6168  
858.642.2058  
tech@eBioscience.com

Clinical Products:  
877.726.8559  
858.642.2058  
tech@eBioscience.com

#### Customer Service:

888.999.1371  
858.642.2058  
info@eBioscience.com

#### Fax:

858.642.2046

### Europe/International\*

#### Technical Support:

+43 1 796 40 40-120  
tech@eBioscience.com

#### Customer Service:

+43 1 796 40 40-304  
europe@eBioscience.com

#### Fax:

+43 1 796 40 40-400



Bender MedSystems GmbH  
Campus Vienna Biocenter 2  
1030 Vienna, Austria  
www.eBioscience.com

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## 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 100 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).**