

KAMIYA BIOMEDICAL COMPANY

Human TNF α ELISA

**For the quantitative determination of TNF α
in human cell culture supernates, serum and plasma (heparin, EDTA, citrate)**

Cat. No. KT-1489

For Research Use Only. Not for diagnostic use in the U.S.

PRODUCT INFORMATION**Human TNF α ELISA
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INTRODUCTION

Tumor necrosis factor-alpha (TNF-alpha, or TNF) is secreted by macrophages in response to inflammation, infection and cancer. Human Tumor Necrosis Factor (TNF) and Lymphotoxin (TNF-beta) are cytotoxic proteins which have similar biological activities and share 30% amino acid homology. TNF-alpha is produced by monocytes, which can stimulate endothelial cells to produce the multilineage growth factor granulocyte-macrophage colony-stimulating factor and extend the role of this immunoregulatory protein to the regulation of hematopoiesis in vitro. TNF is a soluble protein that causes damage to tumor cells but has no effect on normal cells. Human TNF has been purified to apparent homogeneity as a 17.3-kilodalton protein from HL-60 leukemia cells and has showed cytotoxic and cytostatic activities against various human tumor cell lines. The human TNF cDNA is 1585 base pairs in length and encodes a protein of 233 amino acids. The mature protein begins at residue 77, leaving a long leader sequence of 76 amino acids. TNF-alpha has been mapped to human chromosome 6.

PRINCIPLE

The human TNF α ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A monoclonal antibody from mouse specific for TNF α has been precoated onto 96-well plates. Calibrators (E.coli,V77-L233) and test samples are added to the wells, a biotinylated detection polyclonal antibody from goat specific for TNF α is added subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the human TNF α amount of sample captured in plate.

COMPONENTS

1. 96-well plate precoated with anti-human TNF α antibody: 1
2. lyophilized recombinant human TNF α calibrator: 10 ng/tube x 2
3. biotinylated anti-human TNF α antibody: 130 μ L (dilution 1:100)
4. Avidin-Biotin-Peroxidase Complex (ABC): 130 μ L (dilution 1:100)
5. sample diluent buffer: 30 mL
6. antibody diluent buffer: 12 mL
7. ABC diluent buffer: 12 mL
8. TMB color developing agent: 10 mL
9. TMB stop solution: 10 mL

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader in standard size.
2. Automated plate washer.
3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.

4. Clean tubes and Eppendorf tubes.

5. Washing buffer (neutral PBS or TBS).

-Preparation of 0.01 M **TBS**: Add 1.2 g Tris, 8.5 g NaCl; 450 μ L of purified acetic acid or 700 μ L of concentrated hydrochloric acid to 1,000 mL H_2O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1 L.

-Preparation of 0.01 M **PBS**: Add 8.5 g sodium chloride, 1.4 g Na_2HPO_4 and 0.2 g NaH_2PO_4 to 1,000 mL distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1 L.

PRECAUTIONS

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using calibrators and a small number of samples is recommended.
2. The TMB Color Developing agent is colorless and transparent before using, contact us freely if it is not the case.
3. Before using the Kit, spin tubes and bring down all components to the bottom of tubes.
4. Duplicate well assay is recommended for both calibrator and sample testing.
5. Don't let 96-well plate dry, for dry plate will inactivate active components on plate.
6. Don't reuse tips and tubes to avoid cross contamination.
7. Avoid using the reagents from different batches together.
8. In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution will be pre-warmed in 37°C for 30 min before using.

REAGENT PREPARATION

1. Sample Preparation and Storage

Store samples to be assayed within 24 hours at 4°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

-**Cell culture supernates**: Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.

-**Serum**: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature or coat at 4°C overnight. Centrifuge at approximately 1,000 X g for 10 min. Analyze the serum immediately or aliquot and store samples at -20°C.

-**Plasma**: Collect plasma using **heparin, EDTA or citrate** as an anticoagulant. Centrifuge for 10 min at 1,000 x g within 30 min of collection at 4°C. Assay immediately or aliquot and store samples at -20°C.

2. Sample Dilution Guideline

The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the calibration curve. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. **The sample must be well mixed with the diluents buffer.**

-**High target protein concentration (10-100 ng/mL)**. The working dilution is 1:100. i.e. Add 1 μ L sample into 99 μ L sample diluent buffer.

-**Medium target protein concentration (1-10 ng/mL)**. The working dilution is 1:10. i.e. Add 10 μ L sample into 90 μ L sample diluent buffer.

-**Low target protein concentration (15.6-1,000 pg/mL)**. The working dilution is 1:2. i.e. Add 50 μ L sample to 50 μ L sample diluent buffer.

-**Very Low target protein concentration (\leq 15.6 pg/mL)**. No dilution necessary, or the working dilution is 1:2.

3. Reagent Preparation and Storage

A. Reconstitution of the human TNF α calibrator: TNF α calibrator solution should be prepared no more than 2 hours prior to the experiment. Two tubes of TNF α calibrator (10 ng per tube) are included in each kit. Use one tube for each experiment.

- a. 10,000 pg/mL of human TNF α calibrator solution: Add 1 mL sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
- b. 1,000 pg/mL of human TNF α calibrator solution: Add 0.1 mL of the above 10 ng/mL TNF α calibrator solution into 0.9 mL sample diluent buffer and mix thoroughly.
- c. 500 pg/mL \rightarrow 15.6 pg/mL of human TNF α calibrator solutions: Label 6 Eppendorf tubes with 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL respectively. Aliquot 0.3 mL of the sample diluent buffer into each tube. Add 0.3 mL of the above 1,000 pg/mL TNF α calibrator solution into 1st tube and mix. Transfer 0.3 mL from 1st tube to 2nd tube and mix. Transfer 0.3 mL from 2nd tube to 3rd tube and mix, and so on.

Note: The calibrator solutions are best used within 2 hours. The 10 ng/mL calibrator solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

B. Preparation of biotinylated anti-human TNF α antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.

- a. The total volume should be: 0.1 mL/well x (the number of wells). (Allowing 0.1-0.2 mL more than total volume)
- b. Biotinylated anti-human TNF α antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1 μ L Biotinylated anti-human TNF α antibody to 99 μ L antibody diluent buffer.)

C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.

- a. The total volume should be: 0.1 mL/well x (the number of wells). (Allowing 0.1-0.2 mL more than total volume)
- b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1 μ L ABC to 99 μ L ABC diluent buffer.)

STORAGE

Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles.

ASSAY PROTOCOL

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Calibrator TNF α detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of TNF α amount in samples.

1. Aliquot 0.1 mL per well of the 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL human TNF α calibrator solutions into the precoated 96-well plate. Add 0.1 mL of the sample diluent buffer into the control well (Zero well). Add 0.1 mL of each properly diluted sample of human cell culture supernates, serum or plasma (heparin) to each empty well. **See “Sample Dilution Guideline” above for details.** It is recommended that each human TNF α calibrator solution and each sample be measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1 mL of biotinylated anti-human TNF α antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash plate 3 times with 0.01 M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (**Plate Washing Method:** Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 mL PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)
6. Add 0.1 mL of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.
7. Wash plate 5 times with 0.01 M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method).
8. Add 90 μ L of prepared TMB color developing agent into each well and incubate plate at 37°C in dark for 25-30 min (**Note:** For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated human TNF α calibrator solutions; the other wells show no obvious color).
9. Add 0.1 mL of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450 nm in a microplate reader within 30 min after adding the stop solution.

For calculation, (the relative O.D.₄₅₀) = (the O.D.₄₅₀ of each well) – (the O.D.₄₅₀ of Zero well). The calibration curve can be plotted as the relative O.D.₄₅₀ of each calibrator solution (Y) vs. the respective concentration of the calibrator solution (X). The human TNFα concentration of the samples can be interpolated from the calibration curve.

Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

SUMMARY

1. Add samples and calibrators and incubate the plate at 37°C for 90 min. Do not wash.
2. Add biotinylated antibodies and incubate the plate at 37°C for 60 min. Wash plate 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the plate at 37°C for 30 min. Wash plate 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the plate at 37°C in dark for 25-30 min.
5. Add TMB stop solution and read.

PERFORMANCE CHARACTERISTICS

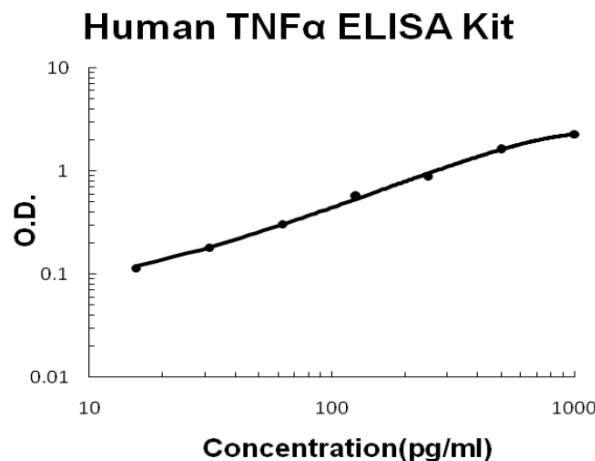
Typical Data Obtained from Human TNFα

(TMB reaction incubate at 37°C for 25 min)

Concentration(pg/ml)	0	15.6	31.2	62.5	125	250	500	1000
O.D	0.046	0.114	0.180	0.306	0.577	0.883	1.640	2.250

Typical Human TNFα ELISA Kit Calibration Curve

This calibration curve was generated for demonstration purpose only. A calibration curve must be run with each assay.



Range: 15.6 pg/mL - 1,000 pg/mL (cell culture superantes)

7.8 pg/mL - 500 pg/mL (serum, plasma)

Sensitivity: <1 pg/mL

Specificity: Natural and Recombinant Human TNFα

Cross-reactivity: No detectable cross-reactivity with other relevant proteins.

Intra-Assay Precision (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
Sample	1	2	3	1	2	3
n	16	16	16	24	24	24
Mean(pg/ml)	93	327	608	102	319	613
Standard deviation	5.1	15.4	31	7.65	15.3	35
CV(%)	5.5	4.7	5.1	7.5	4.8	5.7

FOR RESEARCH USE ONLY**KAMIYA BIOMEDICAL COMPANY**

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