CELL APPLICATIONS, INC.

Human TNFα ELISA Kit

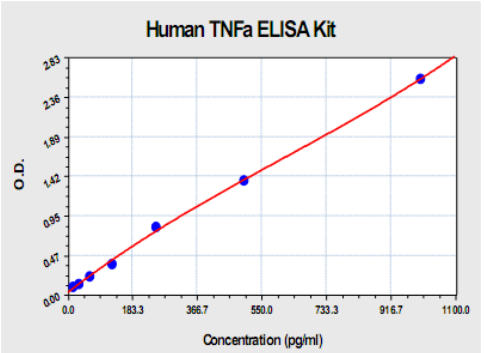
Cat. No. CL0525
96-wells

Target Protein Species: Human
Range: 15.6pg/ml-1000pg/ml (Body fluids, tissue lysates, cell culture supernates)
7.8pg/ml-500pg/ml (Human sera)
Specificity: No detectable cross-reactivity with other cytokines

KIT COMPONENTS

Component	Amount
96-well plate precoated with Anti-Human TNFα	1 Plate
Protein Standard: Lyophilized recombinant Human TNFα	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-Human TNFα)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethylbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

Storage
Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	15.6	31.2	62.5	125	250	500	1000
Y	O.D.450	0.047	0.100	0.133	0.227	0.373	0.807	1.364	2.573

Figure 1: TNFα Standard Curve. Using the Human TNFα ELISA Kit, O.D. data was graphed against TNFα protein concentration. The TMB reaction was incubated at 37°C for 16 min.

TNFα BACKGROUND

TNF-alpha is the first prototypic member identified in the TNF superfamily (TNFSF). The human TNF superfamily currently has 19 well-characterized members. Other members, such as TNFSF19, TNFSF21, and TNFSF22 have not been well-established. Although each member has its own receptor preference, a functional overlapping, such as induction of apoptosis and NF-kB activation, has been observed among the majority of these members. Except TNFSF1 (lymphotoxin alpha) and TNFSF3 (lymphotoxin beta) that can form either homotrimer or heterotrimer, the active form of other members in this family is homotrimer.[1] TNF-alpha is produced initially in a membrane-associated form, which is then subjected to enzymatic remove of the N-terminal 76 amino acids by TACE/ADAM17, a TNF-alpha converting enzyme, to generate the soluble 17kDa TNF-alpha molecule that forms homotrimer. TNF-alpha is expressed virtually in every type of cells in response to inflammatory signals. The most abundant cellular sources of TNF-alpha are macrophage and monocyte. TNF-alpha can induce apoptotic or necrotic cell death of certain tumor cell lines. In addition, TNF-alpha is also capable of inducing cell proliferation and differentiation in many types of cells under certain circumstances. TNF-alpha can be a pyrogen that causes fever by its direct action or by stimulation of interleukin 1 secretion. Sustained generation of TNF-alpha in a variety of human diseases, especially cancer and severe infection, can cause cachexia-like syndrome. The increased expression of TNF-alpha in adipose tissue was considered to be responsible for the development of obesity or diabetes due to the induction of insulin resistance by TNF-alpha.[2] All of above functional characteristics of TNF-alpha are executed through specific members of the TNF receptor (TNFR) superfamily, mainly TNFR1, the primary receptor for soluble TNF-alpha, and TNFR2, the predominant receptor for membrane-associated TNF-alpha. These receptors trigger several intracellular signaling pathways, most importantly, the IkB kinase (IKK) and mitogen-activated protein kinase (MAPK) cascades, which govern gene expression through NF-kB and AP-1 transcription factors, respectively.[3] Moreover, it has been shown that mutations or polymorphisms in the promoter or coding region of TNF-alpha gene have been associated with asthma, celiac, septic shock susceptibility, silicosis, Psoriasis, GVHD, Leprosy, etc.[4]

ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

References

1. Ware, C.F.: Cytokine Growth Factor Rev. 14:181-4, 2003
2. Aggarwal,B.B. et al: Current drug targets. Inflam. and allergy 1:327-341, 2002
3. Dampsey, P.W. et al: Cytokine Growth Factor Rev. 14:193-209, 2003
4. Brennan, F.M. et al: Br J Rheumatol. 31:293-8, 1992

ELISA PROTOCOL

Preparation of Test Samples

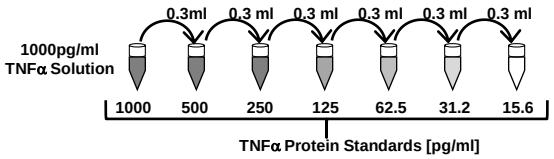
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation, analyze immediately or aliquot and store at -20° C.
   - **Serum**: Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 x g for 10 min. Analyze the serum immediately or aliquot and store frozen at -20°C.
2. Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the 15.6-1000 pg/ml standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
10-100 ng/ml	1:100	1 µl	99 µl
1-10 ng/ml	1:10	10 µl	90 µl
15.6-1000 pg/ml	1:2	50 µl	50 µl
≤15.6 pg/ml	n/a	100µl	n/a

3. If samples will be assayed within 24 hours, store at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

Preparation of Standard Solutions (15.6-1000 pg/ml)

4. Reconstitute the Lypophilized Recombinant Protein to make a 10,000 pg/ml TNFα solution. Add 1 ml Sample Diluent Buffer to a tube of lypophilized protein, keep the tube at room temperature for 10 min. Mix thoroughly.
5. Aliquot 0.9 ml of the Sample Diluent Buffer to an eppendorf tube, and label as 1000 pg/ml Protein Standard.
6. Add 0.1 ml of the mixed 10,000 pg/ml TNFα solution to the eppendorf tube containing 0.9 ml diluent buffer and mix to make a 1000 pg/ml TNFα solution.
7. Label 6 eppendorf tubes with the TNFα protein concentrations to be prepared by serial dilution: 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, 15.6pg/ml.
8. Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
9. Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 1000pg/ml TNFα Solution to the 500pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 500 pg/ml solution to the 250pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 250pg/ml solution to the 125pg/ml tube and mix, and so on to make the 62.5, 31.2, and 15.6 pg/ml solutions.
10. Store at 4°C until use.



Loading the 96-well Plate

11. Aliquot 0.1 ml of the sample diluent buffer into a control well to serve as the Blank. This will yield the O.D.450(Blank) reading.
12. Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (15.6-1000 pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.
13. Aliquot 0.1 ml of each properly diluted test sample to empty wells prepared in **Step 2**. Duplicate measurements of each test sample are recommended.
14. Cover the 96-well plate and incubate at 37°C for 90 min.
15. During the **Step 14** incubation period, prepare a stock of Biotinylated 1:100 Antibody Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the Biotinylated Antibody to 1:100 in Antibody Diluent Buffer and mix thoroughly. Use the working solution within 2 hours.
16. Upon completion of the 90 min incubation of **Step 14**, remove the cover of the 96 well plate and discard plate well contents. Blot the plate onto paper towels or other absorbent material. DO NOT let the wells completely dry at any time.
17. Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 15**) to each well and incubate the plate at 37°C for 60 min.
18. During the incubation period of **Step 17**, prepare a stock of ABC Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the ABC Stock Solution to 1:100 in ABC Diluent Buffer and mix thoroughly. Pre-warm the ABC working solution at 37°C for 30 min before use. Use the working solution within 1 hour.
19. Upon completion of the 60 min incubation of **Step 17**, wash the plate 3 times with 0.3 ml TBS or PBS. For each wash, leave washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
20. Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 18**) to each well and incubate the plate at 37°C for 30 min.
21. During the incubation period of **Step 20**, pre-warm TMB Color Developing Agent at 37°C for 30 min before use.
22. Upon completion of the 30 min incubation of **Step 20**, wash the plate 5 times with 0.3 ml TBS or PBS. For each wash, leave the washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
23. Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37°C for 15-20 min (shades of blue can be seen in the wells with the four most concentrated Protein Standard Solutions; the other control wells should show no obvious color).
24. Add 0.1 ml of the TMB Stop Solution to each well. The acidic stop solution will change the mixture color to yellow. The yellow intensity is proportional to the amount of target protein captured by the plate.
25. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution. These readings are the O.D.450(Reading).

Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):
  O.D.450(Relative) = O.D.450(Reading) – O.D.450(Blank)

- Plot the standard curve: plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.

- The TNFα concentration of the samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.