

PRODUCT INFORMATION & MANUAL

Human TNF- β Instant ELISA

Enzyme-linked Immunosorbent Assay for
quantitative detection of human TNF- β .



*For in-vitro diagnostic
use. Not for therapeutic
procedures.*

REF *BMS202INSTCE*

Σ *128 TESTS*



*Human TNF- β
Instant ELISA
CE-IVD*

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1 Intended Use

The human TNF- β Instant ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human TNF- β . **The human TNF- β Instant ELISA is for in vitro diagnostic use. Not for use in 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 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 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 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 7 human TNF- β standard dilutions and human TNF- β sample concentration determined.

Figure 1

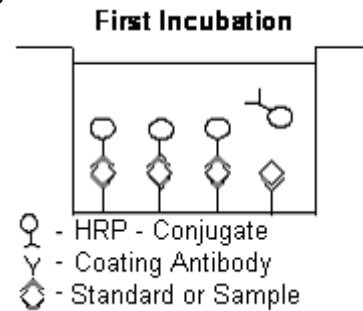


Figure 2

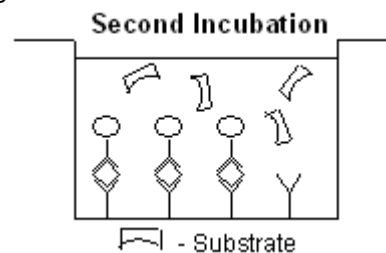
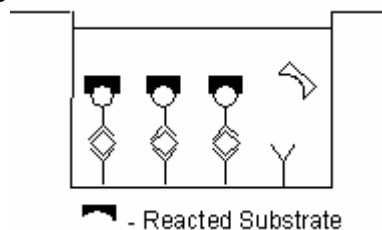


Figure 3



4 Reagents Provided

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human TNF- β , HRP-Conjugate (anti-human 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 (5 ml) **Sample Diluent**
- 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 and plasma (EDTA, citrate, heparin) were tested with this assay. Other body fluids 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 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, the frozen sample 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
- adjustable multichannel micropipettes (for volumes between 50 μ l and 500 μ 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 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 in vitro diagnostic use and are not for use in 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 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 an adhesive film and incubate at room temperature (18°C to 25°C) for 4 hours, if available on a microplate shaker at 400 rpm.
- f. Remove adhesive film and empty wells. **Wash** the microwell strips 6 times with approximately **400 µl** Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.

After the last wash, tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for no longer than 15 minutes. Do not allow wells to dry.

- g. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.
- h. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes. 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 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 (a 5-parameter curve fit is recommended).
- 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 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.

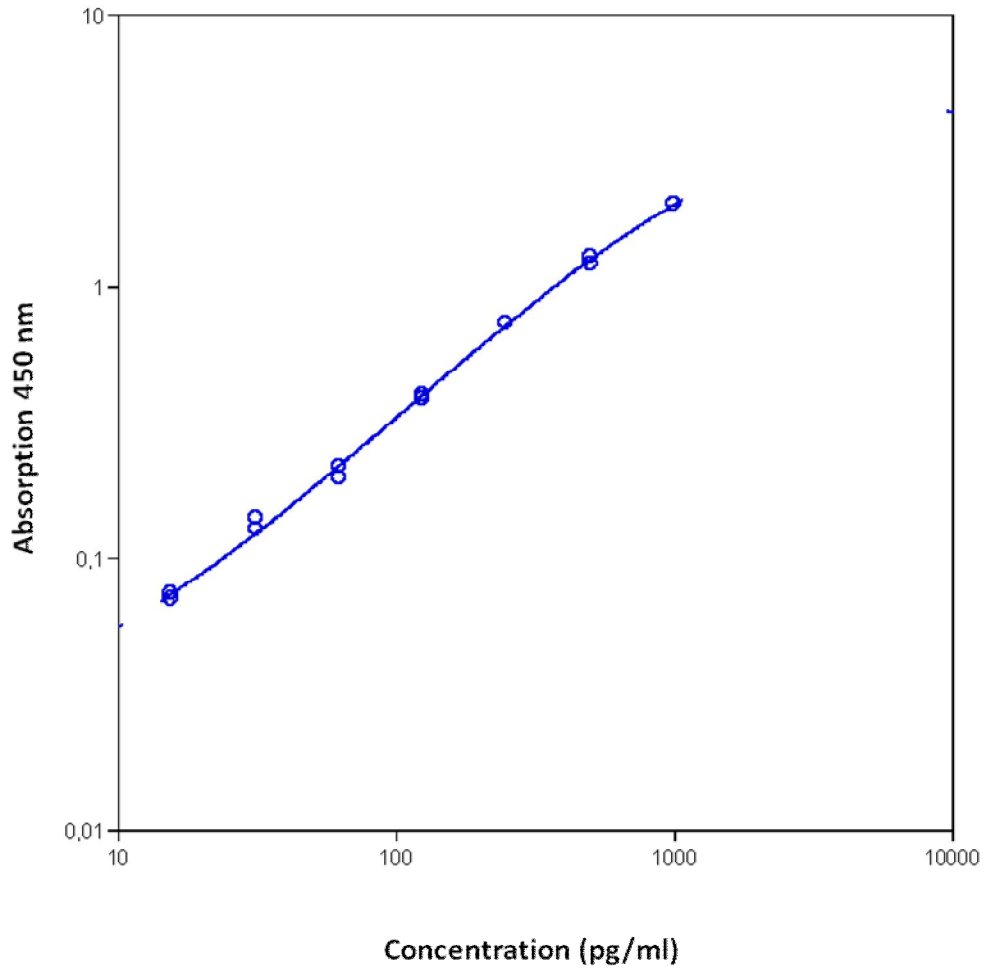


Table 2

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
	2	572	7
	3	560	8
2	1	287	4
	2	281	6
	3	301	7
3	1	145	5
	2	148	3
	3	164	2
4	1	73	5
	2	72	2
	3	80	4
5	1	458	1
	2	447	9
	3	491	5
6	1	242	2
	2	236	3
	3	276	8
7	1	123	3
	2	125	2
	3	150	6
8	1	64	2
	2	69	2
	3	76	4

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 TNF- β . 2 standard curves were run on each plate. Data below show the mean human TNF- β 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 6,4%.

Table 4

The mean human TNF- β concentration and the coefficient of variation of each sample

Sample	Mean 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 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

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	Mean human TNF- β Concentration (pg/ml)		
		Expected Value	Observed Value	% Recovery of Exp. Val.
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 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 TNF- β level determined after 24 h. There was no significant loss of human TNF- β immunoreactivity detected 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 sera samples from randomly selected apparently healthy donors (males and females) 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 309,0 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 6,7 pg human TNF- β .

14 Ordering Information

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* Customers outside North America and Europe may contact their eBioscience distributor listed on our website at www.eBioscience.com/distributors.

15 Reagent Preparation Summary

15.1 Wash Buffer (1x)

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

16 Test Protocol Summary

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

PRODUKTINFORMATION UND HANDBUCH (Deutsch)

1 Mitgelieferte Reagenzien

- 1 Aluminiumbeutel mit **Mikrotiterplatte**, **beschichtet** mit Antikörper (monoklonal) gegen humanes TNF- β , HRP-Konjugat (monoklonaler anti-humaner TNF- β Antikörper) und Verdünnungslösung, lyophilisiert.
- 2 Aluminiumbeutel mit einer human TNF- β -**Standardkurve** (farbig)
- 1 Flasche (25 ml) **Waschpufferkonzentrat 20x** (PBS mit 1% Tween 20)
- 1 Fläschchen (5 ml) **Verdünnungslösung**
- 1 Fläschchen (15 ml) **Substratlösung** (Tetramethylbenzidin)
- 1 Fläschchen (15 ml) **Stopplösung** (1 M Phosphorsäure)
- 2 **Klebefolien**

2 Lagerhinweise

Lagern Sie die ELISA Platte und Standardkurven oder den ganzen Kit bei -20°C . Die Platte und Standardkurven können auch herausgenommen und bei -20°C gelagert werden, die verbleibenden Reagenzien können bei $2-8^{\circ}\text{C}$ gelagert werden. Das Ablaufdatum des Kits und der Reagenzien ist auf den Etiketten angegeben.

Haltbarkeit des Kits und der Komponenten kann nur bei fachgerechter Lagerung garantiert werden, sowie bei mehrfacher Verwendung nur dann, wenn die Reagenzien bei der ersten Verwendung nicht kontaminiert wurden.

3 Sicherheitsvorkehrungen für den Gebrauch

- Alle enthaltenen Reagenzien sollten als potenziell gefährlich betrachtet werden. Daher wird empfohlen, dass dieses Produkt nur von Personen mit labortechnischer Erfahrung und in Übereinstimmung mit GLP Richtlinien verwendet wird. Passende Schutzbekleidung, wie Labormäntel, Sicherheitsbrillen und Laborhandschuhe müssen getragen werden. Vermeiden Sie jeden Kontakt der Reagenzien mit Haut oder Augen. Im Falle des Kontaktes von Reagenzien mit Haut oder Augen, sofort mit Wasser spülen. Bitte entnehmen Sie weitere spezifische Hinweise den Sicherheitsdatenblättern und/oder den Sicherheitsbestimmungen.
- Die Reagenzien sind ausschließlich für Diagnosezwecke bestimmt und nicht für den Einsatz bei Therapien.
- Reagenzien aus verschiedenen Chargen oder anderer Herkunft nicht mischen oder untereinander austauschen.
- Verwenden Sie die Kitreagenzien nicht nach dem Ablaufdatum (siehe Etikett).
- Setzen Sie die Kitreagenzien während der Lagerung oder Inkubation keiner starken Lichteinstrahlung aus.
- Nicht mit dem Mund pipettieren.
- In Bereichen, in denen mit Kitreagenzien oder Proben hantiert wird, nicht essen, trinken oder rauchen.
- Vermeiden Sie den Kontakt der Haut/Schleimhäute mit Kitreagenzien/Proben.
- Tragen Sie während des Hantierens mit Kitreagenzien oder Proben geeignete Gummi- oder Einweghandschuhe.
- Vermeiden Sie den Kontakt zwischen Substratlösung und Oxidationsmitteln/Metallen.
- Vermeiden Sie Verspritzen von Flüssigkeit oder Bildung von Aerosolen.
- Zur Vermeidung von Kontamination mit Mikroben oder Kreuzkontamination der Reagenzien oder Proben, die den Test

ungültig machen könnten, verwenden Sie Einwegpipettenspitzen und/oder Einwegpipetten.

- Verwenden Sie saubere, geeignete Reagenzgefäße für das Dispensieren von Konjugat und Substratreagenzien.
- Für die Reagensherstellung muss destilliertes oder entionisiertes Wasser verwendet werden.
- Die Substratlösung muss vor der Verwendung auf Raumtemperatur gebracht werden.
- Dekontaminieren und entsorgen Sie Proben sowie alle möglicherweise kontaminierten Materialien so, als ob sie Infektionserreger enthalten könnten. Die bevorzugte Dekontaminationsmethode ist Autoklavieren für mind. eine Stunde bei 121,5°C.
- Flüssige Abfälle, die kein Säure enthalten, sowie neutralisierte Abfälle werden zur Dekontamination mit Natrium Hypochlorit versetzt (Endkonzentration von Natrium Hypochlorit 1.0%). Nach 30 min ist eine effektive Dekontamination erreicht. Flüssige Abfälle, die Säure enthalten, müssen vor der Dekontamination neutralisiert werden.

4 Vorbereitung der Reagenzien

Bringen Sie das Pufferkonzentrat auf Raumtemperatur und stellen Sie die Verdünnung vor Beginn des Tests her. Sollten sich im Pufferkonzentrat Kristalle gebildet haben, erwärmen Sie es vorsichtig bis zur vollständigen Auflösung der Kristalle.

4.1 Waschpuffer (1x)

Leeren Sie den gesamten Inhalt (25 ml) des Waschpufferkonzentrats (20x) in einen sauberen 500-ml-Messzylinder. Füllen Sie mit destilliertem oder entionisiertem Wasser auf, bis ein Endvolumen von 500 ml erreicht ist. Vorsichtig mischen, um Schäumen zu vermeiden.

In eine saubere Waschflasche umfüllen und bei 2° bis 25°C lagern. Bitte beachten Sie, dass der Waschpuffer (1x) 30 Tage haltbar ist.

5 Testprotokoll

- **Verwenden Sie die Platte sofort nach Entnahme von -20°C!**
 - **Warten Sie mit dem Auftragen der Proben nicht bis zur vollständigen Auflösung der Lyophilisate – die Bindereaktion in den Standardstreifen beginnt sofort nach Beigabe von Wasser!**
 - **Versuchen Sie nicht, die Lyophilisate mittels Auf- und Abpipettieren in den Vertiefungen aufzulösen – Teile des Lyophilisats könnten dabei an der Spitze hängen bleiben und starke Abweichungen der Ergebnisse bewirken.**
 - **Führen Sie den Waschschrift mit mind. 400 µl Waschpuffer durch wie in der Anleitung beschrieben, oder füllen Sie die Vertiefungen vollständig – andernfalls werden allfällige Lyophilisatrückstände am Rand der Vertiefung nicht entfernt und bewirken starke Abweichungen der Ergebnisse.**
 - **Lassen Sie den Waschpuffer einige Sekunden einwirken bevor Sie ihn absaugen.**
 - **Entfernen Sie die Abdeckungen der Standardstreifen vorsichtig, sodass alle Lyophilisate in den Vertiefungen bleiben.**
- a. Bestimmen Sie die Anzahl der Mikrowellstreifen die für das Testen der gewünschten Anzahl von Proben benötigt werden, sowie die Mikrowellstreifen für Blindwert und Standards (farbig). Probe, Standard und Blindwert immer jeweils doppelt testen. Entfernen Sie die zusätzlichen Mikrowellstreifen von der Halterung und bewahren Sie diese mit dem mitgelieferten Trockenmittel in dem Folienbeutel fest verschlossen bei -20°C auf. Bringen Sie die Mikrowellstreifen mit der Standardkurve in Position A1/A2 bis H1/H2 (siehe Table 6).
- b. Pipettieren Sie **destilliertes Wasser** in alle **Standardvertiefungen und Blindwertvertiefungen** wie auf dem Etikett der Standardstreifen angegeben (A1, A2 bis H1, H2).
- c. Pipettieren Sie in alle **Probenvertiefungen** 50 µl **destilliertes Wasser**.

Table 6

Diagramm mit Beispiel für die Anordnung von Blindwert, Standards und Proben in den Mikrowellstreifen:

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

- d. Pipettieren Sie je 100 µl von jeder **Probe** (Doppelbestimmung) in die **Probenvertiefungen** und mischen Sie den Inhalt durch.
- e. Mit einer Klebefolie abdecken und bei Raumtemperatur (18° bis 25°C) für 4 Stunden inkubieren, wenn möglich auf einem Schüttler bei 200 U/min.
- f. Entfernen Sie die Klebefolie und entleeren Sie die Vertiefungen. Waschen Sie die Mikrowellstreifen 6 mal mit ca. **400 µl** Waschpuffer pro Vertiefung; zwischen den Waschgängen den Inhalt der Vertiefungen gründlich absaugen. Vor dem Absaugen Waschpuffer **10-15 Sekunden** einwirken lassen. Achten Sie darauf, die Oberfläche der Vertiefungen nicht zu zerkratzen.

Leeren Sie die Vertiefungen nach dem letzten Waschschrift und klopfen Sie die Mikrowellstreifen auf einem Saug- oder Papiertuch aus, um überschüssigen Waschpuffer zu entfernen. Verwenden Sie die Mikrowellstreifen sofort nach dem Waschen, oder legen Sie diese für maximal 15 Minuten umgedreht auf ein nasses Saugtuch. Lassen Sie die Vertiefungen nicht austrocknen

- g. Pipettieren Sie in alle Vertiefungen, einschließlich der Blindwertvertiefungen, 100 µl **TMB-Substratlösung**.
- h. Inkubieren Sie die Mikrowellstreifen bei Raumtemperatur (18° bis 25°C) für ca. 10 Minuten. Vermeiden Sie direkte, starke Lichteinstrahlung.

Die Farbentwicklung innerhalb der einzelnen Vertiefungen muss beobachtet und die Substratreaktion gestoppt werden (siehe nächster Protokollpunkt), bevor die gefärbten wells nicht mehr richtig gemessen können.

Die optimale Inkubationszeit für die Farbentwicklung muß bei jedem Versuch neu bestimmt werden.

Es wird empfohlen, die Stopplösung zuzugeben, wenn der höchste Standardpunkt eine dunkelblaue Farbe angenommen hat.

Alternativ kann die Farbentwicklung auch mit einem Photometer bei 620 nm verfolgt werden. Die Substratreaktion sollte gestoppt werden, wenn der höchste Standardpunkt eine OD von 0.9-0.95 erreicht.

- i. Stoppen Sie die Enzymreaktion durch rasches Pipettieren von 100 µl **Stopplösung** in jede Vertiefung, einschließlich der Blindwertvertiefungen. Für eine vollständige Inaktivierung der Enzyme ist es wichtig, die Stopplösung rasch und gleichmäßig in die Vertiefungen zu verteilen. Die OD Werte müssen sofort nach Beigabe der Stopplösung oder innerhalb einer Stunde nach Lagerung der Mikrowellstreifen in Dunkelheit bei 2-8°C gemessen werden.
- j. Messen Sie die Absorption jeder Vertiefung mit einem Spektrophotometer, verwenden Sie dabei 450 nm als primäre Wellenlänge (optional 620 nm als Referenzwellenlänge; 610 nm bis 650 nm sind möglich). Stellen Sie das Plattenmessgerät nach Anleitung des Herstellers und unter Verwendung der

Blindwertvertiefungen auf den Leerwert ein. Bestimmen Sie die Absorption der Proben wie auch der human TNF- β -Standards.

Note: Falls die Platte während der Inkubation nicht geschüttelt wurde, können die erreichten OD Werte niedriger als die oben angeführten sein. Die Ergebnisse sind trotzdem gültig.

INFORMACIÓN Y MANUAL DEL PRODUCTO (Español)

1 Reactivos Suministrados

- 1 bolsa de aluminio que contiene **una placa de micropocillos** recubiertos con anticuerpos monoclonales anti-human TNF- β , HRP-conjugado (anticuerpos monoclonales anti-human TNF- β) y diluyente de muestra, liofilizados
- 2 bolsas de aluminio con **una curva de estándar** human TNF- β (en color)
- 1 frasco (25 ml) de **concentrado de tampón de lavado** 20x (PBS con Tween 20 al 1%)
- 1 vial (5 ml) de **diluyente de muestra**
- 1 vial (15 ml) de **solución de sustrato** (tetrametil-bencidina)
- 1 vial (15 ml) de **solución de parada** (ácido fosfórico 1M)
- 2 **tapas para placas**, adhesives

2 Instrucciones de Conservación

Conservar la placa de ELISA y los estándares o el kit completo a -20°C . La placa y los estándares también pueden ser separados y conservarla a -20°C y conservar los demás reactivos del kit a una temperatura comprendida entre 2 y 8°C . En las etiquetas figuran las fechas de caducidad del kit y de los reactivos.

Sólo se podrá garantizar la fecha de caducidad de los componentes del kit si se conservan adecuadamente y, en caso de uso reiterado de un mismo componente, si el reactivo no queda contaminado en la primera manipulación.

3 Precauciones de uso

- Todos los productos químicos deben considerarse potencialmente peligrosos. Por tanto, recomendamos que este producto sea manipulado únicamente por aquellas personas que hayan sido entrenadas en técnicas de laboratorio y que sea usado de acuerdo con los principios de buenas prácticas de laboratorio. Se debe llevar ropa de protección apropiada como puedan ser las batas de laboratorio, gafas de seguridad y guantes. Se debe trabajar con cuidado para evitar cualquier contacto con piel y ojos. En el caso de que tenga lugar un contacto con piel u ojos, proceder de forma inmediata a lavar la parte afectada con abundante agua. Véase la(s) hoja(s) de seguridad y/o declaraciones de seguridad para recomendaciones específicas.
- Los reactivos están destinados para un uso en diagnóstico in vitro y no se deben usar en procedimientos terapéuticos.
- No mezclar o sustituir los reactivos por los equivalentes de otros lotes u otras fuentes.
- No usar reactivos caducados.
- No exponer los reactivos del kit a una luz intensa durante su almacenamiento o incubación.
- No pipetear con la boca.
- No se recomienda comer o fumar en las zonas donde se manipulen muestras o reactivos.
- Evitar el contacto de los reactivos del kit o de las muestras con piel o mucosas.
- Se recomienda el uso de guantes desechables de goma o látex durante la manipulación de las muestras y reactivos.
- Evitar el contacto de la solución de sustrato con agentes oxidantes y metales.
- Evitar salpicaduras y la generación de aerosoles.

- Con el propósito de evitar una contaminación microbiológica o contaminaciones cruzadas de reactivos y muestras que puedan invalidar el test se recomienda el uso de pipetas y/o puntas de pipetas de un solo uso.
- Usar recipientes limpios y específicos de reactivos para la dispensación de reactivos de sustrato.
- Se debe usar agua destilada o deionizada en la preparación de los reactivos.
- La solución de sustrato debe de estar a temperatura ambiente antes de su uso.
- Descontaminar y disponer las muestras y todos los materiales potencialmente contaminados como si pudieran contener agentes infecciosos. El método preferente de descontaminación es un autoclavado durante un mínimo de 1 hora a 121.5°C.
- Los residuos líquidos que no contengan ácido y los residuos neutralizados pueden ser mezclados con hipoclorito sódico en volúmenes tales que la mezcla final contenga 1.0% de hipoclorito sódico. Dejar actuar durante 30 minutos para una efectiva descontaminación. Los residuos líquidos que contengan ácido deben ser neutralizados previamente a la adición de hipoclorito sódico.

4 Preparación de los Reactivos

test. Si en el concentrado de tampón concentrado se han formado cristales, caliente suavemente hasta su completa disolución.

4.1 Tampón de Lavado (1x)

Vierta todo el contenido (25 ml) del concentrado de tampón de lavado (20x) en un matraz aforado de 500 ml limpio. Enrase en matraz con agua destilada o desionizada. Mezcle suavemente para evitar la formación de espuma.

Transfiera la solución a un frasco de lavado limpio y consérvela a una temperatura entre 2°C y 25°C. El Tampón de lavado (1x) permanece estable durante 30 días.

5 Protocolo de Ensayo

- Utilice la placa inmediatamente después de extraerla del congelador a -20°C
 - Al aplicar las muestras, no espere a la disolución total de las microcápsulas ya que la reacción de unión a las tiras de patrón se inicia inmediatamente después de añadir el agua.
 - No intente disolver las microcápsulas pipeteando por los pocillos ya que algunas partes de las microcápsulas podrían quedar adheridas a la punta y alterar considerablemente los resultados.
 - En la etapa de lavado, emplee al menos $400\ \mu\text{l}$ de tampón de lavado como se indica en el manual o llene los pocillos totalmente ya que, de lo contrario, podrían quedar restos de microcápsulas en los bordes de los pocillos que alterarían considerablemente los resultados.
 - Permitir que el tampón de lavado añadido a los pocillos permanezca unos segundos antes de ser aspirado.
 - Retire las tapas de las tiras de patrón con cuidado para que todas las microcápsulas liofilizadas permanezcan en los pocillos.
- a. Determine el número de tiras necesarias para analizar el número deseado de muestras y además añada las tiras para blancos y patrones (de color). Todas las muestras, estándares y blancos deben ser analizadas por duplicado. Retire del soporte las tiras sobrantes y consérvelas, junto con el desecante suministrado en una bolsa metalizada y cerrada herméticamente, a una temperatura de -20°C . Coloque las tiras que contienen la curva de valoración en las posiciones A1/A2 a H1/H2 (véase la Table 7).
 - b. Añada $50\ \mu\text{l}$ de **agua destilada** a los **pocillos con muestras**.
 - c. Añada **agua destilada** a los **pocillos del estándar y del blanco** como se indica en la etiqueta de las tiras de patrón (A1, A2 a H1, H2).
 - d. Por duplicado, añada $100\ \mu\text{l}$ de cada **muestra** a los **pocillos designados** y mezcle los contenidos.

Table 7

Tabla que representa un ejemplo de la disposición de los blancos, estándares y muestras en las tiras de micropocillos.

	1	2	3	4
A	Estándar 1 (1000.0 pg/ml)	Estándar 1 (1000.0 pg/ml)	Muestra 1	Muestra 1
B	Estándar 2 (500.0 pg/ml)	Estándar 2 (500.0 pg/ml)	Muestra 2	Muestra 2
C	Estándar 3 (250.0 pg/ml)	Estándar 3 (250.0 pg/ml)	Muestra 3	Muestra 3
D	Estándar 4 (125.0 pg/ml)	Estándar 4 (125.0 pg/ml)	Muestra 4	Muestra 4
E	Estándar 5 (62.5 pg/ml)	Estándar 5 (62.5 pg/ml)	Muestra 5	Muestra 5
F	Estándar 6 (31.3 pg/ml)	Estándar 6 (31.3 pg/ml)	Muestra 6	Muestra 6
G	Estándar 7 (15.6 pg/ml)	Estándar 7 (15.6 pg/ml)	Muestra 7	Muestra 7
H	blanco	blanco	Muestra 8	Muestra 8

- e. Cubra la placa con una tapa e incúbela a temperatura ambiente (18°C - 25°C) durante 4 horas (en un agitador mecánico a 400 rpm, si es posible).
- f. Retire la tapa y vacíe los pocillos. Lave 6 veces las tiras con aproximadamente **400 µl** de tampón de lavado por cada pocillo, aspirando completamente el contenido de los pocillos entre cada lavado. Permitir que el tampón de lavado permanezca en los pocillos durante **10-15 segundos** antes de su aspiración. Evite rayar la superficie de los pocillos.

Tras el último lavado, golpee suavemente las tiras contra un papel absorbente o una toallita de papel para eliminar el exceso de tampón de lavado. Utilice las tiras inmediatamente después de lavadas o bien colóquelas boca abajo sobre un papel absorbente húmedo durante como máximo 15 minutos. No deje secar los pocillos.

- g. Pipetee 100 μ l de **solución de sustrato TMB** y viértalos en todos los pocillos, incluidos los del blanco.
- h. Incube las tiras a temperatura ambiente (18°C - 25°C) durante aproximadamente 10 minutos. Evite la exposición directa a la luz intensa.

Deben monitorizarse los valores DO de la placa para detener la reacción del sustrato (véase el siguiente punto de este protocolo) antes de que deje de ser posible registrar correctamente los pocillos positivos.

La determinación del tiempo adecuado para el desarrollo del color, debe realizarse de forma individual para cada ensayo.

Se recomienda añadir la solución de parada cuando el estándar más alto presente un color azul oscuro. Alternativamente el desarrollo de color puede ser monitorizado con un lector de placas de ELISA a 620 nm. La reacción del sustrato debería ser parada cuando este estándar alcance una DO entre 0.9 y 0.95.

- i. Detenga la reacción enzimática pipeteando rápidamente 100 μ l de **solución de parada** en cada pocillo, incluidos los del blanco. Es importante dispensar la solución de parada de forma rápida y uniforme en todos los pocillos para inactivar totalmente la enzima. Los resultados deben leerse inmediatamente después de añadir la solución de parada o, como máximo, en el plazo de 1 hora si las tiras se conservan a una temperatura entre 2 - 8°C en un lugar oscuro.
- j. Lea la absorbancia de cada pocillo en un espectrofotómetro utilizando 450 nm como longitud de onda principal (opcionalmente 620 nm como longitud de onda de referencia; los valores comprendidos entre 610 nm y 650 nm son aceptables). Utilizando los pocillos de blanco, haga el blanco del lector de placas de acuerdo con las instrucciones del fabricante. Determine la absorbancia de las muestras y de los estándar human TNF- β .

Note: En caso de incubar sin agitar, los valores de D.O. pueden ser inferiores a los indicados más abajo. De todas formas los resultados siguen siendo válidos.

INFORMATIONS SUR LE PRODUIT ET MANUEL (Français)

1 Réactifs Fournis

- 1 pochette en aluminium contenant **une plaque de microtitration** recouverte d'anticorps monoclonaux anti-human TNF- β , le conjugué HRP (anticorps monoclonaux anti-human TNF- β) et de diluant d'échantillon, lyophilisés
- 2 pochettes en aluminium contenant **une courbe étalon** human TNF- β (colorée)
- 1 flacon (25 ml) de **tampon de lavage concentré 20 x** (tampon phosphate avec du Tween 20 1%)
- 1 flacon (5 ml) de **diluant d'échantillon**
- 1 flacon (15 ml) de **solution de substrat** (tétraméthyle-benzidine)
- 1 flacon (15 ml) de **solution d'arrêt** (acide phosphorique 1 M)
- 2 **Couvre-plaques** adhésifs

2 Instruction de stockage

Conserver la plaque ELISA et les courbes étalons ou le kit complet à -20°C. La plaque et les courbes peuvent également être retirées pour être conservées à -20°C, le reste du kit étant conservé entre 2 et 8°C. La date de péremption du kit est spécifiée sur les étiquettes. Le délai de péremption du kit ne peut être garanti que si les composants sont conservés correctement et si, en cas d'utilisation répétée d'un composant, le réactif n'a pas été contaminé lors d'une première utilisation.

3 Préventions de Sécurité pour l'Usage

- Tout réactifs doivent être considérés comme potentiellement dangereux. Pour cela il est recommandé que ce produit est utilisé que par des personnes ayant une qualification de laboratoire et qu'il soit utilisé à l'avenant au code GLP. Une tenue correspondante comme des une blouse de travail, des lunettes protectrices et des gants de travail doivent-être portés. Evitez tous contacts de réactifs avec la peau ou les yeux. En cas de contact avec les yeux ou la peau rincez immédiatement avec de l'eau. Veuillez consulter tous conseils spécifiques dans les fiches de données de sécurité et/ou les les règles de sécurité.
- Les réactifs sont réservés exclusivement au diagnostique et non pas au thérapeutique.
- Evitez de mélanger et d'échanger les réactifs de lots différents et de provenance différents.
- Evitez l'utilisation des réactifs périmés (voyez étiquette).
- N'exposez pas les réactifs à la lumière pendant le stockage ou l'incubation.
- Ne pas pipeter avec la bouche
- Ne pas manger, boire ou fumer dans les zones de manipulation de réactifs et d'échantillons.
- Evitez le contact de la peau et des muqueuses avec les réactifs.
- Pendant le travail avec les réactifs, utilisez des gants appropriés.
- Evitez le contact de substrats avec des métaux/oxydant.
- Evitez de gicler des liquides et la formation d'Aérosols.
- A fin d'éviter des contaminations avec microbes ou contaminations de réactifs et d'échantillons qui pourraient rendre le test sans valeur, veuillez utiliser des pointes de pipettes jetables.
- Utilisez des tubes appropriés pour dispenser le conjugué et le substrat.

- Pour la préparation des réactifs de l'eau distillée ou déionisée doit être utilisée.
- La solution de substrat doit être rendue à température ambiante avant usage.
- Décontaminez et éliminez les échantillons et tous matériaux contaminés de manière comme si ils contenaient des germes de maladies infectieuses. La méthode préférée de décontamination est par l'autoclave pour au moins une heure à 121.5 °C.
- Traitez les déchets liquides non-acidiques tel que des déchets neutralisés par l'hypochlorite de sodium (concentration finale d'hypochlorite: 1,0%). Après 30 minutes la décontamination effective est atteinte. Les déchets liquides contenant de l'acide doivent être neutralisés avant la décontamination.

4 Préparation des Réactifs

Mettre le concentré de tampon à une température ambiante et diluer avant de commencer le test. Si des cristaux se sont formés dans le concentré de tampon, chauffer doucement ce dernier jusqu'à la dissolution des cristaux totale.

4.1 Tampon de Lavage (1x)

Verser tout le contenu (25 ml) du concentré de tampon de lavage (20x) dans un cylindre gradué propre de 500 ml. Porter le volume final à 500 ml avec de l'eau distillée dans un alambic en verre ou désionisée. Mélanger doucement pour éviter la formation de mousse.

Transférer tout dans une bouteille de lavage et conserver à une température comprise entre 2° et 25°C. Noter que le tampon de lavage (1x) reste stable pendant 30 jours.

5 Protocole de Test

- **Utiliser la plaque immédiatement après son retrait d'un environnement à -20°C !**
 - **Ne pas attendre que les pastilles soient complètement dissoutes pour appliquer les échantillons. La réaction de liaison dans les barrettes étalons commence immédiatement après l'ajout d'eau !**
 - **Ne pas essayer de dissoudre les pastilles en pipetant de haut en bas dans les puits. Certains fragments des pastilles pourraient se coller à l'embout et induire une forte variation des résultats.**
 - **Procéder à l'étape de lavage avec au moins 400 µl de tampon de lavage comme indiqué dans le manuel ou remplir complètement les puits. Dans le cas contraire, tous les résidus de pastilles collés au bord du puits ne seraient pas éliminés et entraîneraient une forte variation des résultats.**
 - **Laisser le Tampon de lavage dans les puits pendant quelques secondes avant l'aspiration.**
 - **Retirer délicatement les couvre-plaques des barrettes étalons de manière à ce que toutes les pastilles lyophilisées restent dans les puits.**
- a. Déterminer le nombre de barrettes de puits de microtitration nécessaires pour tester le nombre voulu d'échantillons plus les barrettes nécessaires aux contrôles vides et aux étalons (colorés). Chaque échantillon, étalon et contrôle vide doit être testé en double. Retirer les barrettes de microtitration inutiles du support et les stocker à -20°C dans une pochette hermétiquement refermée, avec le dessiccatif fourni. Placer les barrettes de puits de microtitration contenant la courbe étalon en position A1/A2 à H1/H2 (voir la Table 8).
 - b. Ajouter de l' **eau distillée** comme indiqué sur l' étiquette à tous les **puits d'étalon et contrôle vide** (A1, A2 à H1, H2).
 - c. Ajouter 50 µl d'**eau distillée** dans les **puits d'échantillon**.

Table 8

Exemple d'arrangement d'échantillons, de standards et de contrôles vides dans les barrettes de puits de microtitration.

	1	2	3	4
A	Étalon 1 (1000.0 pg/ml)	Étalon 1 (1000.0 pg/ml)	Échantillon 1	Échantillon 1
B	Étalon 2 (500.0 pg/ml)	Étalon 2 (500.0 pg/ml)	Échantillon 2	Échantillon 2
C	Étalon 3 (250.0 pg/ml)	Étalon 3 (250.0 pg/ml)	Échantillon 3	Échantillon 3
D	Étalon 4 (125.0 pg/ml)	Étalon 4 (125.0 pg/ml)	Échantillon 4	Échantillon 4
E	Étalon 5 (62.5 pg/ml)	Étalon 5 (62.5 pg/ml)	Échantillon 5	Échantillon 5
F	Étalon 6 (31.3 pg/ml)	Étalon 6 (31.3 pg/ml)	Échantillon 6	Échantillon 6
G	Étalon 7 (15.6 pg/ml)	Étalon 7 (15.6 pg/ml)	Échantillon 7	Échantillon 7
H	Contrôle vide	Contrôle vide	Échantillon 8	Échantillon 8

- d. Ajouter 100 µl de chaque **échantillon**, en double, dans **les puits assignés** et mélanger le contenu.
- e. Recouvrir avec un couvre-plaque et incuber à température ambiante (entre 18 et 25°C) pendant 4 heures, si possible sur un agitateur rotateur réglé à 400 tr/min.
- f. Retirer le couvre-plaque et vider les puits. Laver 6 fois les barrettes de puits de microtitration avec environ **400 µl** de tampon de lavage pour chaque puits, en aspirant complètement le contenu des puits entre les lavages. Laisser le tampon de lavage dans les puits pendant **10-15 seconds** avant l'aspiration. Veiller à ne pas rayer la surface des puits de microtitration.

Après le dernier lavage, vider les barrettes de puits et les tapoter sur un tampon absorbant ou une serviette en papier pour éliminer l'excès de tampon de lavage. Utiliser les barrettes de micropuits immédiatement après le lavage ou les placer renversées sur un papier absorbant pendant 15 minutes au maximum. Ne pas laisser sécher les puits.

- g. Pipeter 100 µl de **solution de substrat TMB** dans chaque puits, y compris les puits de contrôle vide.
- h. Incuber les puits de microtitration à température ambiante (entre 18 et 25 C) pendant environ 10 minutes. Éviter toute exposition directe à une source de lumière intense.

Les valeurs de densité optique au niveau de la plaque doivent être surveillées et la réaction du substrat stoppée (voyez le point prochain) avant que les puits positifs ne soient plus correctement mesurables.

La durée de l'incubation pour le développement de couleur doit être déterminé pour chaque essai individuellement.

Il est recommandé d'ajouter la solution stop quand une couleur bleu sombre s'est développée dans le point le plus haut de la gamme standard. Une autre alternative consiste à suivre le développement de la couleur en lecteur ELISA à 620 nm. La réaction du substrat doit être arrêtée dès que la DO atteint 0.9 à 0.95

- i. Arrêter la réaction enzymatique en pipetant rapidement 100 µl de **solution d'arrêt** dans chaque puits, y compris les puits de contrôle vide. Il est important que la solution d'arrêt soit répandue rapidement et uniformément dans les puits pour inactiver complètement l'enzyme. Les résultats doivent être lus immédiatement après l'ajout de la solution d'arrêt ou dans l'heure qui suit si les barrettes de microtitration sont conservées à l'obscurité entre 2 et 8 °C.
- j. Lire l'absorbance de chaque puits sur un spectrophotomètre avec 450 nm comme longueur d'onde primaire (éventuellement 620 nm comme longueur d'onde de référence; 610 à 650 nm sont acceptables). Mesurer le contrôle vide du lecteur de plaque conformément aux instructions du fabricant, en utilisant les puits de contrôle vide. Déterminer l'absorbance des échantillons et des étalons human TNF-β.

Note: Si la plaque n'est pas agitée pendant l'incubation, les valeurs de densité optique peuvent être inférieures aux valeurs indiquées plus haut. Néanmoins ces valeurs sont valables.

INFORMAZIONI SUL PRODOTTO E MANUALE (italiano)

1 Reagenti Forniti

- 1 busta d'alluminio contenente **una piastra micropozzetti rivestita** con anticorpo monoclonale verso human TNF- β , coniugato HRP (anticorpo monoclonale verso human TNF- β ,) e diluente del campione, liofilizzato
- 2 busta d'alluminio ognuna con una **curva degli standard** human TNF- β (colorata)
- 1 bottiglia (25 ml) con **tampone di lavaggio concentrato 20x** (soluzione salina tamponata con 1% Tween 20)
- 1 flaconcino (5 ml) con **diluente del campione**
- 1 flaconcino (15 ml) di **soluzione del substrato** (tetrametilbenzidina)
- 1 flaconcino (15 ml) di **soluzione bloccante** (acido fosforico 1M)
- 2 **copripietra adesivi**

2 Istruzioni di Conservazione

Conservare la piastra ELISA ed la curva standard o l'intero kit a -20° C. La piastra ed curva standard possono anche essere rimossi e conservati a -20°C, mentre i rimanenti reagenti del kit devono essere conservati tra 2° e 8° C. La scadenza del kit e dei reagenti è indicata sulle etichette.

La data di scadenza dei componenti del kit può essere garantita solo se questi sono conservati correttamente e, in caso di uso ripetuto di un componente, il reagente non è stato contaminato durante la prima manipolazione.

3 Precauzioni per l'uso

- Tutti i prodotti chimici vanno considerati come potenzialmente pericolosi. Raccomandiamo, perciò, l'utilizzo di questo prodotto solo da personale addestrato alle tecniche di laboratorio e che siano avvezze alle comuni pratiche di laboratorio. Indossare abbigliamento idoneo come camici, guanti ed occhiali. Attenzione ad evitare contatto con la pelle e gli occhi. Nel caso di contatto con pelle o occhi, immediatamente lavare con acqua. Consultare la scheda di sicurezza del prodotto per specifici consigli.
- I reagenti sono per uso in vitro diagnostico e non sono per uso terapeutico.
- Non mischiare tra loro reagenti di diversi lotti o provenienza.
- Non usare i kit dopo la data di scadenza.
- Non esporre i reagenti del kit, durante la conservazione e l'incubazione a forti fonti di luce.
- Non pipettare utilizzando la bocca.
- Non mangiare o fumare nell'area dove sono utilizzati i reagenti dei kit o i campioni.
- Evitare il contatto dei reagenti o campioni con la pelle o le mucose.
- Guanti di gomma o lattice dovrebbero essere sempre indossati quando si usano reagenti e campioni.
- Evitare il contatto tra il substrato del kit e agenti ossidanti e metallo.
- Evitare schizzi o fumi.
- Per evitare contaminazione microbica o cross-contaminazione dei reagenti o dei campioni che invaliderebbero il test, usare sempre pipette e puntali mono-uso.
- Usare vaschette pulite e dedicate per la dispensare il reagente substrato.

- Acqua distillata o de-ionizzata deve essere utilizzata per la preparazione dei reagenti.
- La soluzione di substrato deve essere portata a temperatura ambiente prima dell'utilizzo.
- Decontaminare ed eliminare i campioni e tutto il materiale potenzialmente contaminante perchè potrebbero contenere agenti infettanti. Il metodo preferito per la decontaminazione è l'autoclavaggio per minimo 1 ora a 121,5°C.
- Gli scarti liquidi, non contenenti acido a gli scarti neutralizzati possono essere mischiati con sodio ipoclorido in un volume finale di 1,0%. Lasciare minimo 30 minuti per l'effettiva decontaminazione. Scarti liquidi contenenti acido devono essere neutralizzati prima dell'aggiunta di sodio ipoclorido.

4 Preparazione dei Reagenti

Prima di cominciare con le procedure del test i il concentrato dei tampone devono essere portati a temperatura ambientale e diluiti a concentrazioni adeguati. Se il concentrato dei tampone presenta cristalli in sospensione, riscaldare lievemente il tampone fino a ottenere la completa dissoluzione dei cristalli.

4.1 Tampone di Lavaggio (1x)

Versare l'intero contenuto (25 ml) del tampone di lavaggio concentrato (20x) in un cilindro graduato pulito da 500 ml. Portare il volume finale a 500 ml utilizzando acqua distillata o acqua deionizzata. Mescolare delicatamente per evitare la formazione di schiuma.

Trasferire il prodotto in una bottiglia pulita e conservare a temperature comprese fra 2°C e 25°C. Il tampone di lavaggio (1x) è stabile per 30 giorni.

5 Procedura del Test

- Usare la piastra immediatamente dopo la rimozione dall'ambiente refrigerato a -20°C !
 - Non attendere la completa dissoluzione dei pellet prima di usare i campioni; la reazione di legame nelle strip degli standard inizia immediatamente dopo l'aggiunta di acqua!
 - Non cercare di dissolvere i pellet pipettando su e giù nei pozzetti; particelle del pellet possono aderire al puntale determinando una notevole variabilità dei risultati.
 - Eseguire la fase di lavaggio con almeno $400\ \mu\text{l}$ di tampone di lavaggio come indicato nel manuale o riempire completamente i pozzetti; in caso contrario non sarà possibile rimuovere tutti i residui del pellet adesi al bordo del pozzetto, determinando una notevole variabilità dei risultati.
 - Il tampone di lavaggio deve coprire bene i fondi dei pozzetti e deve essere lasciata per qualche secondo, prima di essere aspirata.
 - Rimuovere con cautela i fogli protettivi delle strip degli standard per lasciare tutti i pellet liofilizzati nei pozzetti.
- a. Stabilire il numero di strip dei micropozzetti necessarie per analizzare la quantità desiderata di campioni più le strip per i bianchi e gli standard (colorate). Tutti i campioni, gli standard ed il bianco devono essere processati in duplicato. Rimuovere dal supporto le strip micropozzetti non utilizzate e conservarle nella bustina metallica contenente la polvere essiccante, mantenendole a -20°C e perfettamente sigillate. Mettere le strip contenenti la curva standard nelle posizioni da A1/A2 a H1/H2 (vedere la Table 9).
 - b. Dispensare **acqua distillata** a tutti i **pozzetti per lo standard ed il bianco** come indicato sull'etichetta degli standard strip (A1, A2 fino a H1, H2)
 - c. Dispensare $50\ \mu\text{l}$ di **acqua distillata** nei **pozzetti dei campioni**.

Table 9

Tabella rappresenta un esempio dell'organizzazione dei bianchi, standards e campioni nelle strip della piastra

	1	2	3	4
A	Standard 1 (1000.0 pg/ml)	Standard 1 (1000.0 pg/ml)	Campione 1	Campione 1
B	Standard 2 (500.0 pg/ml)	Standard 2 (500.0 pg/ml)	Campione 2	Campione 2
C	Standard 3 (125.0 pg/ml)	Standard 3 (125.0 pg/ml)	Campione 3	Campione 3
D	Standard 4 (125.0 pg/ml)	Standard 4 (125.0 pg/ml)	Campione 4	Campione 4
E	Standard 5 (62.5 pg/ml)	Standard 5 (62.5 pg/ml)	Campione 5	Campione 5
F	Standard 6 (31.3 pg/ml)	Standard 6 (31.3 pg/ml)	Campione 6	Campione 6
G	Standard 7 (15.6 pg/ml)	Standard 7 (15.6 pg/ml)	Campione 7	Campione 7
H	bianco	bianco	Campione 8	Campione 8

- d. Dispensare 100 μ l di ciascun **campione**, in duplicato, **nei pozzetti di reazione e mescolare il contenuto**.
- e. Coprire con un copripiastra e incubare a temperatura ambiente (18-25°C) per 4 ore utilizzando, se disponibile, un vortex a 400 rpm.
- f. Rimuovere il copripiastra e svuotare i pozzetti. Lavare 6 volte le strip micropozzetti utilizzando circa **400 μ l** di tampone di lavaggio per pozzetto, aspirando accuratamente il contenuto dei micropozzetti tra un lavaggio e l'altro. Permettere al tampone di lavaggio di rimanere, nei pozzetti, circa **10-15 secondi** prima dell'aspirazione. Evitare di scalfire la superficie dei micropozzetti.

Dopo l'ultimo lavaggio, asciugare le strip micropozzetti con un tampone o carta assorbente per rimuovere il tampone di lavaggio in eccesso. Utilizzare le strip subito dopo il lavaggio o sistemarle capovolte su carta assorbente umida per non più di 15 min. Non lasciar asciugare i pozzetti.

- g. Pipettare 100 μ l di **soluzione substrato TMB** in tutti i pozzetti, inclusi quelli del blank.
Incubare le strip a temperatura ambiente (18-25° C) per circa 10 minuti. Evitare l'esposizione diretta a luci intense.

È necessario monitorare i valori O.D. a livello della piastra e interrompere la reazione del substrato (vedi il punto prossimo del protocollo) prima che i pozzetti positivi cessino di essere appropriatamente registrabili.

La determinazione del tempo necessario per lo sviluppo del colore dev'essere fatto per ogni singolo parametro.

Si raccomanda di aggiungere la soluzione di stop quando lo standard più elevato ha sviluppato un colore blu scuro.

Alternativamente lo sviluppo del colore può essere monitorato con un lettore ELISA a 620 nm. La reazione del substrato deve essere bloccata non appena viene misurato un valore delle OD di 0.9 - 0.95.

- h. Interrompere la reazione enzimatica pipettando rapidamente 100 μ l di **soluzione bloccante** in ciascun pozzetto, inclusi i pozzetti del bianco. È importante che la soluzione bloccante si diffonda rapidamente e uniformemente attraverso i micropozzetti per inattivare completamente l'enzima. I risultati devono essere letti immediatamente dopo l'aggiunta della soluzione bloccante o entro 1 ora se le strip sono conservate in un luogo buio a 2-8° C.
- i. Leggere l'assorbanza di ciascun micropozzetto su uno spettrofotometro che utilizza 450 nm come lunghezza d'onda primaria (620 nm come lunghezza d'onda di riferimento alternativa; valori da 610 nm a 650 nm sono accettabili). Azzerare il lettore della piastra secondo le istruzioni del produttore e utilizzando i pozzetti del bianco. Determinare l'assorbanza sia dei campioni, sia degli standard di human TNF- β .

Note: In caso di incubazione senza agitazione i valori di densità ottica (O.D.) potranno essere più bassi di quanto indicato sotto. Tuttavia i risultati saranno da ritenersi validi.