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Human TNF-α ELISA Set

CATALOG No :	CDK095B
SIZE:	10 x 96 tests
SPECIFICITY :	Recognizes both natural and recombinant human TNF-α
RANGE :	25 pg/ml to 800 pg/ml
SENSITIVITY :	<10 pg/ml
INCUBATION :	From sample to end: 2 hr 45 min
SAMPLE TYPES :	Serum Plasma Cell culture supernatant
SAMPLE SIZE :	100 µl
CROSS REACTION :	No cross reactivity with other human cytokines Cross reactivity with Rhesus macaque TNF-alpha No interference with soluble TNFR I and TNFR II

Intended Use

The TNFα ELISA Set is intended for use in a 'do it yourself' solid phase sandwich ELISA for the *in vitro* qualitative and quantitative determination of TNFα in supernatants, buffered solutions, serum, plasma samples and other body fluids. This assay will recognize both natural and recombinant human TNFα. This kit has been configured for research use only. Not for human use.

Introduction

1. Background

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. 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. Activation of B-cells by the Epstein Barr virus can be inhibited by TNFα. Due to its varied actions throughout the immune system, TNFα may play a role in the pathogenesis of many disease states.

TNF-α production is mediated by the action of lymphokines and endotoxins on the macrophage. Purified monocytes produce TNFα within four hours of stimulation by recombinant IL-2 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. 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.

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2. Basic principle of the ELISA method

A capture antibody highly specific for TNFα is coated to the wells a microtiter strip plate. Binding of TNFα samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti-TNFα secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue colored complex with the conjugate. The color development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced colored complex is directly proportional to the concentration of TNFα present in the samples and standards. The absorbance of the color complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of TNFα in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store @ 2-8°C)	**Quantity** 10 x 96 tests	**Reconstitution**
TNFα Standard: 800 pg/ml	10 vials	Reconstitute as directed on the vial. (see assay preparation, section 9)
Capture Antibody	2 vials (0.5 ml)	Sterile, dilute prior to use. (see plate preparation, section 8)
Detection Antibody	2 vials	Biotinylated anti-TNFα Reconstitute with 0.55 ml of reconstitution buffer prior to use. (see assay preparation, section 9)
Streptavidin-HRP	2 vials (25 µl)	Dilute prior to use. (see assay preparation, section 9)
TMB Substrate	4 vials (25 ml)	Ready to use.

4. Materials required but not provided

- 96 well Microtiter plates (e.g. Nunc Maxisorp Cat # 468667)
  Note: the use of ELISA plates which are not high affinity binding will result in lower performances.
- Reconstitution Buffer (1x PBS, 0.09% Azide)
- Coating Buffer (1x PBS, pH 7.2-7.4)
- Wash Buffer (1x PBS, 0.05% Tween-20)
- Blocking Buffer (1x PBS, 5% BSA)
- Standard Dilution (1x PBS, 1% BSA)
- Buffer Secondary Antibody Dilution Buffer (1x PBS, 1% BSA)
  Note: Supplementation with 10% Animal Serum (e.g. FCS) for serum, plasma or other body fluids samples may be necessary
- HRP Diluent Buffer (1x PBS, 1% BSA, 0.1% Tween-20)
- Stop Reagent (1M Sulfuric Acid)
- Microtiter plate reader with appropriate filters (450 nm required with optional 630 nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000 µl adjustable single channel micropipettes with disposable tips
- 50-300 µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs

- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiration date of the reagents is stated on box front labels. The expiration of the 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.

Reconstitution Buffer: Once prepared store at 2-8°C for up to one week.

Coating Buffer: Once prepared store at 2-8°C for up to one week.

Wash Buffer: Once prepared use immediately.

Blocking Buffer: Once prepared store at 2-8°C for up to one week.

Standard and Secondary Antibody Dilution Buffer: Once prepared store at 2-8°C for up to one week.

HRP Diluent Buffer: Once prepared store at 2-8°C for up to one week.

Reconstituted Biotinylated anti TNFα Detection Antibody: Once prepared store at 2-8°C for up to one year.

Reconstituted TNFα Standard: Discard after use.

6. Specimen collection, processing & 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: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following 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 repeated 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 use. When possible avoid use of badly hemolyzed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

• Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984.

• Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water.

• Do not eat, drink, smoke or apply cosmetics where kit reagents are used.

• Do not pipette by mouth.

• When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottle labels.

• All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.

• Cover or cap all reagents when not in use.

• Do not mix or interchange reagents between different lots.

• Do not use reagents beyond the expiration date of the kit.

• Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H2SO4 and substrate solution, avoid pipettes with metal parts.

• Use a clean plastic container to prepare the washing solution.

• Thoroughly mix the reagents and samples before use by agitation or swirling.

• 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 insert absorbent paper directly into the wells.

• The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent color development. Warning TMB is toxic avoid direct contact with hands. Dispose of properly.

• If a dark blue color develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance within 1 hour after completion of the assay.

• When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.

• Follow incubation times described in the assay procedure.

• Dispense the TMB solution within 15 minutes of washing the microtiter plate.

8. Plate Preparation

8.1 Capture Antibody

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom. For one plate add 100 µl of Capture Antibody into 10 mL of Coating Buffer.

8.2 Preparation method

1.	Addition	Add 100 µl of diluted **Capture Antibody** to every well
2.	Incubation	Cover with a plastic plate cover and incubate at 4℃ overnight
3.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.4 ml of **washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c
4.	Addition	Add 250 µl of **Blocking Buffer** to every well
5.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25℃) for **2 hours**.
6.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.4 ml of washing solution into each well c) Aspirate the contents of each well d) Repeat step b and c

For Immediate use of the plate(s) continue to section 9.
To store the coated and blocked plates for future use, bench dry each plate at room temperature (18 to 25℃) for 24 hours. Cover the plates and then store at 2-8℃ in a sealed plastic bag with desiccant for up to 12 months.

9. Assay Preparation
Bring all reagents to room temperature before use.

9.1 Assay Design
Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**.

Example of plate layout (example shown for a 6 point standard curve)

	Standards		**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	zero	zero										
H												

All remaining empty wells can be used to test samples in duplicate.

9.2 Preparation of Standard
Standard vials must be reconstituted with the volume of standard dilution buffer shown on the vial immediately prior to use. This reconstitution gives a stock solution of 800pg/ml of TNFα. Mix the reconstituted standard gently by inversion only. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 800 to 25 pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200 µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration of standard at 800 pg/ml.
- Add 100 µl of appropriate standard dilution buffer to the remaining standard wells B1 and B2 to F1 and F2.
- Transfer 100 µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells.
- Continue this 1:1 dilution using 100 µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 800 pg/ml to 25 pg/ml.
- Discard 100 µl from the final wells of the standard curve (F1 and F2).

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

9.3 Preparation of Biotinylated anti-human TNFα Detection Antibody
It is recommended this reagent is prepared immediately before use. Dilute the reconstituted biotinylated anti-human TNFα with the biotinylated antibody dilution buffer in an appropriate clean glass vial.

For one plate add 100 µl of the reconstituted detection antibody into 5 mL of biotinylated antibody dilution buffer.

9.4 Preparation of Streptavidin-HRP
It is recommended to centrifuge vial for a few seconds in a micro centrifuge to collect all material at the bottom.

Dilute 5 µl of Streptavidin-HRP into 0.5 ml of HRP diluent buffer immediately before use.
Take 150 µl of the diluted HRP solution into 10 mL of HRP diluent buffer.

Do not keep these solutions for future experiments.

10. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Note: Final preparation of Biotinylated anti-TNFα and Streptavidin-HRP should occur immediately before use.

Assay Step		Details
1.	Preparation	**Prepare Standard curve** as shown in section 9.2 above.
2.	Addition	Add 100 µl of each **standard, sample and zero** (Standard Dilution Buffer) in duplicate to appropriate number of wells.
3.	Addition	Add 50 µl of diluted **Detection Antibody** to all wells.
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **3 hours.**
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.4 ml of **washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c
6.	Addition	Add 100 µl of **Streptavidin-HRP** solution into all wells.
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min.**
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100 µl of ready-to-use **TMB Substrate Solution** into all wells.
10.	Incubation	Incubate in the dark for **5-15 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
11.	Addition	Add 100 µl of **H_2SO_4: Stop Reagent** into all wells.
Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optimally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).		

Read the absorbance value of each well immediately after step 11 on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm is acceptable).

**Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the color development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range.*

11. Data Analysis

Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding TNFα standard concentration on the horizontal axis.

The amount of TNFα in each sample is determined by extrapolating OD values against TNFα standard concentrations using the standard curve**.**

12. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer; fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore a fresh standard curve must be prepared and run for every assay.

13. Performance Characteristics

Sensitivity

The sensitivity, or minimum detectable dose, of this TNFα antibody pair was determined using the Cell Sciences Human TNFα ELISA kit (which contains the same antibodies) and was found to be **<8 pg/ml**. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 40 times.

Specificity

The assay recognizes natural human TNFα. To define specificity of this TNFα antibody pair, several proteins were tested for cross reactivity using the Cell Sciences Human TNFα ELISA kit (which contains the same antibodies). There was no cross reactivity observed for any protein tested (IL-1β, IL-6, IL-12, IL-4, IL-2, IFNγ, IL-10, IL-8, and IL-13).

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