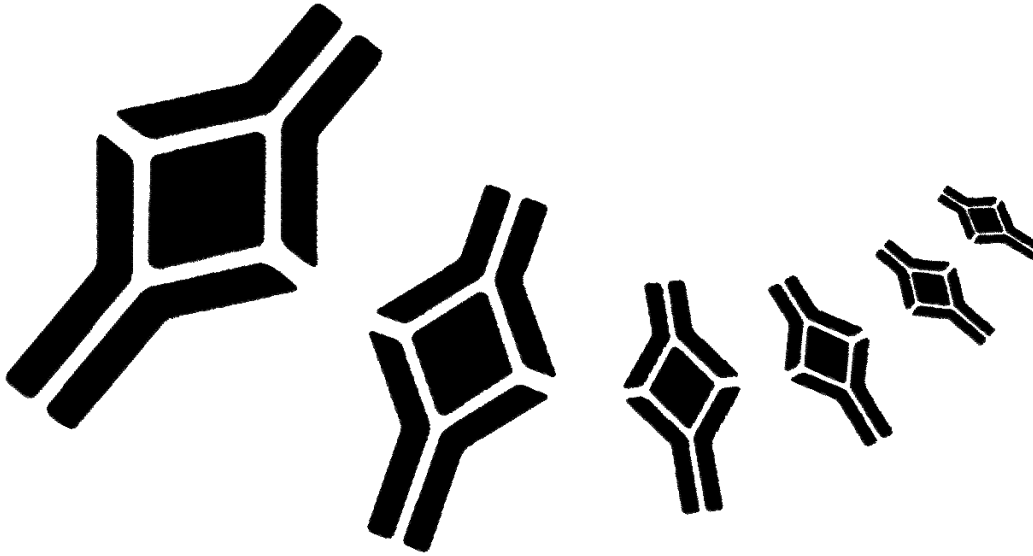


BioVendor

Research
and Diagnostic Products



HUMAN TNF- β ELISA

Product Data Sheet

Cat. No.: RBMS202R

For Research Use Only

CONTENTS

1.	INTENDED USE	2
2.	SUMMARY	2
3.	PRINCIPLES OF THE TEST	5
4.	REAGENTS PROVIDED	6
5.	STORAGE INSTRUCTIONS	7
6.	SPECIMEN COLLECTION	7
7.	MATERIALS REQUIRED BUT NOT PROVIDED	8
8.	PRECAUTIONS FOR USE	9
9.	PREPARATION OF REAGENTS	11
10.	TEST PROTOCOL	15
11.	CALCULATION OF RESULTS	19
12.	LIMITATIONS	22
13.	PERFORMANCE CHARACTERISTICS	23
14.	REFERENCES	25
15.	REAGENT PREPARATION SUMMARY	28
16.	TEST PROTOCOL SUMMARY	29

**»» This kit is manufactured by:
BioVendor – Laboratorní medicína, a.s.**

»» Use only the current version of Product Data Sheet enclosed with the kit!

1 INTENDED USE

The TNF- β ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of tumor Necrosis Factor- β (Lymphotoxin) levels in cell culture supernatants, human serum, plasma, or other body fluids. **The TNF- β ELISA is for research use only. Not for use in 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 (3, 7, 18). The TNF family consists of two proteins designated TNF- α , also called cachectin (5), and TNF- β , also called lymphotoxin (19), 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 (19). Soluble TNF- β is a T-cell derived glycoprotein of 25 kD encoded by a gene within the MHC (22). The molecule consists of a 17.5 to 18 kD polypeptide core and 7kD of N-linked carbohydrate (16, 19). 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 (10, 16). 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 (11, 20, 23). Recently, an ELISA has been established by Adolf and Apfler (1) 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 (6), 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 (3, 19).

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 (21). The release of IL-1 from human endothelial cells is also induced by TNF- α and TNF- β (17). 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.

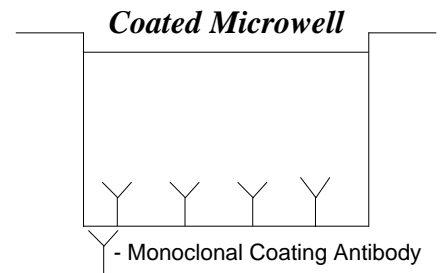
Recently, TNF- β was found to belong to the group of endogenous pyrogens/sleep factors (13). Elevated TNF- β levels were also found in patients with adult T-cell leukemia and hypercalcemia (12), diabetes (15) as well as malaria (8).

While both TNFs inhibit growth of tumor cells, they stimulate the growth of human lung fibroblasts and dermal fibroblasts (24). 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 (25). Moreover, TNF- β contributes to the defense against parasitic infections and induces osteoclastic bone resorption and inhibition of bone collagen synthesis (4).

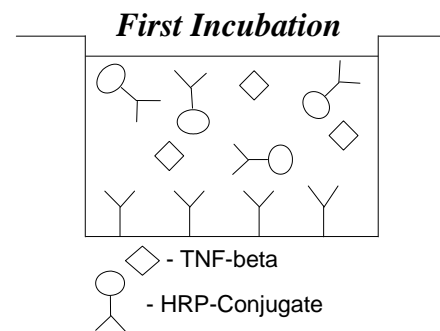
The elucidation of the physiological and pathophysiological role was limited due to a lack of adequate assay systems. The present assay developed by Adolf and Lamche (2) provides a simple and rapid method for determination of serum levels of TNF- β with a minimal detectable dose as low as 7 pg/ml serum. 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.

3 PRINCIPLES OF THE TEST

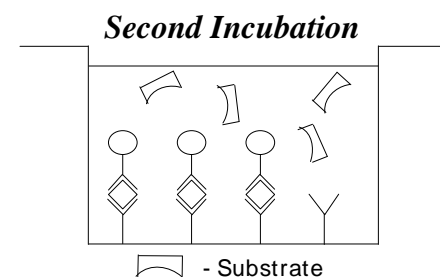
An anti-TNF- β monoclonal coating antibody is adsorbed onto microwells.



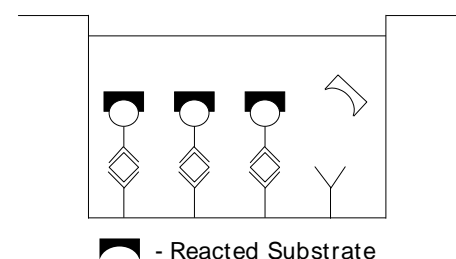
TNF- β present in the sample or standard binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-TNF- β antibody is added and binds to TNF- β captured by the first antibody.



Following incubation unbound enzyme conjugated anti-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 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 eight TNF- β standard dilutions and TNF- β sample concentration determined.



4 REAGENTS PROVIDED

- 1 aluminium pouch with a **Antibody Coated Microtiter Strips** with Monoclonal Antibody (murine) to human TNF- β
- 1 vial (0.2 ml) **HRP-Conjugate** anti-TNF- β monoclonal (murine) antibody
- 2 vials (0.05 ml) 10 ng/ml **TNF- β Standard** concentrate
- 1 bottle (50 ml) **Concentrate Wash Buffer** (PBS with 1 % Tween 20)
- 1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1 % Tween 20 and 10 % BSA)
- 1 bottle (5 ml) **Sample Diluent** (protein matrix)
- 1 vial (7 ml) **Substrate Solution I** (tetramethyl-benzidine)
- 1 vial (7 ml) **Substrate Solution II** (0.02 % buffered hydrogen peroxide)
- 1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)
- 2 vials (0.4 ml each) **Blue-Dye, Green-Dye**
- 2 adhesive **Plate Covers**

Reagent Labels

5 STORAGE INSTRUCTIONS

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.

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 and plasma, or other biological samples like cerebrospinal fluid will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, 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.

Clinical samples should be kept at 2° to 8°C and separated rapidly (within half an hour) before storing at -20°C to avoid loss of bioactive TNF- β (9). Addition of protease inhibitors may account for better stability of samples. Avoid repeated freeze-thaw cycles.

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 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 solutions 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 reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- The protein matrix used as sample diluent is tested and found negative for HBsAg by the most sensitive techniques (third generation). Similarly, they are tested and found negative in tests for anti-HIV.

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

Except for HRP-Conjugate (reagent C., see page 12), the TNF- β Standard (reagent D., s. page 12), and the TMB Substrate Solution (reagent E., s. page 13) the reagents should be prepared before starting with the test procedure.

A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 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 is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

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

B. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

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

C. Preparation of HRP-Conjugate

The HRP-Conjugate must be diluted 1:100 with **Assay Buffer** (reagent B) just prior to use in a clean plastic test tube.

Please note that the HRP-Conjugate should be used within 30 minutes after dilution. HRP-Conjugate may be prepared as needed according to the following table:

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

D. Preparation of TNF- β Standard

Add **Sample Diluent** or respective diluent if other samples than sera are tested (e.g. cell culture medium) to one vial of concentrated **TNF- β Standard** as needed. The volume is stated on the label of the standard vial. Shake gently to mix. Store diluted Standard promptly at -20°C after use. Discard after one week.

E. TMB Substrate Solution

Using clean pipettes and containers known to be metal free, dispense an equal volume of **Substrate Solution I** into **Substrate Solution II** and swirl gently to mix. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect product performance. A blue colour present in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. The TMB Substrate Solution must be used within a few minutes after mixing. Warm to room temperature before use. Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation.

Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

F. Addition of colour-giving reagents: **Blue-Dye**, **Green-Dye**

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**) can be added to the reagents according to the following guidelines:

- 1. Diluent:** Before 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 Diluent	20 µl Blue-Dye
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- 2. HRP-Conjugate:** Before dilution of the concentrated conjugate, add the **Green-Dye** at a dilution of 1:100 (see table below) to the Assay Buffer used for the final conjugate dilution. Proceed after addition of **Green-Dye** according to the instruction booklet, preparation of HRP-conjugate.

3 ml Assay Buffer	30 µl Green-Dye
6 ml Assay Buffer	60 µl Green-Dye
12 ml Assay Buffer	120 µl Green-Dye

10 TEST PROTOCOL

- a. Mix all reagents thoroughly without foaming before use.
- b. 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 coated with Monoclonal Antibody** (murine) to human TNF- β from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- c. Wash the microwell strips twice with approximately 300 μ l 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 not longer than 15 minutes. Do not allow wells to dry.

- d. Add 100 μ l of **Sample Diluent** or respective diluent if other samples than sera are tested, in duplicate, to the standard wells, leaving the first wells (1000 pg/ml) empty. Prepare standard dilutions by pipetting 200 μ l of **TNF- β Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents by repeated aspiration and ejection, and transfer 100 μ l to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Mix the contents by repeated aspiration and ejection, and transfer 100 μ l to well C1 and C2, respectively. Continue this procedure five times, creating two rows of TNF- β standard dilutions ranging from 1000 to 8 pg/ml. Discard 100 μ l of the contents from the last microwells (H1, H2) used.

Figure 1. Preparation of TNF-β standard dilutions:

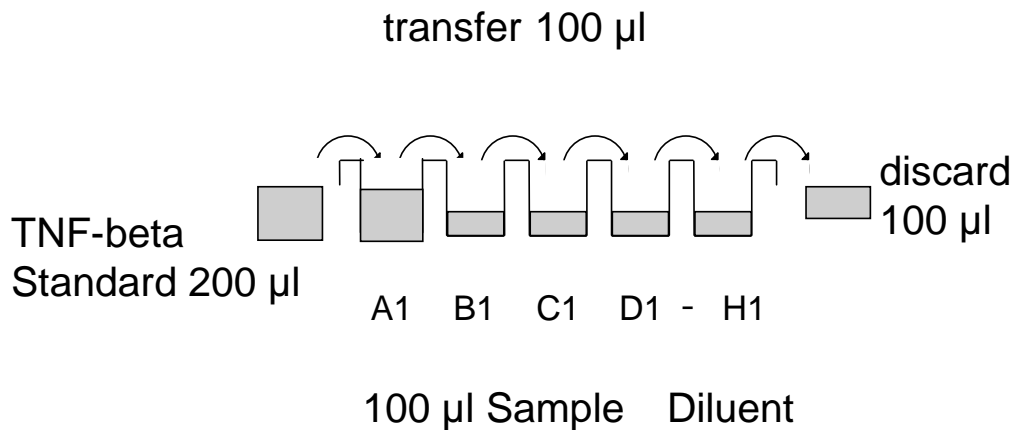


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (1000 pg/ml)	Standard 1 (1000 pg/ml)	Blank	Blank
B	Standard 2 (500 pg/ml)	Standard 2 (500 pg/ml)	Sample	1
C	Standard 3 (250 pg/ml)	Standard 3 (250 pg/ml)	Sample	2
D	Standard 4 (125 pg/ml)	Standard 4 (125 pg/ml)	Sample	3
E	Standard 5 (63 pg/ml)	Standard 5 (63 pg/ml)	Sample	4
F	Standard 6 (31 pg/ml)	Standard 6 (31 pg/ml)	Sample	5
G	Standard 7 (16 pg/ml)	Standard 7 (16 pg/ml)	Sample	6
H	Standard 8 (8 pg/ml)	Standard 8 (8 pg/ml)	Sample	7

- e. Add 100 µl of **Sample Diluent** or respective diluent if other samples than sera are tested, in duplicate, to the blank wells.
- f. Add 100 µl of each **Sample**, in duplicate, to the designated wells.
- g. Prepare **HRP-Conjugate** (refer to preparation of reagents 9.C.).
- h. Add 50 µl of 1:100 diluted **HRP-Conjugate** to all wells, including the blank wells.
- i. Cover with a **Plate Cover** and incubate at room temperature (18 to 25°C) for 4 hours, if available on a rotator set at 100 rpm.
- j. Prepare **TMB Substrate Solution** a few minutes prior to use. (Refer to preparation of reagents 9.E.)
- k. Remove **Plate Cover** and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- l. Pipette 100 µl of mixed **TMB Substrate Solution** to all wells, including the blank wells.
- m. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see point o. of this protocol) before positive wells are no longer properly recordable.

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 620nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

- o. Stop the enzyme reaction by quickly pipetting 100 μ l 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

- p. 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 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.

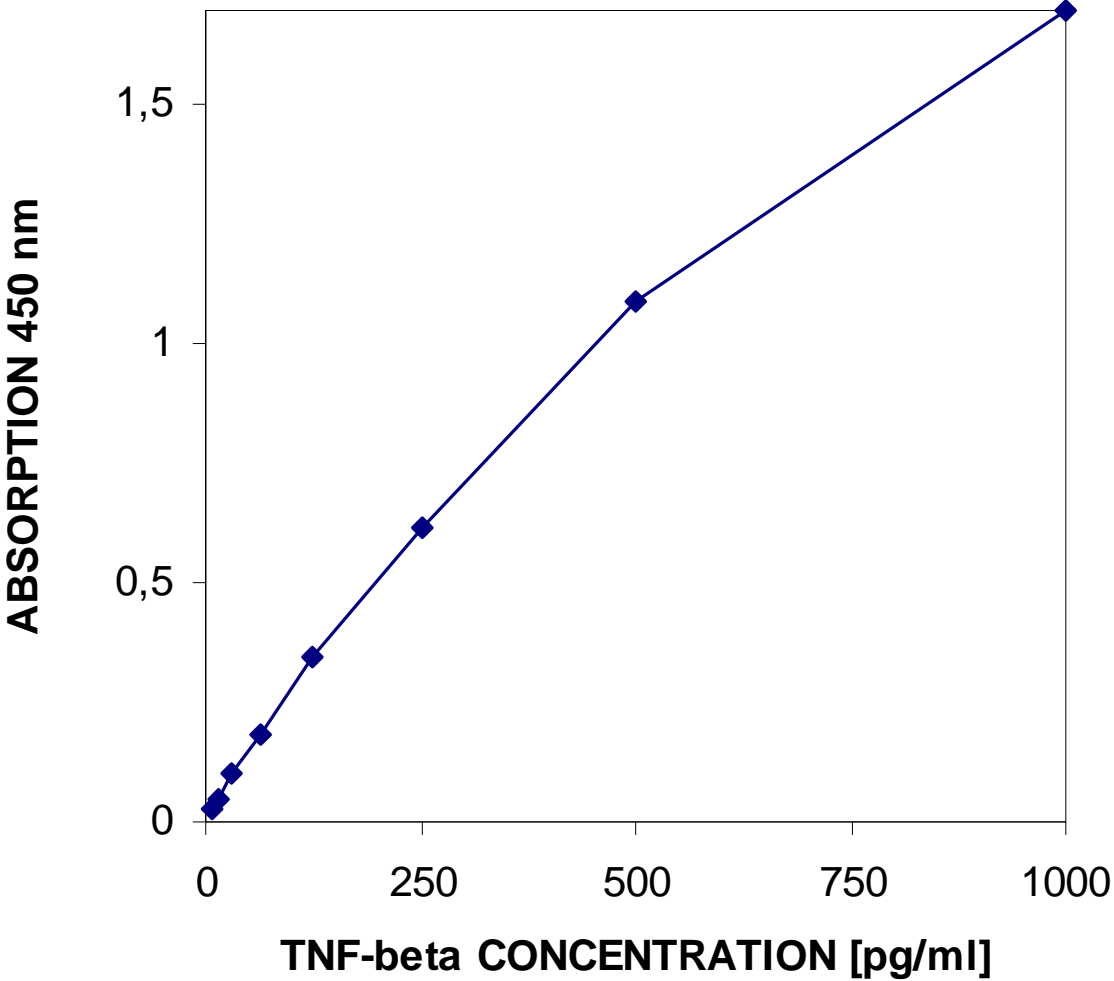
11 CALCULATION OF RESULTS

- 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 TNF- β concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating 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 TNF- β concentration.
- **For samples which have been diluted according to the instructions given in this manual the concentration read from the standard curve must be multiplied by the dilution factor.**

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low TNF- β levels. Such samples require further dilution of 1:2 - 1:4 with Sample Diluent in order to precisely quantitate the actual TNF- β level.

- It is suggested that each testing facility establishes a control sample of known 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 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for TNF-β ELISA. Recombinant soluble TNF-β was diluted in serial two-fold steps in Sample Diluent; symbols represent the mean of three 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.



Typical data using the TNF- β ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	TNF- β Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	1000	1.687	1.693	0.5
	1000	1.700		
2	500	1.112	1.086	3.3
	500	1.061		
3	250	0.605	0.612	1.6
	250	0.619		
4	125	0.345	0.344	0.2
	125	0.344		
5	63	0.183	0.179	3.2
	63	0.175		
6	31	0.105	0.100	7.1
	31	0.095		
7	16	0.046	0.046	0.0
	16	0.046		
8	8	0.029	0.028	5.1
	8	0.027		
Blank	0	0.014	0.015	
	0	0.015		

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 detergent before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, 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 antibody (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 analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Sample Diluent.

13 PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection for recombinant TNF- β , spiked into normal human serum, defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations) was determined to be 4.6 pg/ml (mean of three independent assays).

B. Reproducibility

Intra-/ Inter-assay

To determine the reproducibility of the assay, artificial samples were generated by spiking recombinant TNF- β into pooled normal human serum. These samples were tested in five independent assays (three plates per assay, four replicates per plate). Two standard curves were run on each plate. Nominal spike concentrations were 40 pg/ml (‘low’), 200 pg/ml (‘medium’), and 750 pg/ml (‘high’) respectively.

	Coefficient of Variation (%)
Intra-assay precision	
‘low’ level sample	8.5
‘medium’ level sample	8.2
‘high’ level sample	7.3
Inter-assay precision	
‘low’ level sample	10.6
‘medium’ level sample	11.2
‘high’ level sample	8.7

C. Specificity

The TNF- β ELISA is specific for human TNF- β . Only biologically active protein is recognized by the antibodies, since inactivation of TNF- β measured by bioassay results in a parallel decrease in immunoreactivity.

Natural, glycosylated TNF- β shows the same reactivity as recombinant, unglycosylated protein. The test is unaffected by the presence of denatured TNF- β , recombinant human TNF- α even at the highest concentration tested (0.5 mg/ml), or recombinant human interferon- α 2c at a concentration up to 1 mg/ml.

Unexpectedly, recombinant human interferon-gamma, which is unrelated to TNF- β in its primary structure, showed a very low but reproducible cross-reactivity of 0.00012 %. Since serum levels of interfer-gamma are well below 1 ng/ml, this cross-reactivity is irrelevant for all practical purposes. There was no interference with sTNF-R (60 kDa) up to a concentration of 400 ng/ml.

D. Correlation

Thermal inactivation of TNF- β was used to investigate the correlation of the ELISA with a widely used cytotoxicity bioassay for TNF- β (2, 14). Recombinant TNF- β was denatured at 80°C for various time periods; samples were tested in the ELISA as well as in the cytotoxicity bioassay on L-M cells. The correlation coefficient was 0.996.

E. Recovery Studies

Spiked samples were prepared by adding TNF- β to five sera from healthy donors at two dose levels. Recoveries determined by ELISA varied from 87 % to 130 %; with a calculated mean recovery of 105 %.

F. Expected Values

A panel of 40 sera from healthy blood donors (male and female) was tested for TNF- β . The detected 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.

G. This immunoassay is calibrated with highly purified recombinant 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 TNF- β .

14 REFERENCES

- 1) Adolf, G.R., and I. Apfler. (1991). A monoclonal antibody-based enzyme immunoassay for quantitation of human tumor necrosis factor binding protein I, a soluble fragment of the 60 kDa TNF-receptor in biological fluids. *J. Immunol. Meth.* 143, 127-136.
- 2) Adolf, G.R., and H.R. Lamche. (1990). Highly sensitive enzyme immunoassay for human lymphotoxin (tumor necrosis factor β) in serum. *J. Immunol. Meth.* 130, 177-185.
- 3) Aggarwal, B.B. (1987). Tumor necrosis factors - TNF- α and TNF- β : their structure and pleiotropic biological effects. *Drugs Future* 12, 891-898.
- 4) Bertolini, D.R., G.E. Nedwin, T.S. Bringman, D.D. Smith, and G.R. Mundy. (1986). Stimulation of bone resorption and inhibition of bone formation in vitro by human tumor necrosis factors. *Nature* 319, 516-518.
- 5) Beutler, B., and A. Cerami. (1987). Cachectin: more than a tumor necrosis factor. *N. Engl. J. Med.* 316, 379-385.
- 6) Broudy, V.C., J.M. Harlan, and J.W. Adamson. (1987). Disparate effects of tumor necrosis factor- α /cachectin and tumor necrosis factor- β /lymphotoxin on haematopoietic growth factor production and neutrophil adhesion molecule expression by cultured human endothelial cells. *J. Immunol.* 138, 4298-4302.
- 7) Carswell, E.A., L.J. Old, R.L. Kassel, S. Green, N. Fiore, and B. Williamson. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA* 72, 3666-3670.
- 8) Clark, I.A., K.M. Gray, E.J. Rockett, W.B. Cowden, K.A. Rockett, A. Ferrante, and B.B. Aggarwal. (1992). Increased lymphotoxin in human malarial serum, and the ability of this cytokine to increase plasma interleukin-6 and cause hypoglycaemia in mice – implications for malarial pathology. *Trans. Roy. Soc. Trop. Med. Hyg.* 86, 602-607.

- 9) Exley, A.R., and J. Cohen. (1980). Optimal collection of blood samples for the measurement of tumor necrosis factor α . *Cytokine* 2, 353-356.
- 10) Gray, P.W., B.B. Aggarwal, C.V. Benton, T.S. Bringman, W.J. Henzel, J.A. Jarrett, D.W. Leung, B. Moffat, P. Ng, L.P. Svedersky, M.A. Palladino, and G.E. Nedwin. (1984). Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumor necrosis activity. *Nature* 312, 721-724.
- 11) Himmler, A., I. Maurer-Fogy, M. Krönke, P. Scheurich, K. Pfizenmaier, M. Lantz, I. Olsson, R. Hauptmann, C. Stratowa, and G.R. Adolf. (1980). Molecular cloning and expression of human and rat tumor necrosis factor receptor chain (p60) and its soluble derivative, tumor necrosis factor-binding protein. *DNA Cell Biol.* 10, 705-715.
- 12) Ishibashi, K., K. Ishitsuka, Y. Chuman, M. Otsuka, Y. Kuwazuru, M. Iwahashi, A. Utsunomiya, S. Hanada, T. Sakurami, and T. Arima. (1991). Tumor necrosis factor- β in the serum of adult T-cell leukemia with hypercalcemia. *Blood* 77, 2451-2455.
- 13) Kapas L., and J.M. Krueger. (1992). Tumor necrosis factor-beta induces sleep, fever, and anorexia. *Am. J. Physiol.* 263, R703-R707.
- 14) Kramer, S., and M.E. Carver. (1986). Serum-free in vitro bioassay for the detection of tumor necrosis factor. *J. Immunol. Meth.* 93, 201-206.
- 15) Mooradian, A.D., R.L. Reed, M.L. Duerr, C. Valdez, and P. Scuderi. (1992). The effect of age and diabetes mellitus on serum levels of lymphotoxin. *Age* 15, 95-99.
- 16) Li, C.B., P.W. Gray, P.F. Lin, K.M. McGrath, F.H. Ruddle, and N.H. Ruddle. (1987). Cloning and expression of murine lymphotoxin cDNA. *J. Immunol.* 138, 4496-4501.
- 17) Locksley, R.M., F.P. Heinzl, H.M. Shepard, J. Agosti, T.E. Eessalu, B.B. Aggarwal, and J.M. Harlan. (1987). Tumor necrosis factors α and β differ in their capacities to generate interleukin 1 release from human endothelial cells. *J. Immunol.* 139, 1891-1895.
- 18) Old, L.J. (1985). Tumor necrosis factor (TNF). *Science* 230, 630-632.

- 19) Paul, N.L., and N.H. Ruddle. (1988). Lymphotoxin. *Ann. Rev. Immunol.* 6, 407-438.
- 20) Scheurich, P., B. Thoma, U. Ucer, and K. Pfizenmaier. (1987). Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)- α : induction of TNF receptors on human T cells and TNF- α mediated enhancement of T cell responses. *J. Immunol.* 138, 1786-1790.
- 21) Shalaby, M.R., B.B. Aggarwal, E. Rinderknecht, L.P. Svedersky, B.S. Finkle, and M.A. Palladino. (1985). Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. *J. Immunol.* 135, 2069-2073.
- 22) Spies, T., C.C. Morton, S.A. Nedospasov, W. Fiers, D. Pious, and J. L. Strominger. (1986). Genes for the tumor necrosis factors α and β are linked to the human major histocompatibility complex. *Proc. Natl. Acad. Sci. USA* 83, 8699-8702.
- 23) Sprang, S.R. (1990). The divergent receptors for TNF. *TIBS* 15, 366-368.
- 24) Vilcek, J., V.J. Palombella, D. Henriksen-DeStefano, C. Swenson, R. Feinman, M. Hirai, and M. Tsujimoto. (1986). Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. *J. Exp. Med.* 163, 632-643.
- 25) Wong, G.H.W., and D.V. Goeddel. (1986). Tumor necrosis factors α and β inhibit virus replication and synergize with interferons. *Nature* 323, 819-822.

16 REAGENT PREPARATION SUMMARY

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

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

C. HRP-Conjugate	Number of Strips	HRP-Conjugate (ml)	Assay Buffer (ml)
	1 - 6	0.03	2.97
	1 - 12	0.06	5.94

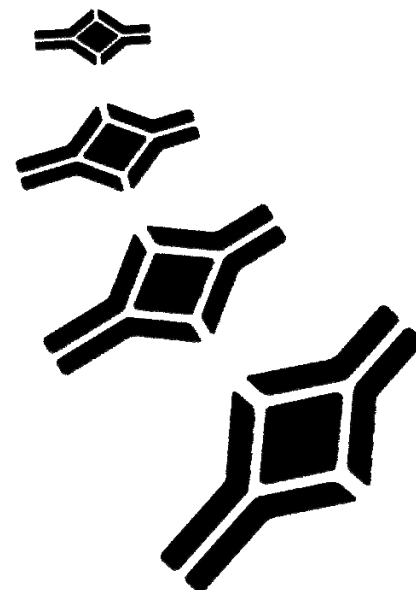
D. Standard Add **Sample Diluent** as stated on the label of the standard vial to each vial of concentrated **TNF- β Standard** as needed.

E. TMB Substrate Solution	Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
	1 - 6	3.0	3.0
	1 - 12	6.0	6.0

17 TEST PROTOCOL SUMMARY

- Wash Microwell Strips twice with **Wash Buffer**.
- Add 100 μ l **Sample Diluent**, in duplicate, to standard wells except the first wells (1000 pg/ml).
- Pipette 200 μ l diluted **TNF- β Standard** into the first wells and create standard dilutions ranging from 1000 to 8 pg/ml by transferring 100 μ l from well to well. Discard 100 μ l from the last wells.
- Add 100 μ l **Sample Diluent**, in duplicate, to the blank wells.
- Add 100 μ l **Sample**, in duplicate, to designated wells.
- Prepare **HRP-Conjugate**.
- Add 50 μ l **HRP-Conjugate** to all wells.
- Cover microwell strips and incubate 4 hours at room temperature (18° to 25°C).
- Prepare **TMB Substrate Solution** few minutes prior to use.
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 μ l of mixed **TMB Substrate Solution** to all wells including blank wells.
- Incubate the microwell strips for about 15 minutes at room temperature (18° to 25°C).
- Add 100 μ l **Stop Solution** to all wells including blank wells.
- Blank microwell reader and measure colour intensity at 450 nm.

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low TNF- β levels. Such samples require further dilution of 1:2, 1:4 with Sample Diluent in order to precisely quantitate the actual TNF- β level.



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