Human TNFα ELISA

For research use only

950 090 096 **1 x 96 tests**
950 090 192 **2 x 96 tests**

INTENDED USE

The Human TNFα ELISA is to be used for the in-vitro quantitative determination of Tumor Necrosis Factor in human serum, plasma, buffered solutions or cell culture medium. The assay will recognize both natural and recombinant human TNFα. **TNFα ELISA is for research use only.**

SUMMARY

Tumor Necrosis Factor (TNFα), 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). TNFα 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. TNFα 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 TNFα (15). Due to its varied actions throughout the immune system, TNFα may play a role in the pathogenesis of many disease states.
TNFα production is mediated by the action of lymphokines and endotoxins on the macrophage. Purified monocytes produce TNFα within four hours of stimulation by recombinant IL-2 (9) and there is some in vitro evidence to suggest that TNFα is expressed at high levels and with prolonged kinetics in T cells stimulated by both CD2 and CD28 (5). Secretion of TNFα is enhanced by gamma interferon. TNFα 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).

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

Waage et al. (25) found that increased levels of TNFα 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 TNFα levels correlated with the number of risk factors involved in children with gram-negative sepsis and purpura fulminians. Elevated levels of TNFα were also found in individuals suffering from myocarditis (11).

Recently, a growing body of information has pointed to a role for TNFα 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 TNFα in vitro than those HIV positive individuals without infection and HIV negative controls (14, 16). Krishnan et al. (16) report that higher TNFα production by AM was associated with lower counts of pneumocystis carinii in broncheoalveolar lavage fluid, indicating that TNFα 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 TNFα than p24- alveolar macrophages and controls. Reddy et al. (20) found persistently elevated levels of circulating TNFα in HIV seropositive individuals and suggest a possible involvement of this cytokine in the development of AIDS.

Measurement of TNFα levels has also been shown to be useful in transplant research, where Maury et al. (18) and McLaughlin et al. (19). both reported TNFα to be markedly elevated in renal allograft rejection episodes. Recent evidence has been presented on increased TNFα 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 TNFα levels significantly increase over controls (13).

PRINCIPLES OF THE TEST

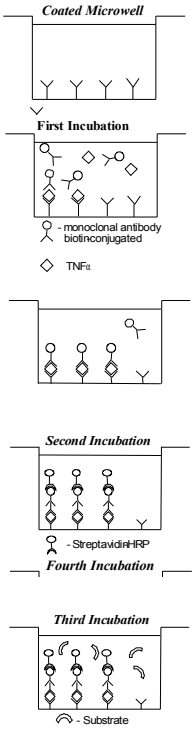
A monoclonal antibody specific for TNFα has been coated onto the wells of the microtiter strips provided.

During the first incubation, TNFα present in the sample or standard and a monoclonal anti TNFα antibody conjugated to biotin are simultaneaously incubated.

Following incubation unbound biotinylated anti-TNFα is removed during a wash step.

Streptavidin-HRP is added and binds to the biotinylated anti TNF α. After incubation and a wash step a substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of TNFα present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm.



REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	COLOUR CODE	QUANTITY 1X96 Tests 950 090 096	QUANTITY 2X96 Tests 950 090 192	RECONSTITUTION
96-wells precoated microtiter plate		1	2	Ready-to-use
Plate covers		2	4	
TNFα Standard: 800 pg/ml	Yellow	2 vials	4 vials	Reconstitute with the volume of standard diluent indicated on the vial (see reagent preparation on page 4
Control	Silver	2 vials	4 vials	Reconstitute with the volume of standard diluent indicated on the vial (see reagent preparation on page 4
Standard Diluent Buffer	Black	1 bottle	1 bottle	(25 ml) 10X concentrate. Dilute in distilled water
Standard Diluent : human serum	Black	1 bottle	2 bottles	(7 ml) ready to use
Biotinylated anti TNFα	Red	1 vial	2 vials	(0.4 ml) Dilute in biotinylated antibody diluent
Biotinylated Antibody Diluent	Red	1 bottle (7 ml)	1 bottle (13 ml)	Ready-to-use
Streptavidin-HRP		2 vials	4 vials	(5 µl) Add 0.5 ml of HRP-Diluent before further dilutions
HRP Diluent	Red	1 bottle	1 bottle	(23 ml) Ready to use
Washing Buffer	White	1 bottle	2 bottles	(10 ml) 200X concentrate. Dilute in distilled water
Substrate Solution ; chromogen TMB		1 bottle (11ml)	1 bottle (24 ml)	Ready-to-use
Stop Reagent H_2SO_4	Black	1 bottle	2 bottles	(11 ml) Ready-to-use

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Vortex, Mixer
- 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

SAFETY

- Reagents are intended for in vitro use and are not for use in therapeutic procedures.
- All chemicals in this kit should be considered as potentially hazardous. We therefore recommend that this product is handled only by persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice.
- The human serum included in this kit have been tested and found non reactive for HbsAg, anti HIV1 & 2 and anti VHC. Nevertheless, no known method can offer complete assurance that human blood derivatives will not transmitted hepatitis, AIDS or other infections. Therefore handling of reagents, serum or plasma specimens should be in accordance with local safety 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.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- Reagents containing preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Respect incubation times described in the assay procedure.

- 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 a clean plastic container to prepare the washing solution.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never invert the absorbent paper directly into the wells.
- 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.

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 is stated on box front 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.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture supernatants, human serum, plasma or other biological samples 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.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.
Serum: Avoid any inintentional stimulation of the cells by the procedure. Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. For that, after clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.
Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.
Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before assaying

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

For sample stability, please refer to Performance Characteristics, Sample stability.

PREPARATION OF REAGENTS

1. Washing Buffer

Dilute the **Washing Buffer Concentrate** (200X) in a clean graduated cylinder. Mix gently to avoid foaming.

Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 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. Washing Buffer may be prepared as needed according to the following table:

Number of Strips	Washing Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	5	995
1 - 12	10	1,990

2. Preparation of Standard Diluent Buffer

Add the content of the vial (10X) to 225 ml distilled water before use.

3. Preparation of TNFα Standards

Depending on the type of samples you are assaying, the kit includes two standard diluents. Because biologicals fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine you want to measure. You should reconstitute standard vials with the most appropriate Standard Diluent.
For serum and plasma samples: use Standard Diluent : human serum
For cells culture supernatants: use Standard Diluent Buffer

Reconstitute TNFα Standard by addition of appropriate Standard Diluent. Reconstitute volume is stated on the label of the standard vial.
This reconstitution produces a stock solution of 800 pg/ml TNFα. Allow standard to stand for 5 minutes with gentle swirling prior to making dilutions. Serial dilutions of standard must be made before each assays and cannot be stored.

4. Preparation of Controls

Freeze-dried control vials should also be reconstituted with the most appropriate Standard Diluent to your samples.
For serum and plasma samples: use Standard Diluent : human serum
For cells culture supernatants: use Standard Diluent Buffer.

Control have to be reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution for which the TNFα concentration is stated on the vial. Allow control to stand for 5 minutes with gentle swirling prior to distribute in control wells. Do not store after use.

5. Preparation of biotinylated anti TNFα

Preparation immediately before use is recommended. Dilute the biotinylated anti-TNFα with the biotinylated antibody diluent in a clean glass vial. Biotinylated anti TNFα may be prepared as needed according the following table. Extemporaneous preparations are recommended.

Number of Strips	Biotinylated Antibody Concentrate (µl)	Biotinylated Antibody Diluent (µl)
2	40	1,060
3	60	1,590
4	80	2,120
6	120	3,180
12	240	6,360

6. Preparation of Streptavidin-HRP

Dilute the Streptavidin-HRP 1:100 just prior to use by adding 0.5 ml of HRP diluent to the vial containing Streptavidin-HRP concentrate. DO NOT KEEP THIS DILUTION FOR FURTHER EXPERIMENTS.
Make a further dilution with HRP-Diluent in a clean glass vial as needed according to the following table:

Number of Strips	Pre-diluted Streptavidin-HRP (µl)	HRP Diluent (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	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 control samples should be assayed in duplicate. Remove sufficient **Microwell Strips coated with Antibody to human TNFα** from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly.

c. Add 100µl of of **appropriate Standard Diluent** (see preparation of reagents) to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2.
Reconstitute **standard vial** with the appropriate volume as described in the chapter preparation of reagents. Pipette 200 µl of standard into wells A1 and A2 (see Figure 1 and 2). Transfer 100 µl from A1 and A2 to B1 and B2 wells. Mix the contents by repeated aspirations and ejections. Take care not to scratch the inner surface of microwells. Repeat this procedure from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and so on creating two parallel rows of TNFα standard dilutions ranging from 800 to 25 pg/ml. Discard 100 µl from the content of the last microwells used (F1, F2).
Alternatively these dilutions can be done in separate tube and diluted standard pipetted directly into wells.

Figure 1. Preparation of TNFα standard dilutions:

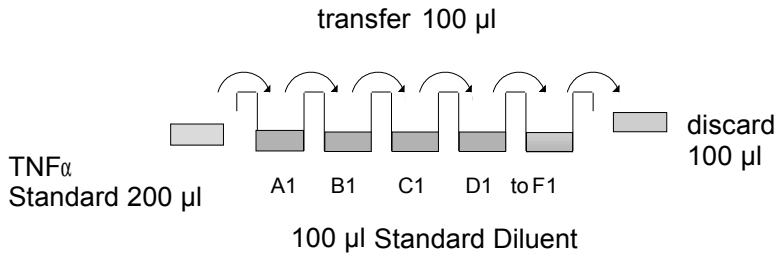


Figure 2. Diagram depicting an example of the arrangement of blanks, standards, samples and controls in the microwell strips:

Standard Concentrations pg/mL			Sample wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	800	800										
B	400	400										
C	200	200										
D	100	100										
E	50	50										
F	25	25										
G	Blank	Blank										
H	Ctrl	Ctrl										

d. Add 100 µl of **appropriate Standard Diluent** in duplicate, to the blank wells (G1, G2).

e. Add 100 µl of **Sample** to sample wells, in duplicate, to the designated wells and 100 µl of **reconstituted control vial,** in duplicate, to control wells (H1, H2).

f. Prepare biotinylated anti TNFα (refer to Preparation of reagents 5.).

g. Add 50 µl of **diluted biotinylated anti TNFα** to all wells.

h. Cover with a Plate Cover and incubate at room temperature (18° to 25°C) for 3 hours.

i. Remove the cover and wash the plate as follows:
   1. Aspirate the liquid from each well;
   2. Dispense 0.3 ml of washing solution into each well;
   3. Aspirate again the content of each well;
   4. Repeat step 2. and 3. two times

j. Prepare Streptavidin-HRP solution just before use: (refer to Preparation of reagents 6.)

k. Distribute 100 µl of **Streptavidin-HRP** solution to all wells, including blanks.

l. Cover the plate and incubate the plate at room temperature (18°C to 25°C) for 30 min.

m. Remove the cover and empty wells. Wash microwell strips according to step i. Proceed immediately to the next step.

n. Pipette 100 µl of ready-to-use **TMB Substrate Solution** to all wells, including the blank wells and incubate in the dark for about 12-15 minutes at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.

o. Incubation time of the substrate solution is usually determined by the ELISA reader performances. Many ELISA readers record absorbance only up to 2.0 O.D. **Therefore the colour development within individual microwells must be watched by the person running the assay, and the substrate reaction stopped before positive wells are no longer properly recordable.**

p. The enzyme-substrate reaction is stopped by quickly pipetting 100 µl of **H_2SO_4 : Stop Reagent** into each well, including the blank wells to completely and uniformly inactivate the enzyme. Results must be read immediately after the H_2SO_4 : Stop Reagent is added.

q. 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, controls and the TNFα standards.

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards, samples and controls. Duplicates should be within 20 per cent of the mean.

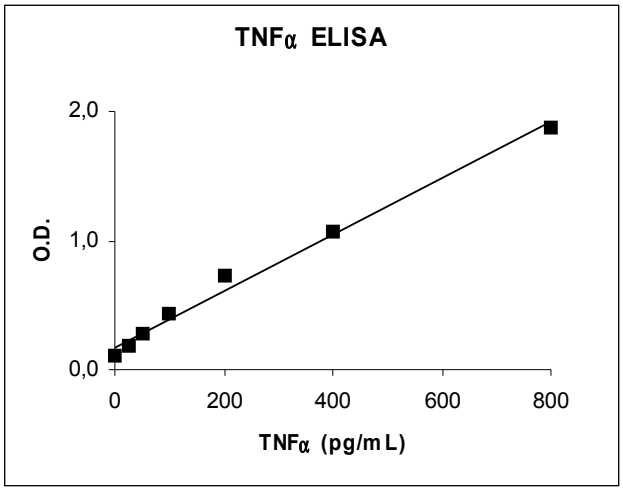
- Create a linear standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the TNFα standard concentration on the abscissa.

- To determine the concentration of TNFα in each sample, first find the mean OD 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 TNFα concentration.

  Note: Do not extrapolate the standard curve beyond the 800 pg/ml standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples (> 800 pg/ml) have to be diluted with standard diluent or with your own sample buffer. During analysis, multiply results by the appropriate dilution factor.

- 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 TNFα ELISA ranging from 25 to 800 pg/ml.
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 TNFα ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	TNFα Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	800 800	1.834 1.932	1.883	3.7
2	400 400	0.993 1.158	1.076	10.8
3	200 200	0.702 0.746	0.724	4.3
4	100 100	0.407 0.452	0.430	7.4
5	50 50	0.276 0.277	0.277	0.3
6	25 25	0.167 0.193	0.180	10.2
Blank	0 0	0.105 0.099	0.102	4.2

LIMITATIONS OF THE PROCEDURE

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either 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. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection for TNFα, defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus three standard deviations) was determined to be less than 8 pg/ml. The sensitivity was determined by evaluation of 40 blanks (80 replicates) in one session.

Reproducibility

a. Intra-assay

Reproductibility within the assay will be evaluated in three independent experiments. Each assay will be carried out with 6 replicates (3 duplicates) of 3 spiked human pooled serum samples containing different concentrations of TNFα. **The overall intra-assay coefficient of variation has been calculated to be 3.3%.**

Session	Samples	Assay 1 [TNFα] pg/ml	Assay 2 [TNFα] pg/ml	Assay 3 [TNFα] pg/ml	Mean intra assay	SD intra assay	CV Intra assay
1	1	837.0	870.0	781.0	829.3	45.0	5.43
	2	531.0	518.0	539.0	529.3	10.6	2.00
	3	199.0	203.0	201.0	201.0	2.0	1.00
2	1	817.0	814.0	790.0	807.0	14.8	1.8
	2	447.0	470.0	449.0	455.3	12.5	2.8
	3	168.0	165.0	182.0	171.7	9.1	5.3
3	1	827.0	845.0	827.0	833.0	10.4	1.2
	2	501.0	492.0	476.0	489.7	12.7	2.6
	3	170.0	175.0	173.0	172.7	2.5	1.5

Example operator A, Overall CV = 2.6%

b. Inter-assay

Assay to assay reproductibility within one laboratory will be evaluated in four independent experiments by two technicians. Each assay will be carry out with 6 replicates of 3 spiked human pooled serum samples containing different concentration of TNFα. **The calculated overall coefficient of variation was 9%.**

Operator	Session	Sample 1 [TNFα] pg/ml	Sample 2 [TNFα] pg/ml	Sample 3 [TNFα] pg/ml
A	1	837	531	199
		870	518	203
		781	539	201
	2	817	446	168
		814	469	164
		790	449	182
	3	827	501	170
		845	492	175
		827	476	173
	4	843	429	149
		810	412	148
		869	392	164
B	1	848	443	157
		860	455	155
		778	492	158
	2	817	431	161
		820	466	158
		815	438	144
	3	772	393	156
		771	381	140
		711	388	134
	4	787	444	138
		781	420	142
		758	442	162
Mean		810	452	163
SD		38	44	19
CV		5	10	12

Spike Recovery

The spike recovery was evaluated by spiking three concentrations of recombinant TNFα in human pooled serum. Recoveries were determined in two independent experiments with 6 replicates each. Recoveries ranged from 74% to 90% with an overall mean recovery of 81%.

TNFα (pg/ml)	Mean Recovery (%) TNFα
800	74
400	81
200	90

Dilution Parallelism

Four serum samples with different levels of TNFα were analysed at different serial two fold dilutions (1:2 – 1:8) with 4 replicates each (2 duplicates). Recoveries ranged from 101% to 115% with an overall mean recovery of 107%.

		TNFα concentration (pg/ml)		
Sample	Dilution	Expected Value	Observed Value	% Recovery of Exp. Value
1	1:2	*	479	--
	1:4	259	240	108
	1:8	133	130	103
2	1:2	*	443	--
	1:4	254	222	115
	1:8	141	127	101
3	1:2	*	486	--
	1:4	279	243	115
	1:8	141	140	101
4	1:2	*	629	--
	1:4	347	315	110
	1:8	178	174	103

Sample stability

a. Freeze-Thaw Stability

Three Aliquots of 2 samples (spiked) were stored at -20°C and thawed up to 4 times, and the TNFα levels determined. As shown in the table below, there was no significant loss of TNFα after 5 cycles of freezing and thawing.

N° of Freeze	TNFα Sample 1		TNFα Sample 2	
Thaw cycles	Concentration (pg/ml)	Recovery (%)	Concentration (pg/ml)	Recovery (%)
0	329	-	214	-
1	382	116	233	109
3	369	112	212	99
5	307	93	197	92

b. Storage stability

Aliquots of two serum samples (spiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the TNFα level determined after 24 h. As shown in the table below, there is no significant loss of TNFα immunoreactivity during storage at 2-8°C and RT, but there is a significant loss of immunoreactivity when the sample is stored at 37°C

Storage	TNFα Sample 1		TNFα Sample 2	
Temperature	Concentration (pg/ml)	Recovery (%)	Concentration (pg/ml)	Recovery (%)
-20°C	357	-	199	-
2-8°C	324	91	195	98
RT	295	83	176	89
37°C	192	54	100	50

Specificity

The assay recognizes both natural and recombinant human TNFα. To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1β, IL-12, IFNγ, IL-4, IL-6, IL-8, IL-2, IL-10 and IL-13).

Expected serum values

A panel of 50 human sera was tested for TNFα. All were below the detection level < 8 pg/ml

Standard Calibration

This immunoassay is calibrated against the International Reference Standard NIBSC E. coli 87/650. NIBSC 87/650 is quantitated in International Units (IU), 1IU corresponding to 75 pg Diaclone TNFα

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REAGENT PREPARATION SUMMARY

1. Washing Buffer Add **Washing Buffer Concentrate** 200 X (10 ml) to 1990 ml distilled water

2 Standard Diluent Buffer Add **Standard Diluent Buffer Concentrate** 10 X (25 ml) to 225 ml distilled water

3. TNFα Standard Reconstitute **TNFα Standard** by addition of appropriate Standard Diluent as stated on vial label.

4. Controls Reconstitute **controls** by addition of appropriate Standard Diluent as stated on vial label.

5. Biotinylated anti TNFα

Number of Strips	Biotinylated Antibody Concentrate (µl)	Biotinylated Antibody Diluent (µl)
2	40	1,060
3	60	1,590
4	80	2,120
6	120	3,180
12	240	6,360

6. Streptavidin-HRP

Number of Strips	Pre-Diluted Streptavidin-HRP (µl)	HRP-Diluent (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

TEST PROTOCOL SUMMARY: Total procedure length: 3h45mn

- Add 100 µl of appropriate Standard Diluent Buffer, in duplicate, to standard wells (B1 to F2)
- Pipette 200 µl reconstituted TNFα Standard in wells A1 and A2 and create standard dilutions ranging from 800 to 25 pg/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells.
- Add 100 µl of appropriate **Standard Diluent Buffer** , in duplicate, to the blank wells.
- Add 100 µl of **Sample**, in duplicate, to designated wells and 100 µl of reconstituted **control**, in duplicate, to control wells.
- Prepare Biotinylated anti TNFα
- Add 50 µl of **diluted biotinylated anti TNFα** to all wells
- Cover microwell strips and incubate 3 hours at room temperature (18-25°C)
- Empty and wash microwell strips 3 times with **Washing Buffer**
- Prepare Streptavidin-HRP
- Add 100 µl of **diluted Streptavidin-HRP** to all wells
- Incubate 30 minutes covered at room temperature (18° to 25°C).
- Empty and wash microwell strips 3 times with **Washing Buffer**
- Add 100 µl of ready-to-use **TMB solution** to all wells including blank wells.
- Incubate the microwell strips for about 12-15 minutes at room temperature (18° to 25°C) in the dark.
- Add 100 µl H_2SO_4: **Stop Solution** to all wells including blank wells.
- Measure colour intensity at 450 nm and optinally at 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

Note: Calculation of samples with an O.D. exceeding the range of the standard curve may result in incorrect, low TNFα levels. Such samples require further dilution with appropriate Standard Diluent Buffer in order to precisely quantitate the actual TNFα level.