

PRODUCT INFORMATION AND MANUAL

human TNF- α Instant ELISA

BMS223INST

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

For research use only.

Not for diagnostic or therapeutic procedures.

128 Tests

**human TNF- α
BMS223INST**



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1 Intended Use

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

2 Summary

Tumor Necrosis Factor α (TNF- α), also known as cachectin, is a polypeptide cytokine produced by monocytes and macrophages. It functions as a multipotent modulator of immune response and further acts as a potent pyrogen (4, 17). TNF- α circulates throughout the body responding to stimuli (infectious agents or tissue injury), activating neutrophils, altering the properties of vascular endothelial cells, regulating metabolic activities of other tissues, as well as exhibiting tumoricidal activity by inducing localized blood clotting. TNF- α also inhibits lipoprotein lipase activity resulting in cachexia, a physical wasting condition (4, 17). Activation of B-cells by the Epstein Barr virus can be inhibited by TNF- α (15). Due to its varied actions throughout the immune system, TNF- α may play a role in the pathogenesis of many disease states.

TNF- α production is mediated by the action of lymphokines and endotoxins on the macrophage. Purified monocytes produce TNF- α within four hours of stimulation by recombinant IL-2 (9) and there is some *in vitro* evidence to suggest that TNF- α is expressed at high levels and with prolonged kinetics in T cells stimulated by both CD2 and CD28 (5). Secretion of TNF- α is enhanced by gamma interferon. TNF then induces or enhances the specific production of Class I MHC antigen, GM-CSF, and IL-1. Recent evidence has suggested an intracellular role for this peptide (23).

TNF- α may play a significant role in the pathogenesis of inflammatory disease of the joints and other tissues. Chin et al. (6) found that TNF- α , along with gamma interferon and IL-1 β increased cell surface expression of ICAM-1 on synovial fibroblasts. Alvaro-Garcia et al. (3) report that TNF- α stimulates synovial proliferation.

Waage et al. (25) found that increased levels of TNF- α in patients with septicemia and meningococcal disease correlated with fatal outcome. Scuderi et al. (22) suggest that increased levels of this cytokine may play a role in the host defense mechanism against parasitic infections. Girardin et al. (12) reported that increased serum TNF- α levels correlated with the number of risk factors involved in children with gram-negative sepsis and *purpura fulminans*. Elevated levels of TNF- α were also found in individuals suffering from myocarditis (11).

Recently, a growing body of information has pointed to a role for TNF- α in the pathogenesis of AIDS. Alveolar macrophages (AM) from HIV positive individuals with opportunistic lung infections have been shown to spontaneously produce higher levels of TNF- α *in vitro* than those HIV positive individuals without infection and HIV negative controls (14, 16). Krishnan et al. (16) report that higher TNF- α production by AM was associated with lower counts of *pneumocystis carinii* in bronchoalveolar lavage fluid, indicating that TNF- α may play a role in the control of this infection in AIDS. Israel-Biet et al. (14) also reported in *in vitro* studies, that AM that express HIV(p24+) released significantly higher levels of TNF- α than p24- alveolar macrophages and controls. Reddy et al. (20) found persistently elevated levels of circulating TNF- α in HIV seropositive individuals and suggest a possible involvement of this cytokine in the development of AIDS.

Measurement of TNF- α levels has also been shown to be useful in transplant research, where Maury et al. (18) and McLaughlin et al. (19). both reported TNF- α to be markedly elevated in renal allograft rejection episodes. Recent evidence has been presented on increased TNF- α levels in Bone Marrow Transplant (BMT) (13, 21). BMT patients with major transplant related complications such as interstitial pneumonitis and severe acute graft-versus-host disease had TNF- α levels significantly increase over controls (13).

3 Principles of the Test

An anti-human TNF- α monoclonal coating antibody is adsorbed onto microwells. human TNF- α present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated polyclonal anti-human TNF- α antibody binds to human TNF- α captured by the first antibody. Streptavidin-HRP binds to the biotin conjugated anti-human TNF- α .

Following incubation unbound biotin conjugated anti human TNF- α 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 TNF- α 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- α standard dilutions and human TNF- α sample concentration determined.

Figure 1

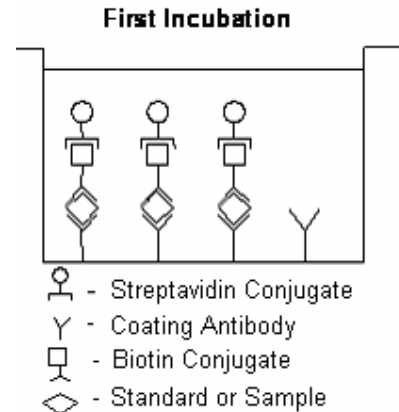


Figure 2

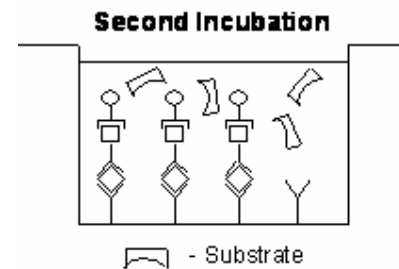
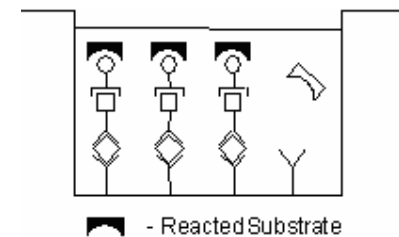


Figure 3



4 Reagents Provided

- 1 aluminium pouch with a **Microwell Plate coated with Monoclonal Antibody** (murine) to human TNF- α , **Biotin-Conjugate** (anti-TNF- α polyclonal antibody), Sample diluent and **Streptavidin-HRP**, lyophilized
- 2 aluminium pouches with a **human TNF- α 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 (12 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, amniotic fluid or other body fluids are 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- α . 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 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 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

9.1 Wash Buffer

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

Pour entire contents (25 ml) of the Wash Buffer Concentrate 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. 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.

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 100 µl of distilled water to the sample wells.
 - c. Add distilled water to all standard and blank wells as indicated on the label of the standard strips (A1, A2 to H1, H2).

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 |

- d. Add 50 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 3 hours, if available on a microplate shaker at 200 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 0.6 – 0.65.

- 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- α 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- α concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating human TNF- α 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- α concentration.
- ***Samples have been diluted 1:2, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**
- It is suggested that each testing facility establishes a control sample of known human TNF- α 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 μ l to the final volume per well. These 100 μ l are composed of 50 μ l of sample diluent plus 50 μ l of the sample. This is a 1:2 dilution.

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

50 μ l sample diluent and 50 μ l conjugate results in 100 μ l reconstitution volume, addition of 50 μ l sample (50 μ l + 50 μ l = 1:2 dilution)

Figure 4

Representative standard curve for human TNF- α Instant ELISA. human TNF- α 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.

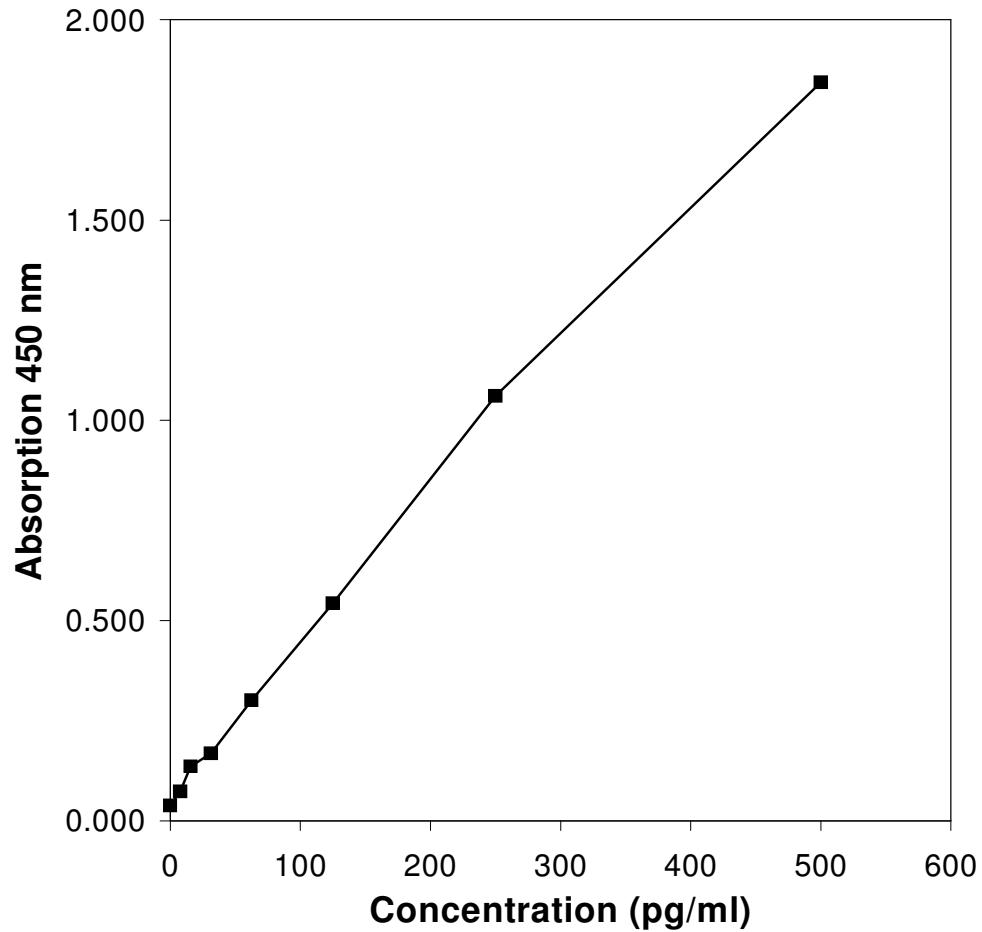


Table 2

Typical data using the human TNF- α INSTANT ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

| Standard | human TNF- α Concentration (pg/ml) | O.D. (450 nm) | O.D. Mean | C.V. (%) |
|----------|---|------------------|--------------|-------------|
| 1 | 500.0 | 1.893 | 1.844 | 3.8 |
| | 500.0 | 1.794 | | |
| 2 | 250.0 | 1.048 | 1.061 | 1.7 |
| | 250.0 | 1.074 | | |
| 3 | 125.0 | 0.578 | 0.543 | 9.1 |
| | 125.0 | 0.503 | | |
| 4 | 62.5 | 0.310 | 0.301 | 4.2 |
| | 62.5 | 0.292 | | |
| 5 | 31.3 | 0.172 | 0.168 | 3.8 |
| | 31.3 | 0.163 | | |
| 6 | 15.6 | 0.138 | 0.136 | 2.6 |
| | 15.6 | 0.133 | | |
| 7 | 7.8 | 0.075 | 0.073 | 3.9 |
| | 7.8 | 0.071 | | |
| Blank | 0 | 0.041 | 0.038 | |
| | 0 | 0.034 | | |

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- α 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 1.65 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 7 serum samples containing different concentrations of human TNF- α . 2 standard curves were run on each plate. Data below show the mean human TNF- α concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 6.0%.

Table 3

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

| Positive Sample | Experiment | human TNF- α Concentration (pg/ml) | Coefficient of Variation (%) |
|-----------------|------------|---|------------------------------|
| 1 | 1 | 868 | 4 |
| | 2 | 956 | 7 |
| | 3 | 972 | 7 |
| 2 | 1 | 528 | 8 |
| | 2 | 584 | 4 |
| | 3 | 468 | 7 |
| 3 | 1 | 226 | 7 |
| | 2 | 238 | 9 |
| | 3 | 180 | 5 |
| 4 | 1 | 82 | 6 |
| | 2 | 93 | 6 |
| | 3 | 85 | 4 |
| 5 | 1 | 29 | 7 |
| | 2 | 30 | 8 |
| | 3 | 26 | 4 |
| 6 | 1 | 22 | 5 |
| | 2 | 23 | 6 |
| | 3 | 22 | 6 |
| 7 | 1 | 237 | 5 |
| | 2 | 292 | 4 |
| | 3 | 228 | 7 |

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 7 serum samples containing different concentrations of human TNF- α . 2 standard curves were run on each plate. Data below (see Table 4) show the mean human TNF- α concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall coefficient of variation was 9.3%.

Table 4

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

| Sample | human TNF- α Concentration (pg/ml) | Coefficient of Variation (%) |
|--------|--|---------------------------------|
| 1 | 942 | 6.8 |
| 2 | 527 | 10.9 |
| 3 | 252 | 13.9 |
| 4 | 215 | 14.2 |
| 5 | 87 | 8.6 |
| 6 | 28 | 7.8 |
| 7 | 22 | 2.7 |

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human TNF- α 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. Average recovery ranged from 95% to 122% with an overall mean recovery of 109%.

13.4 Dilution Parallelism

4 serum samples with different levels of human TNF- α were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 82% to 116% with an overall mean recovery of 91% (see Table 5).

Table 5

| Sample | Dilution | human TNF- α Concentration (pg/ml) | | % Recovery of Exp. Val. |
|--------|----------|---|----------------|-------------------------|
| | | Expected Value | Observed Value | |
| 1 | 1:2 | -- | 1845 | -- |
| | 1:4 | 923 | 800 | 87 |
| | 1:8 | 461 | 373 | 81 |
| 2 | 1:2 | -- | 2215 | -- |
| | 1:4 | 1107 | 965 | 87 |
| | 1:8 | 554 | 481 | 87 |
| 3 | 1:2 | -- | 2006 | -- |
| | 1:4 | 1003 | 1162 | 116 |
| | 1:8 | 502 | 419 | 83 |
| 4 | 1:2 | -- | 1867 | -- |
| | 1:4 | 934 | 963 | 103 |
| | 1:8 | 467 | 384 | 82 |

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- α levels determined. There was no significant loss of human TNF- α 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 TNF- α level determined after 24 h. There was no significant loss of TNF- α 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- α 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 for TNF- β . Interference with the TNF-receptors TNF-R (60kDa) and TNF-R (80kDa) depends on the respective sample as shown in Table 6.

Table 6

| | Concentration of TNF-R (ng/ml) | Detected concentration of TNF- α (pg/ml) | |
|---------------|--------------------------------|---|----------|
| | | Sample 1 | Sample 2 |
| TNF-R | 0 | 138.7 | 579.4 |
| TNF-R (60kDa) | 10 | 104.9 | 508.4 |
| TNF-R (80kDa) | 10 | 54.8 | 507.0 |

13.8 Expected Values

A panel of 8 randomly selected sera from apparently healthy blood donors (males and females) was tested for human TNF- α . The detected human TNF- α levels ranged between less than 5 and 66 pg/ml with a mean level of 19.8 pg/ml. The normal levels measured may vary with the sample collective used.

14 Bibliography

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15 Ordering Information

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For technical information please contact:

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Cat.No. BMS223INST human TNF- α INSTANT ELISA

16 Reagent Preparation Summary

16.1 Wash Buffer

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

17 Test Protocol Summary

- Place standard strips in position A1/A2 to H1/H2.
- Add 100 µl **distilled water** to sample wells.
- Add **distilled water**, in duplicate, to all standard and blank wells as indicated on the label of the standard strips.
- Add 50 µl **Sample** to designated wells.
- Cover microwell strips and incubate 3 hours at room temperature (18° to 25°C) on a microplate shaker at 200 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).