

Human TNF-alpha Sandwich ELISA Kit Datasheet

Please read it entirely before use

Catalogue Number: KE00154

Size: 96T

Sensitivity: 8.6 pg/mL

Range: 15.6-500 pg/mL

Usage: For the quantitative detection of human TNF-alpha concentrations in serum, plasma and cell culture supernatant.

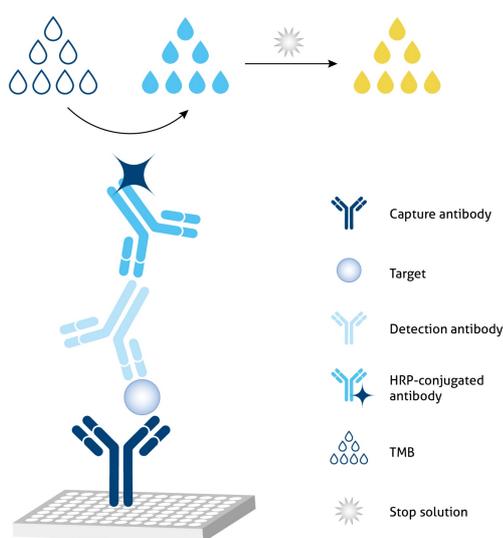
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1. Background

TNF, as also known as TNF-alpha, or cachectin, is a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. It is expressed as a 26 kDa membrane bound protein and is then cleaved by TNF-alpha converting enzyme (TACE) to release the soluble 17 kDa monomer, which forms homotrimers in circulation. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFR2. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. Dysregulation of TNF production has been implicated in a variety of human diseases including Alzheimer's disease, cancer, major depression and inflammatory bowel disease (IBD).

2. Principle



Sandwich ELISA structure (HRP conjugated secondary antibody)

A capture antibody is pre-coated onto the bottom of wells which binds to analyte of interest. A detection antibody also binds to the analyte. Horseradish peroxidase (HRP)-conjugated secondary antibody binds to the detection antibody. TMB acts as the HRP substrate and the solution color will change from colorless to blue. A stop solution containing sulfuric acid turns solution yellow. The color intensity is proportional to the quantity of bound protein which is measurable at 450 nm with the correction wavelength set at 630 nm.

3. Required Materials

- 3.1 A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.
- 3.2 Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.
- 3.3 Plate washer: automated or manual.
- 3.4 Absorbent paper towels.
- 3.5 Glass or plastic tubes to prepare standard and sample dilutions.
- 3.6 Beakers and graduated cylinders.
- 3.7 Log-log or semi-log graph paper or computer and software for ELISA data analysis. A four-parameter logistic (4-PL) curve-fit is recommended.

4. Kit Components and Storage

Microplate - antibody coated 96-well microplate (8 well × 12 strips)	1 plate	Unopened Kit: Store at 2-8°C for 6 months or -20°C for 12 months. Opened Kit: All reagents stored at 2-8°C for 7 days. Please use a new standard for each assay.
Protein standard - 1000 pg/bottle; lyophilized	2 bottles	
Detection antibody (100×) - 120 µL/vial*	1 vial	
HRP-conjugated antibody (100×) - 120 µL/vial*	1 vial	
Sample Diluent PT 1-ec - 30 mL/bottle. For human serum and human plasma	1 bottle	
Sample Diluent PT 6 - 30 mL/bottle. For cell culture supernatant	1 bottle	
Detection Diluent - 30 mL/bottle	1 bottle	
Wash Buffer Concentrate (20×) - 30 mL/bottle	1 bottle	
Tetramethylbenzidine Substrate (TMB) - 12 mL/bottle	1 bottle	
Stop Solution - 12 mL/bottle	1 bottle	
Plate Cover Seals	4 pieces	

* Centrifugation immediately before use

5. Safety Notes

- 5.1 Avoid any skin and eye contact with Stop Solution and TMB. In case of contact, wash thoroughly with water.
- 5.2 Do not use the kit after the expiration date.
- 5.3 Do not mix or substitute reagents or materials from other kit lots or other sources.
- 5.4 Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.
- 5.5 When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer to improve assay precision

6. Sample Collection and Storage

- 6.1 Serum: Allow blood samples to clot for 30 minutes, followed by centrifugation for 15 minutes at 1000×g. Clear serum can be assayed immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.
- 6.2 Plasma: Use EDTA, heparin, or citrate as an anticoagulant for plasma collection. Centrifuge for 15 minutes at 1000×g within 30 minutes of collection. The plasma can be assayed immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.
- 6.3 Cell Culture Supernatant: Remove particulates by centrifugation for 5 minutes at 500×g and assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

7. Regent Preparation

7.1 Wash Buffer (1X): If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate(20X) to 570 mL deionized or distilled water to prepare 1X Wash Buffer.

7.2 Detection Antibody (1X): Dilute 100X Detection Antibody 1:100 using Detection Diluent prior to assay. Suggested 1:100 dilution: 10 µL 100X Detection Antibody + 990 µL Detection Diluent (Centrifuge the 100 X Detection Antibody solution for a few seconds prior to use).

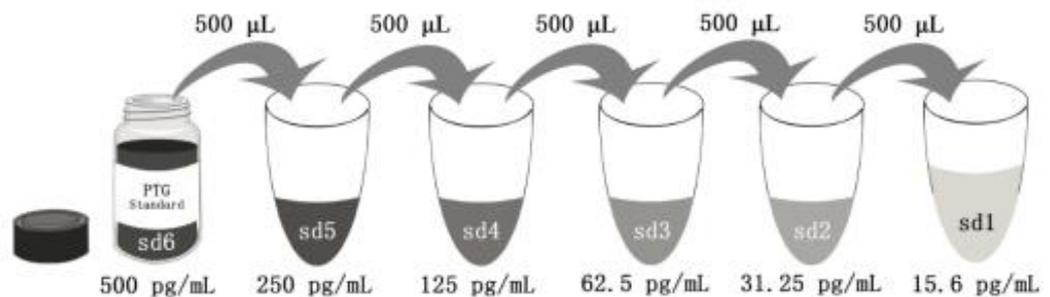
7.3 HRP-conjugated antibody (1X): Dilute 100X HRP-conjugated antibody 1:100 using Detection Diluent prior to assay. Suggested 1:100 dilution: 10 µL 100X HRP-conjugated antibody + 990 µL Detection Diluent (Centrifuge the 100X HRP-conjugated antibody solution for a few seconds prior to use).

7.4 Sample Dilution: Different samples should be diluted with corresponding Sample Diluent, samples may require further dilution if the readout values are higher than the highest standard OD reading. Variations in sample collection, processing and storage may affect the results of the measurement.

Recommended Dilution for different sample types: 1:2 or 1:4 is recommended for human serum, human plasma and cell culture supernatant.

7.5 Standard Serial Dilution:

For human serum and plasma samples, add 2 mL Sample Diluent PT 1-ec in protein standard; For cell culture supernatant, add 2 mL Sample Diluent PT 6 in protein standard.



Add # µL of Standard diluted in the previous step	—	500 µL				
# µL of Sample Diluent PT1-ec or PT 6	2000 µL	500 µL				
	"sd6"	"sd5"	"sd4"	"sd3"	"sd2"	"sd1"

8. Assay Procedure Summary

Bring all reagents to room temperature before use (Detection antibody and HRP-conjugated antibody can be used immediately). To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

8.1 Take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

8.2 Preset the layout of the microplate, including control group, standard group and sample group, add 100 µL of each standard and sample to the appropriate wells. (Make sure sample addition is uninterrupted and completed within 5 to 10 minutes, it is recommended to assay all standards, controls, and samples in duplicate).

8.3 Seal plate with cover seal, pressing it firmly onto top of microwells. Incubate the plate for 2 hours at 37°C.

8.4 Wash

1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels.

2) Wash 4 times with 1X Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

8.5 Add 100 µL of 1X Detection Antibody solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate for 1 hour at 37°C.

8.6 Repeat wash step in 8.4.

8.7 Add 100 µL of 1X HRP-conjugated antibody solution (refer to Reagent Preparation 7.3) to each well. Seal plate with cover seal and incubate the plate for 40 minutes at 37°C.

8.8 Repeat wash step in 8.4.

8.9 Signal development: Add 100 µL of TMB substrate solution to each well, protected from light. Incubate for 15 to 20 minutes. Substrate Solution should remain colorless until added to the plate.

8.10 Quenching color development: Add 100 µL of Stop Solution to each well in the same order as addition of the TMB substrate. Mix by tapping the side of the plate gently. NB: Avoid skin and eye contact with the Stop solution.

8.11 Read results: Immediately after adding Stop solution read the absorbance on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

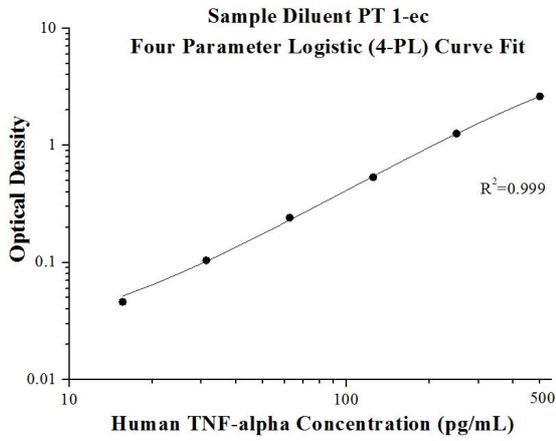
8.12 Data analysis: Calculate the average of the duplicate readings (OD value) for each standard and sample, and subtract the average of the zero standard absorbance. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, use four-parameter logistic curve-fit (4-PL) analysis to do this. If the samples have been diluted, the OD readout from the standard curve must be multiplied by the dilution factor used.

Step	Reagent	Volume	Incubation	Wash	Notes
1	Standard and Samples	100 µL	120 min	4 times	Cover Wells incubate at 37°C
2	Diluent Antibody Solution	100 µL	60 min	4 times	Cover Wells incubate at 37°C
3	Diluent HRP Solution	100 µL	40 min	4 times	Cover Wells incubate at 37°C
4	TMB Substrate	100 µL	15-20 min	Do not wash	Incubate in the dark at 37°C
5	Stop Solution	100 µL	0 min	Do not wash	-
6	Read plate at 450 nm and 630 nm immediately after adding Stop solution. DO NOT exceed 5 minutes.				

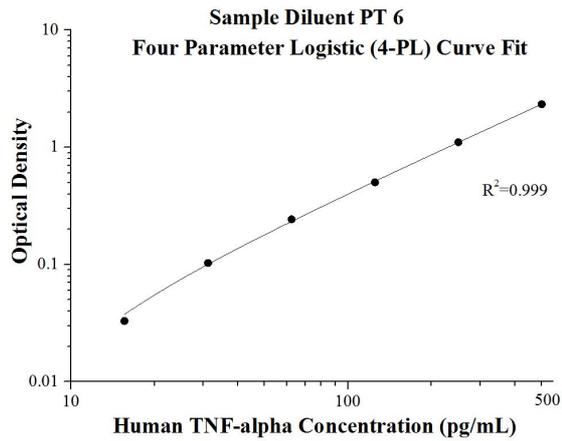
9. Validation Data

9.1 Standard curve

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D	Average	Corrected
0	0.04 0.04	0.040	-
15.6	0.087 0.085	0.086	0.046
31.25	0.142 0.146	0.144	0.104
62.5	0.275 0.287	0.281	0.241
125	0.561 0.584	0.573	0.533
250	1.291 1.304	1.298	1.258
500	2.623 2.692	2.658	2.618



(pg/mL)	O.D	Average	Corrected
0	0.076 0.084	0.080	-
15.6	0.106 0.119	0.113	0.033
31.25	0.190 0.175	0.183	0.103
62.5	0.320 0.325	0.323	0.243
125	0.582 0.582	0.582	0.502
250	1.186 1.182	1.184	1.104
500	2.381 2.439	2.410	2.330

9.2 Precision

Intra-assay Precision (Precision within an assay) Three samples of known concentration were tested 20 times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays) Three samples of known concentration were tested in 24 separate assays to assess inter-assay precision.

Intra-assay Precision					Inter-assay Precision				
Sample	n	Mean (pg/mL)	SD	CV%	Sample	n	Mean (pg/mL)	SD	CV%
1	20	52.5	2.0	3.8	1	24	51.3	2.4	4.6
2	20	87.3	2.8	3.2	2	24	86.2	2.1	2.5
3	20	184.8	5.8	3.1	3	24	184.2	5.1	2.7

9.3 Recovery

The recovery of human TNF-alpha spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type		Average% of Expected	Range (%)
Human plasma	1:2	109	103-117
	1:4	94	82-101
Cell culture supernatant	1:16	96	85-106
	1:32	94	88-97

9.4 Sample values

Twenty-four serum samples from healthy volunteers were evaluated for human TNF-alpha in this assay. All samples measured less than the lowest standard, 15.6 pg/mL. No medical histories were available for the donors used in this study.

Cell Culture supernatant - Human peripheral blood mononuclear cells (PBMC) (1×10^6 cells/mL) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin sulfate. The cell culture supernatants were stimulated with 10 µg/mL of PHA. An aliquot of the cell culture supernatant was removed, assayed for human TNF-alpha.

Stimulated conditions	Day 1 (pg/mL)	Day 3 (pg/mL)
PHA 10µg/mL	2,474.8	2,029.4
Unstimulated	405.2	94.1

Cell Culture Supernatant - Human peripheral blood mononuclear cells (PBMC) (1×10^6 cells/mL) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were stimulated with 10 ng/mL of PMA and 500 ng/mL of Ionomycin (MCE[®], Catalog #HY-1343/CS-0006887) for 24 hours. An aliquot of the culture supernates were removed, assayed for levels of human TNF-alpha.

Stimulated conditions	Day 1 (pg/mL)
10 ng/mL of PMA+ 500 ng/mL of Ionomycin	432
Unstimulated	55

9.5 Sensitivity

The minimum detectable dose of human TNF-alpha is 8.6 pg/mL. This was determined by adding two standard deviations to the concentration corresponding to the mean O.D. of 20 zero standard replicates.

9.6 Linearity

To assess the linearity of the assay, three samples were spiked with high concentrations of human TNF-alpha in human plasma and diluted with **Sample Diluent PT 1-ec** to produce samples with values within the dynamic range of the assay. Cell culture supernatant was diluted with **Sample Diluent PT6** to produce samples with values within the dynamic range of the assay. (The cell culture supernatant samples were initially diluted 1:2)

		Human plasma (Sample Diluent PT 1-ec)	Cell culture supernatant (Sample Diluent PT 6)
1:2	Average% of Expected	98	98
	Range (%)	90-106	80-112
1:4	Average% of Expected	91	97
	Range (%)	85-96	86-107
1:8	Average% of Expected	93	100
	Range (%)	92-94	95-103
1:16	Average% of Expected	97	99
	Range (%)	93-101	82-109

9.7 Specificity

This assay recognizes natural and recombinant human TNF-alpha.

The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference.

Recombinant human:	Recombinant mouse:	Recombinant rat:
TNF- β	TNF- α	TNF- α
TNF RI		
TNF RII		
TNFSF13		

10. References

1. Agbanoma G. et al. (2012) J Immunol. 188: 1307-17
2. Kriegler M. et al. (1988) Cell. 53: 45-53.
3. Theiss AL. et al. (2005) J Biol Chem. 280: 36099-109
4. Swardfager W. et al. (2010) Biol Psychiatry. 68:930-41
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6. provided by RefSeq, Jul 2008