

PRODUCT INFORMATION & MANUAL

Human TNF- β Instant ELISA

BMS202INST

Enzyme-linked immunosorbent assay for
quantitative detection of human TNF- β .

For research use only.

Not for diagnostic or therapeutic procedures.

128 Tests



*Human TNF- β
Instant ELISA*

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TABLE OF CONTENTS

1	Intended Use	3
2	Summary	3
3	Principles of the Test	6
4	Reagents Provided	7
5	Storage Instructions	8
6	Specimen Collection	8
7	Materials Required But Not Provided	9
8	Precautions for Use	10
9	Preparation of Reagents and Samples	12
10	Test Protocol	12
11	Calculation of Results	17
12	Limitations	20
13	Performance Characteristics	21
14	Ordering Information	26
15	Reagent Preparation Summary	27
16	Test Protocol Summary	28

1 Intended Use

The human TNF- β Instant ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human Tumor Necrosis Factor β in cell culture supernatants, human serum, plasma, urine, synovial fluid, amniotic fluid or other body fluids. The human TNF- β Instant ELISA is **for research use only. Not for diagnostic or therapeutic procedures.**

2 Summary

Tumor Necrosis Factor (TNF) was originally discovered as a serum protein with necrotizing effects on certain transplantable mouse tumors in vivo and cytotoxic effects against some transformed cells in vitro. The TNF family consists of two proteins designated TNF- α , also called cachectin, and TNF- β , also called lymphotoxin, which are pleiotropic cytokines that can mediate a wide variety of biological effects.

TNF- β is produced by activated lymphocytes, whereas TNF- α is mostly produced by activated macrophages. Soluble TNF- β is a T-cell derived glycoprotein of 25 kD encoded by a gene within the MHC. The molecule consists of a 17.5 to 18 kD polypeptide core and 7kD of N-linked carbohydrate. TNF- β is 28 % structurally homologous with the macrophage produced non-glycoprotein TNF- α . The genes for TNF- α and TNF- β are closely linked, and the proteins share biological activities. TNF has been shown to interact with a cell through specific high-affinity receptors with a few hundred up to more than 20.000 copies per cell. In a variety of cell lines, two different TNF-receptor proteins have been identified and the cDNAs cloned. Recently, an ELISA has been established by Adolf and Apfler which provides a simple, rapid, and highly sensitive method for the determination of soluble TNF-R (60 kDa) levels in body fluids or cell culture supernatants.

TNF- β is induced in an antigen-specific MHC restricted fashion from class I and class II restricted T cells. Viral infection is also associated with TNF- β production by lymphoid cells. TNF- β has several effects on target cells including killing, growth stimulation, induction of adhesion molecule (ICAM-1) expression, and induction of differentiation. The mechanisms of TNF- β effects involve receptor binding and internalization and several sequelae including changes in prostaglandins

and chromosome integrity. TNF- β participates in tumor immunity, and it has been reported to inhibit carcinogenesis as well as growth of some tumors in vivo.

Recent studies have demonstrated that both TNF- α and TNF- β are capable of activating neutrophils in vitro. The exposure of neutrophils to TNF- α or TNF- β causes the production of superoxide radicals, induces phagocytic response and enhances antibody dependent cell cytotoxicity. The release of IL-1 from human endothelial cells is also induced by TNF- α and TNF- β . All the in vitro studies suggest that TNF- β may play an important role in immunoregulation. In fibroblasts TNF- β induces the synthesis of colony-stimulating factors, IL-1, collagenase and prostaglandin-E2. Monocytes are stimulated for terminal differentiation. On B-cells TNF- β acts as mitogen. As TNF- β exerts proliferative capacity on fibroblasts it may participate in the process of wound healing.

Recently, TNF- β was found to belong to the group of endogenous pyrogens/sleep factors. Elevated TNF- β levels were also found in patients with adult T-cell leukemia and hypercalcemia, diabetes as well as malaria.

While both TNFs inhibit growth of tumor cells, they stimulate the growth of human lung fibroblasts and dermal fibroblasts. TNF- β also acts as antiviral agent against a variety of RNA (VSV and EMCV) and DNA (Ad-2 and HSV-2) viruses and its activity is potentiated by interferon-gamma in a synergistic fashion. Moreover, TNF- β contributes to the defense against parasitic infections and induces osteoclastic bone resorption and inhibition of bone collagen synthesis.

The elucidation of the physiological and pathophysiological role was limited due to a lack of adequate assay systems. The present assay developed by Adolf and Lamche provides a simple and rapid method for determination of serum levels of TNF- β with a minimal detectable dose as low as 7 pg/ml serum. This assay will help to clarify the possible diagnostic and prognostic value of circulating TNF- β in various neoplastic and inflammatory diseases. The assay detects recombinant, unglycosylated lymphotoxin with the same sensitivity as the natural, glycosylated protein, shows good correlation with the standard

cytotoxicity bioassay, and is specific for biologically active TNF- β without TNF- α cross reactivity.

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

3 Principles of the Test

An anti-human TNF- β monoclonal coating antibody is adsorbed onto microwells. Human TNF- β present in the sample or standard binds to antibodies adsorbed to the microwells; an HRP-conjugated monoclonal anti-human TNF- β antibody binds to human TNF- β captured by the first antibody. Following incubation unbound enzyme conjugated anti-human TNF- β 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 TNF- β present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven human TNF- β standard dilutions and human TNF- β sample concentration determined.

Figure 1

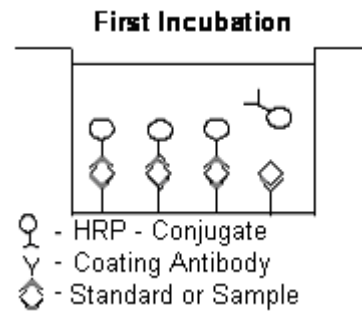


Figure 2

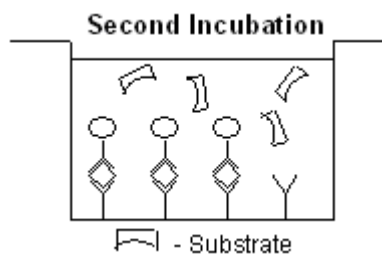
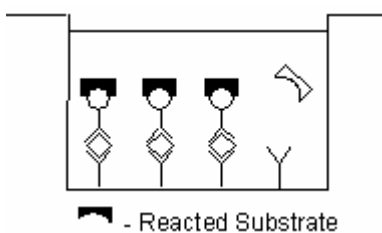


Figure 3



4 Reagents Provided

- 1 aluminium pouch with a **Microwell Plate coated with Monoclonal Antibody** (murine) to human TNF- β , **HRP-Conjugate** (anti-TNF- β monoclonal antibody), and Sample diluent, lyophilized
- 2 aluminium pouches with a **human TNF- β Standard curve (coloured)**
- 1 bottle (25 ml) **Wash Buffer Concentrate 20x**
(phosphate-buffered saline with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (5 ml) **Sample Diluent**
(Use when an external predilution of the samples is needed)
- 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
- 2 adhesive **Plate Covers**

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 supernatants, human serum, plasma, urine, synovial fluid and amniotic fluid were tested with this assay. Other biological samples might be suitable for use in the assay. Remove the serum or plasma from the clot or red 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 TNF- β . 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
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis

8 Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate 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 in order that all the lyophilised 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, 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 -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 50 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 (1000.0 pg/ml)	Standard 1 (1000.0 pg/ml)	Sample 1	Sample 1
B	Standard 2 (500.0 pg/ml)	Standard 2 (500.0 pg/ml)	Sample 2	Sample 2
C	Standard 3 (250.0 pg/ml)	Standard 3 (250.0 pg/ml)	Sample 3	Sample 3
D	Standard 4 (125.0 pg/ml)	Standard 4 (125.0 pg/ml)	Sample 4	Sample 4
E	Standard 5 (62.5 pg/ml)	Standard 5 (62.5 pg/ml)	Sample 5	Sample 5
F	Standard 6 (31.3 pg/ml)	Standard 6 (31.3 pg/ml)	Sample 6	Sample 6
G	Standard 7 (15.6 pg/ml)	Standard 7 (15.6 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- d. Add 100 µl of each **Sample**, in duplicate, to the designated wells and mix the contents.
- e. Cover with a **Plate Cover** and incubate at room temperature (18°C to 25°C) for 4 hours, if available on a microplate shaker at 400 rpm.
- f. Remove **Plate Cover** 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. 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 minutes. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see point i. 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 TNF- β 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 TNF- β concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating human TNF- β 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 TNF- β concentration.
- ***As the samples have not been diluted, the value read from the standard curve is the actual concentration of human TNF- β in the sample.**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human TNF- β levels. Such samples require further external predilution according to expected human TNF- β values with Sample Diluent in order to precisely quantitate the actual human TNF- β level.**
- It is suggested that each testing facility establishes a control sample of known human TNF- β 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: The samples contribute 100 μ l to the final volume per well. The remaining 50 μ l to give 150 μ l are due to the addition of 50 μ l conjugate to all wells.

Figure 4

Representative standard curve for human TNF- β Instant ELISA. Human TNF- β 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.

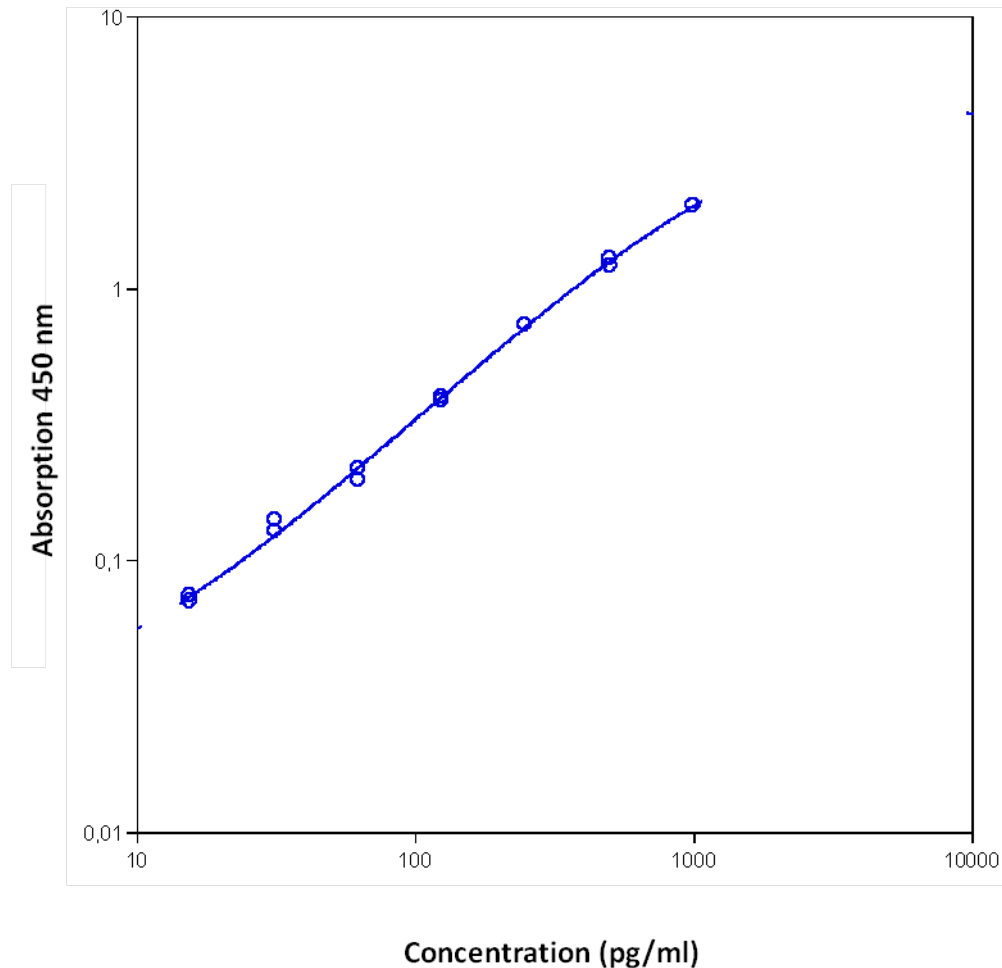


Table 2

Typical data using the human TNF- β INSTANT ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	human TNF- β Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	1000.0	2.006 2.003	2.005	2.7
2	500.0	1.281 1.210	1.245	3.6
3	250.0	0.735 0.737	0.736	1.4
4	125.0	0.398 0.389	0.394	1.4
5	62.5	0.218 0.198	0.208	4.2
6	31.3	0.140 0.127	0.134	7.4
7	15.6	0.074 0.071	0.073	9.3
Blank	0.0	0.020 0.021	0.021	3.4

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 TNF- β 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 4.6 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 TNF- β . 2 standard curves were run on each plate. Data below show the mean human TNF- β concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 4.1%.

Table 3

The Mean human TNF- β concentration and the coefficient of variation for each sample.

Positive Sample	Experiment	human TNF- β Concentration (pg/ml)	Coefficient of Variation (%)
1	1	585	1.0
	2	572	7.0
	3	560	8.0
2	1	287	4.0
	2	281	6.0
	3	301	7.0
3	1	145	5.0
	2	148	3.0
	3	164	2.0
4	1	73	5.0
	2	72	2.0
	3	80	4.0
5	1	458	1.0
	2	447	9.0
	3	491	5.0
6	1	242	2.0
	2	236	3.0
	3	276	8.0
7	1	123	3.0
	2	125	2.0
	3	150	6.0
8	1	64	2.0
	2	69	2.0
	3	76	4.0

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 TNF- β . 2 standard curves were run on each plate. Data below (see Table 4) show the mean human TNF- β concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall coefficient of variation was 6.4%.

Table 4

The mean human TNF- β concentration and the coefficient of variation calculated on 18 determinations of each sample.

Sample	human TNF- β Concentration (pg/ml)	Coefficient of Variation (%)
1	572	2.2
2	290	3.6
3	152	6.8
4	75	5.6
5	465	4.8
6	251	8.7
7	133	11.2
8	70	8.4

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human TNF- β into pooled normal human serum. The unspiked serum was used as blank in these experiments. Average recovery ranged from 82% to 103% with an overall mean recovery of 88%.

13.4 Dilution Parallelism

4 serum samples with different levels of human TNF- β were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 98% and 108% with an overall recovery of 104% (see Table 5).

Table 5

Sample	Dilution	TNF- β Concentration (pg/ml)		% Recovery of Exp. Val.
		Expected Value	Observed Value	
1	-	-	1145	-
	1:2	572	580	101
	1:4	290	305	105
2	-	-	930	-
	1:2	465	503	108
	1:4	251	265	106
3	-	-	1209	-
	1:2	605	650	108
	1:4	325	328	101
4	-	-	1319	-
	1:2	660	671	102
	1:4	336	330	98

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed 5 times, and the human TNF- β levels determined. There was no significant loss of human TNF- β concentrations between 0 and 5 freeze-thaw cycles.

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 TNF- β level determined after 24 h. There was no significant loss of human TNF- β immunoreactivity during storage under above conditions.

13.6 Comparison of Serum and Plasma

From two individuals, serum as well as EDTA and citrate, and heparin plasma obtained at the same time point were evaluated. Human TNF- β

concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

13.7 Specificity

To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed.

13.8 Expected Values

A panel of 40 randomly selected sera from healthy blood donors (male and female) was tested for human TNF- β . The detected human TNF- β levels ranged between 1.0 and 1125.5 pg/ml with a mean level of 140.4 pg/ml and a standard deviation of ± 309 pg/ml.

13.9 Calibration

The immunoassay is calibrated with highly purified recombinant human TNF- β which has been evaluated against the international Reference Standard NIBSC 87/640 and has been shown to be equivalent. NIBSC 87/640 is quantitated in International Units (IU), 1IU corresponding to 6.7pg human TNF- β .

14 Ordering Information

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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 50 µl distilled water to sample wells.
- Add 100 µl Sample to designated wells.
- Cover microwell strips and incubate 4 hours at room temperature (18° to 25°C) 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.