

# Human TNF-alpha HP ELISA Kit

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This package insert must be read in its entirety  
before using this product

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## **Background**

Tumor Necrosis Factor alpha (TNF- $\alpha$ ), also known as cachectin and TNFSF1A, is the prototypic ligand of the TNF superfamily. It is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism. TNF- $\alpha$  is also involved in a number of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, neuropathic pain, obesity, type 2 diabetes, septic shock, autoimmunity, and cancer. Human TNF- $\alpha$  is synthesized as a 26 kDa type II transmembrane protein that consists of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 177 aa extracellular domain (ECD). Within the ECD, human TNF- $\alpha$  shares 97% aa sequence identity with rhesus monkey, and 71%-92% aa identity with bovine, canine, cotton rat, equine, feline, mouse, porcine, and rat TNF- $\alpha$ . It is produced by a wide variety of immune, epithelial, endothelial, and tumor cells. TNF- $\alpha$  is assembled intracellularly to form a noncovalently linked homotrimer which is expressed on the cell surface. Cell surface TNF- $\alpha$  can both induce the lysis of tumor cells and virus infected cells, and generate its own downstream cell signaling following ligation by soluble TNF RI.

## **Introduction**

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of TNF-alpha in human serum, cell culture supernatants.

<b>Features</b>	<b>Specifications</b>
Sensitivity	0.51pg/mL
Detection Range	3.13-200pg/mL
Suitable Sample	serum, plasma, cell lysates, cell culture
Specificity	No significant cross-reactivity of similar compounds was found (see Specificity)
Recommended dilution	See sample preparation section below for details

## **Principle of the Assay**

This assay employs the quantitative sandwich enzyme immunoassay technique. An monoclonal antibody specific for human TNF-alpha has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-alpha present is bound by the immobilized antibody. After washing away any unbound substances, and then a detection antibody specific for TNF-alpha is added to the wells and binds to the combination of capture antibody TNF-alpha in sample. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Following incubation and wash steps, a substrate solution is added to the wells and color develops in proportion to the amount of TNF-alpha bound in the initial step. The color development is stopped and the absorbance is measured.

## **Material Provided & Storage Conditions**

Unopened kits can be stored at 2-8°C for 1 year, and opened products must be used within 1 month.

<b>Part</b>	<b>Size</b>	<b>Cat. No.</b>	<b>Storage of opened/reconstituted material</b>
Human TNF-alpha Microwell Plate Coated	8x12	RM96680	Put the unused slats back in the aluminum foil bag with the desiccant and reseal them. They can be stored at 2-8°C for 1 month.
Human TNF-alpha Standard Lyophilized	200pg*4 vials	RM96681	It is not recommended to use again after redissolving.
Human TNF-alpha Concentrated Biotin Conjugate Antibody (100x)	1 x120ul	RM96682	Store at 2-8°C for 1 month *

Streptavidin-HRP Concentrated (100x)	1 x120ul	RM96683	Store at 2-8°C for 1 month *
Quality Control	12.5pg*2 支	RM96681 C	It is not recommended to use again after redissolving.
Standard/Sample Diluent (R1)	1 x20mL	RM00023	Store at 2-8°C for 1 month *
Biotin-Conjugate Antibody Diluent (R2)	1 x12mL	RM00024	
Streptavidin-HRP Diluent(R3)	1 x12mL	RM00025	
Wash Buffer(20x)	1 x30mL	RM00026	
TMB Substrate	1 x12mL	RM00027	
Stop Solution	1 x6mL	RM00028	
Plate Sealers	4 Strips		
Specification	1		



## **Other Supplies Required**

1. Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 630 nm or 570 nm.
2. Pipettes and pipette tips.
3. Deionized or distilled water.
4. Squirt bottle, manifold dispenser, or automated microplate washer.
5. Incubator.
6. Test tubes for dilution of standards and samples.

## **Precautions**

1. Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
2. Variations in sample collection, processing, and storage may cause sample value differences.
3. Reagents may be harmful, if ingested, rinse it with an excess amount of tap water.
4. Stop Solution contains strong acid. Wear eye, hand, and face protection.
5. Please perform simple centrifugation to collect the liquid before use.
6. Do not mix or substitute reagents with those from other lots or other sources.
7. Adequate mixing is particularly important for good result. Use a mini-vortexer at the lowest frequency.
8. Mix the sample and all components in the kits adequately, and use clean plastic container to prepare all diluents.
9. Both the sample and standard should be assayed in duplicate, and reagents should be added in sequence in accordance with the requirement of the specification.
10. Reuse of dissolved standard is not recommended.

11. The kit should not be used beyond the expiration date on the kit label.
12. The kit should be away from light when it is stored or incubated.
13. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma, and other biological fluids in accordance with NCCLS regulations.
14. To avoid cross contamination, please use disposable pipette tips.
15. Please prepare all the kit components according to the Specification. If the kits will be used several times, please seal the rest strips and preserve with desiccants. Do use up within 2 months.
16. This assay is designed to eliminate interference by other factors present in biological samples.
17. Until all factors have been tested in this assay, the possibility of interference cannot be excluded.
18. The 48T kit is also suitable for the specification.

## **Sample Collection & Storage**

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

**Cell Culture Supernatant:** Remove particulates by centrifugation. Assay immediately or aliquot and store samples at  $\leq -20$  °C. Avoid repeated freeze-thaw cycles.

**Serum:** Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at  $\leq -20$  °C. Avoid repeated freeze-thaw cycles.

**Plasma:** Collect plasma using EDTA or Heparin as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay immediately or aliquot and store samples at  $\leq -20$ °C. Avoid repeated freeze-thaw cycles. (Note: Citrate plasma has not been validated for use in this assay.)

**Cell Lysates :** Cells need to be lysed before assaying according to the following directions. Adherent cells should be washed by cold PBS gently, and then detached with trypsin, and collected by centrifugation at 1,000 x g for 5 minutes (suspension cells can be collected by centrifugation directly).

Wash cells three times in cold PBS. Resuspend cells in fresh lysis buffer with concentration of  $10^7$  cells/mL. If it is necessary, the cells could be subjected to ultrasonication till the solution is clarified. Centrifuge at  $1,500 \times g$  for 10 minutes at  $2-8\text{ }^{\circ}\text{C}$  to remove cellular debris. Assay immediately or aliquot and store at  $\leq -20\text{ }^{\circ}\text{C}$ .

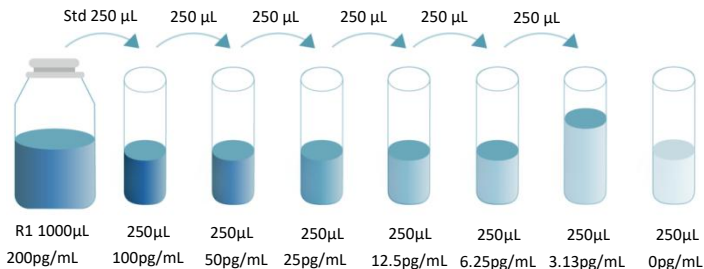
**Note:** It is suggested that all samples in one experiment be collected at the same time of the day. Avoid hemolytic and hyperlipidemia sample for serum and plasma.

## **Reagent Preparation**

Bring all reagents to room temperature before use. If crystals have formed in the Wash Buffer(20x), Bring the reagent to room temperature and mix gently until the crystals have completely dissolved.

**Standard** - Reconstitute the Standard Lyophilized with 1.0mL Standard/Sample Diluent(R1). This reconstitution produces a stock solution of 200pg/mL. Mix gently until completely dissolved and allow the lyophilisate to dissolve to a concentration of 1000pg/mL prior to making dilutions.

Use the 200pg/mL standard stock to produce a dilution series (below) with Standard/Sample Diluent(R1). Mix each tube thoroughly and change pipette tips between each transfer (recommended concentration for standard curve: 200,100,50, 25, 12.5, 6.25, 3.13,0pg/mL). Use diluted standards within 60 minutes of preparation.



**Human TNF-alpha Control** - Reconstitute the Standard Lyophilized with 1.0mL Standard/Sample Diluent(R1), Mix gently until completely dissolved.

**Working Biotin Conjugate Antibody** - Dilute 1:100 of Concentrated Biotin Conjugate Antibody (100x) with Biotin-Conjugate Antibody Diluent (R2) before use, for example: Add 20 µL of Concentrated Biotin Conjugate Antibody (100x) to 1980 µL Biotin-Conjugate Antibody Diluent (R2) to prepare 2000 µL Working Biotin Conjugate Antibody Buffer.

**Working Streptavidin-HRP** - Dilute 1:100 of Concentrated Streptavidin-HRP (100x) with Streptavidin-HRP Diluent (R3) before use, for example: Add 20 µL of Concentrated Streptavidin-HRP (100x) to 1980µL Streptavidin-HRP Diluent (R3)

to prepare 2000 $\mu$ L Working Streptavidin-HRP Buffer.

**1 x Wash Buffer-** If crystals have formed in the Wash Buffer(20x), warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 1:20 with double distilled or deionized water before use, for example : Add 20 mL of Wash Buffer Concentrate to 380 mL of deionized or distilled water to prepare 400 mL of Wash Buffer.

## **Sample preparation**

For different samples, the appropriate dilution level should be chosen on a case-by-case basis.

1. Cell supernatant: As cell supernatant samples vary considerably depending on the experimental conditions, it is recommended to carry out a pre-test to determine the appropriate dilution.

2.Serum: Samples are tested and 2-fold dilution of normal serum/plasma samples is recommended to eliminate quantitative inaccuracies caused by sample matrix interference.



Samples containing more than 200 pg/mL of human TNF-alpha should be diluted 4-fold or more with Standard/Sample Diluent (R1) or PBS until the diluted sample falls within the detection range of the kit.

### **Dilution Method**

For 10 fold dilution: One-step dilution. Add 50  $\mu$ L sample to 450  $\mu$ L sample diluent to yield 10 fold dilution. .

For 100 fold dilution: One-step dilution. Add 5  $\mu$ L sample to 495  $\mu$ L sample diluent to yield 100 fold dilution.

For 1000 fold dilution: Two-step dilution. Add 5  $\mu$ L sample to 95  $\mu$ L sample diluent to yield 20 fold dilution, then add 5  $\mu$ L 20 fold diluted sample to 245  $\mu$ L sample diluent, after this, the neat sample has been diluted at 1000 fold successfully.

Each dilution step should be performed at a minimum of 3  $\mu$ L and at a maximum of 100-fold dilution. Each dilution step should be mixed well to avoid foaming.

## **Assay Procedure**

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal properly.
3. Add 100  $\mu$ L Standard/sample Diluent (R1) in a blank well.
4. Add 100  $\mu$ L different concentration of standard, Control or sample in other wells, Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. record the plate layout of standards and sample assay.
5. Prepare the Concentrated Biotin Conjugate Antibody (100x) Working Solution 15 minutes early before use.
6. Add wash buffer 350  $\mu$ L/well, aspirate each well after holding 40 seconds, repeating the process two times for a total of three washes. Pat the plate on paper towel to remove residual liquid in the wells.
7. Add 100  $\mu$ L Working Biotin Conjugate Antibody in each well, cover with new adhesive Sealer provided. Incubate

for 1 hour at 37°C.

8. Prepare the Streptavidin-HRP Concentrated (100x) Working Solution 15minutes early before use.
9. Repeat the aspiration/wash as in step 6.
10. Add 100  $\mu$ L Working Streptavidin-HRP in each well, cover with new adhesive Sealer provided.Incubate for 0.5 hour at 37°C.
11. Repeat the aspiration/wash as in step 6.
12. During the incubation, turn on the microplate reader to warm up for 30 minutes before measuring.
13. Add 100  $\mu$ L TMB Substrate to each well. Incubate for 15 minutes at 37°C .Protect from light.
14. Add 50  $\mu$ L Stop Solution, determine the optical density of each well within 5 minutes, using a Microplate reader set to 450 nm. If wavelength correction is available, set to 570 nm or 630 nm. This subtraction will correct for optical imperfections in the plate.

## **Assay Procedure Summary**

Prepare the standard and reagents



Add 100ul of standards or test samples to each well

Incubate for 2 hours at 37°C, then wash 3 times,

Completely pat dry after plate washing



Add 100ul Working Biotin Conjugate Antibody

Incubate for 1 hour at 37°C, then wash 3 times,

Completely pat dry after plate washing



Add 100ul Working Streptavidin-HRP

Incubate for 0.5 hour at 37°C, then wash 3 times,

Completely pat dry after plate washing



Add 100ul Substrate Solution

Incubate for 15-20 min at 37°C under dark condition



Add 50ul Stop Solution



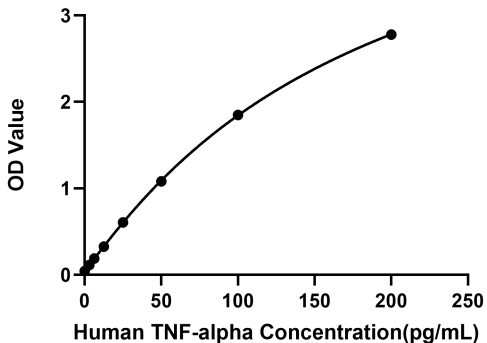
Detect the optical density within 5 minutes under 450nm.

Correction Wavelength set at 570nm or 630nm

## **Calculation of Results**

1. Average the duplicate readings for each standard, control and sample, and the OD for each test should be subtracted from the OD for the sub-wavelengths.
2. Create a standard curve by reducing the data using computer software capable of generating a four-parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the Y-axis against the concentration on the X-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the TNF-alpha concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.
3. The sample read-outs from the curve must be multiplied by the dilution factor of the sample in order to obtain the concentration in the original sample.
4. Samples that fall above this interval should be diluted accordingly and re-analyzed. Samples that fall below this interval are too low to be accurately quantified by this method.

## Typical Data



pg/mL	Repeat 1 OD450-OD630	Repeat 2 OD450-OD630	Average
0	0.043	0.0499	0.0455
3.125	0.1145	0.1063	0.1104
6.25	0.1887	0.1775	0.1831
12.5	0.3267	0.3134	0.32005
25	0.6064	0.6138	0.6101
50	1.0813	1.0222	1.05175
100	1.8496	1.7655	1.80755
200	2.7798	2.771	2.7754

The standard curves are provided for demonstration only. The concentration calculations should be based on difference data

## **Sensitivity**

The minimum detectable dose (MDD) of TNF-alpha typically less than 0.51pg/mL. The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

## **Specificity**

This assay recognizes both recombinant and natural human TNF-alpha. The factors listed below were prepared at 2ng/mL and assayed for cross-reactivity. No significant cross-reactivity was observed with the following:

<b>Recombinant human</b>	<b>Recombinant mouse</b>	<b>Recombinant rat</b>
NFKB-1	TNF- $\alpha$	TNF- $\alpha$

### **Note:**

Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between TNF-alpha and all the analogues, therefore, cross reaction may still exist.



## Precision

### Intra-plate Precision

Samples containing 5 different concentrations of the target were tested with the same kit, and each sample was analysed in the same replicate 3 times for 2 days.

Three batches of kits were sampled with an Intra-Assay:  
CV<10%

Lot 1	LLOQ	LQC	MQC	HQC	ULOQ
Number of Replicates	3	3	3	3	3
Mean(pg/mL)	2.93	6.97	26.04	126.82	201.69
Standard deviation	0.14	0.23	1.48	9.01	3.92
Coefficient of Variation (%)	4.79%	3.25%	5.67%	7.11%	1.94%
Lot 2	LLOQ	LQC	MQC	HQC	ULOQ
Number of Replicates	3	3	3	3	3
Mean(pg/mL)	3.26	7.36	25.19	138.22	202.14
Standard deviation	0.22	0.36	1.36	5.69	7.23
Coefficient of Variation (%)	6.77%	4.89%	5.40%	4.11%	3.58%

Lot 3	LLOQ	LQC	MQC	HQC	ULOQ
Number of Replicates	3	3	3	3	3
Mean(pg/mL)	3.01	7.97	24.27	129.35	193.35
Standard deviation	0.25	0.49	2.21	10.63	4.60
Coefficient of Variation (%)	8.24%	6.14%	9.10%	8.22%	2.38%

### Inter-plate Precision

Five samples of known concentration were tested six times with each of the three batches of kits and the coefficient of variation (CV) of the concentration was calculated.

Inter-Assay: CV<15%

	LLOQ	LQC	MQC	HQC	ULOQ
Number of Replicates	3	3	3	3	3
Mean(pg/mL)	3.07	7.43	25.17	131.47	199.06
Standard deviation	0.23	0.54	1.68	9.14	6.37
Coefficient of Variation (%)	7.56%	7.30%	6.67%	6.95%	3.20%

## **Recovery**

Matrices listed below were spiked with certain level of TNF-alpha and the recovery rates were calculated by comparing the measured value to the expected amount of TNF-alpha in samples.

Sample Type	Recovery Range (LQC, %)	Recovery Range (MQC, %)	Recovery Range (HQC, %)
Serum(n=5)	95-110	97-108	108-114

## Linearity

Five samples were spiked with known concentrations of target proteins, and serially diluted 2-64 times. The read-out (pg/mL) for each measurement was multiplied by its respective dilution factor and the mean concentration of each sample was calculated from all values within the calibrator curve quantification range. Linearity was calculated for each measurement by relating it to its respective mean.

Dilution		Serum(n=5)
1: 2	Recovery(%)	102.15%
1: 4	Recovery(%)	99.88%
1: 8	Recovery(%)	101.02%
1: 16	Recovery(%)	100.92%
1: 32	Recovery(%)	93.27%
1: 64	Recovery(%)	91.24%

## **CALIBRATION**

This immunoassay is calibrated against a highly purified recombinant human TNF-alpha produced at ABclonal.

The NIBSC/WHO TNF-alpha(12/154) was evaluated in this kit. The dose response curve of the International Standard ((12/154) parallels the kit standard curve. To convert sample values obtained with the Human TNF-alpha kit to approximate NIBSC 89/548 units, use the equation below.

NIBSC/WHO (12/154) approximate value (U/mL) = 0.038 ×  
Human TNF-alpha value (pg/mL)

## **SAMPLE VALUES**

**Serum/Plasma-** Samples from apparently healthy volunteers were evaluated for the presence of human TNF-alpha in this assay. No medical histories were available for the donors used in this study.

Sample Type (n=10)	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum	108.7	100%	60.99-194.54

ND=Non-detectable

## Trouble Shooting

<b>Problem</b>	<b>Possible Cause</b>	<b>Solution</b>
High Background	Insufficient washing	Sufficiently wash plates as required. Ensure appropriate duration and number of washes. Ensure appropriate volume of wash buffer in each well.
	Incorrect incubation procedure	Check whether the duration and temperature of incubation are set up as required.
	Cross-contamination of samples and reagents	Be careful of the operations that could cause cross-contamination. Use fresh reagents and repeat the tests.
No signal or weak signal	Incorrect use of reagents	Check the concentration and dilution ratio of reagents. Make sure to use reagents in proper order.
	Incorrect use of microplate reader	Warm the reader up before use. Make sure to set up appropriate main wavelength and correction wavelength.
	Insufficient colour reaction time	Optimum duration of colour reaction should be limited to 15-25 minutes.

	Read too late after stopping the colour reaction	Read the plate in 5 minutes after stopping the reaction.
	Matrix effect of samples	Use positive control.
Too much signal	Contamination of TMB substrate	Check if TMB substrate solution turns blue. Use new TMB substrate solution.
	Plate sealers reused	Use a fresh new sealer in each step of experiments.
	Protein concentration in sample is too high	Do pre-test and dilute samples in optimum dilution ratio.
Poor Duplicates	Uneven addition of samples	Check the pipette. Periodically calibrate the pipette.
	Impurities and precipitates in samples	Centrifuge samples before use.
	Inadequate mixing of reagents	Mix all samples and reagents well before loading.

\*For research purposes only. Not for therapeutic or diagnostic purposes.