

Human HS-TNF- α (High sensitive Tumor Necrosis Factor Alpha)Accquant® ELISA Kit

Catalogue No.: AQ-H0302-B

Revision: V 2.1

Size: 48T/96T

Reactivity: Human

Range: 3.1-200pg/ml

Sensitivity: 1.875pg/ml

Application: For quantitative detection of TNF- α in serum, plasma, tissue homogenates and other biological fluids.

Storage: 2-8°C

Expiry Date: see kit label

Principle: Sandwich

NOTE: FOR RESEARCH USE ONLY.

Kit Components

No.	Item	Specifications(48T/96T)	Storage
E001	ELISA Microplate(Dismountable)	8×6/8×12	2-8°C/-20°C
E002	Lyophilized Standard	1vial/2vial	2-8°C/-20°C
E039	Sample Dilution Buffer	5ml/10ml	2-8°C
E003	Biotin-labeled Antibody(Concentrated)	60ul/120ul	2-8°C(Avoid Direct Light)
E004	Assay Dilution	2.5ml/5ml	2-8°C
E040	Antibody Dilution Buffer	5ml/10ml	2-8°C
E034	HRP-Streptavidin Conjugate(SABC)	60ul/120ul	2-8°C(Avoid Direct Light)
E049	SABC Dilution Buffer	5ml/10ml	2-8°C
E024	TMB Substrate	5ml/10ml	2-8°C(Avoid Direct Light)
E026	Stop Solution	5ml/10ml	2-8°C
E038	Wash Buffer(25X)	15ml/30ml	2-8°C

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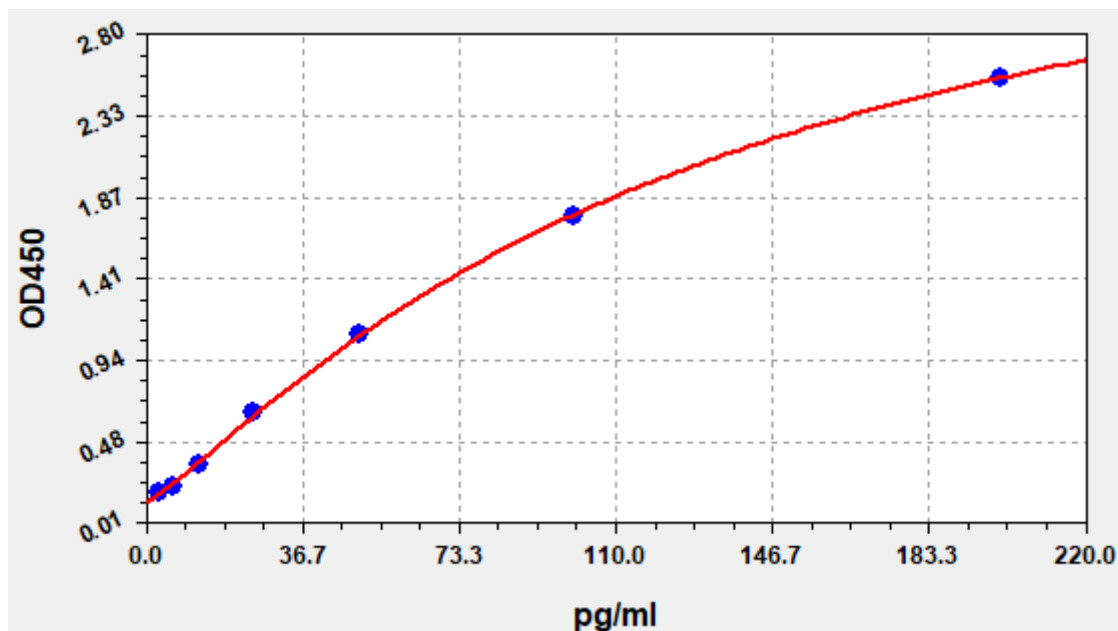
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E006	Plate Sealer	3/5pieces	
E007	Product Description	1copy	

Typical Data & Standard Curve

Results of a typical standard operation of a TNF- α ELISA Kit are listed below. This standard curve was generated at our lab for demonstration purpose only. Users shall obtain standard curve as per experiment by themselves. (N/A=not applicable)

STD.(pg/ml)	OD-1	OD-2	Average	Corrected
0	0.144	0.151	0.147	0.000
3.1	0.192	0.200	0.196	0.049
6.25	0.222	0.231	0.227	0.080
12.5	0.346	0.361	0.354	0.207
25	0.637	0.663	0.650	0.503
50	1.070	1.114	1.092	0.945
100	1.730	1.800	1.765	1.618
200	2.504	2.607	2.555	2.408



Specificity

This assay has high sensitivity and excellent specificity for detection of TNF- α . No significant cross-reactivity or interference between TNF- α and analogues was observed.

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

Recovery

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

Matrix	Recovery Range (%)	Average (%)
Serum(n=5)	90-99	94
EDTA Plasma(n=5)	89-102	95
Heparin Plasma(n=5)	88-100	94

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of TNF- α and their serial dilutions. The results were demonstrated by percentage of calculated concentration to the expectation.

Sample	1:2	1:4	1:8
Serum(n=5)	89-102%	90-98%	91-101%
EDTA Plasma(n=5)	87-100%	86-103%	88-102%
Heparin Plasma(n=5)	88-98%	89-99%	90-101%

Precision

Intra-Assay: CV<8%

Inter-Assay: CV<10%

Stability

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage condition.

Standard(n=5)	37°C for 1 month	2-8°C for 6 months

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Average (%)	80	95-100
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To minimize extra influence on performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is strongly suggested that the same operator performs the whole assay from the beginning to the end.

Sample values

Sample values Cell Culture Supernates - Human peripheral blood mononuclear cells (1 x 10⁶ cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100μg/mL streptomycin sulfate and stimulated for 24h with 10μg/mL PHA. Aliquots of the culture supernates were removed and assayed for levels of human TNF-α.

Condition	Con.(pg/mL)
Treated	1500
untreated	400

Operation Procedure

Background

Tumor necrosis factor alpha (TNF-alpha), also known as cachectin and TNFSF2, 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 assembled intracellularly to form a noncovalently linked homotrimer which is expressed on the cell surface. Cell surface TNF-alpha can induce the lysis of neighboring tumor cells and virus infected cells, and it can generate its own downstream cell signaling following ligation by soluble TNFR I. Shedding of membrane bound TNF-alpha by TACE/ADAM17 releases the bioactive cytokine, a 55kDa soluble trimer of the TNF-alpha extracellular domain. TNF-alpha binds the ubiquitous 55-60kDa TNF RI and the hematopoietic cell-restricted 80 kDa TNF RII, both of which are also expressed as homotrimers. Both type I and type II receptors bind TNF-alpha with comparable affinity, although only TNF RI contains a cytoplasmic death domain which triggers the activation of apoptosis. Soluble forms of both types of receptors are released and can neutralize the biological activity of TNF-alpha..

Principle of the Assay

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Capture antibody was pre-coated onto 96-well plates. And the biotin conjugated antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were 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 target amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of target can be calculated.

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Precautions

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.
2. After opening and before using, keep plate dry.
3. Before using the kit, spin tubes and bring down all components to the bottom of tubes.
4. Storage TMB reagents avoid light.
5. Washing process is very important, not fully wash easily cause a false positive and high background.
6. Duplicate well assay is recommended for both standard and sample testing.
7. Don't let microplate dry at the assay, for dry plate will inactivate active components on plate.
8. Don't reuse tips and tubes to avoid cross contamination.
9. Avoid using the reagents from different batches together.
10. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

Material Required but Not Supplied

1. Microplate reader (wavelength:450nm)
2. 37°C incubator
3. Automated plate washer
4. Precision single and multi-channel pipette and disposable tips
5. Clean tubes and Eppendorf tubes
6. Deionized or distilled water

Washing

Manual: Discard the solution in the plate without touching the side walls. Clap the plate on absorbent filter papers or other absorbent material. Fill each well completely with 350ul wash buffer and soak for 1 to 2 minutes, then aspirate contents from the plate, and clap the plate on absorbent filter papers or other absorbent material.

Automatic: Aspirate all wells, and then wash plate with 350ul wash buffer. After the final wash, invert plate, and clap the plate on absorbent filter papers or other absorbent material. It is recommended that the washer shall be set for soaking 1 minute. (**Note:** set the height of the needles; be sure the fluid can be sipped up completely)

Sample Collection and Storage (universal)

- **Serum:** Place whole blood sample at room temperature for 2 hours or put it at 2-8°C overnight and centrifugation for 20 minutes at approximately 1000×g, Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.
- **Plasma:** Collect plasma using EDTA-Na₂ or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2-8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.
- **Tissue Homogenates:** As hemolysis blood has relation to assay result, it is necessary to remove residual blood by washing tissue with pre-cooling PBS buffer (0.01M, pH=7.4). Mince tissue after weighing it and get it homogenized in PBS (the volume depends on the weight of the tissue. Normal, 9mL PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The

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homogenates are then centrifuged for 5 minutes at 5000×g to get the supernatant. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3mg.

- **Cell Culture Supernatant:** Centrifuge supernatant for 20 minutes at 1000×g at 2-8°C to remove insoluble impurity and cell debris. Collect the clear supernatant and carry out the assay immediately.
- **Cell Culture Lysate:** Commercial RIPA kits are recommended to follow the instructions provided. Generally, 0.5ml RIPA lysis buffer would be appropriate to 2×10^6 cells, DNA must be removed. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3mg.
- **Other Biological Fluids:** Centrifuge samples for 20 minutes at 1000×g at 2-8°C. Collect supernatant and carry out the assay immediately.

Note: Samples to be used within 5 days can be stored at 2-8°C, besides that, samples must be stored at -20°C (assay ≤1 month) or -80°C (assay ≤2 months) to avoid loss of bioactivity and contamination. Avoid multiple freeze-thaw cycles. The hemolytic samples are not suitable for this assay.

Sample Dilution

The user should estimate the concentration of target protein in the test sample, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit. Dilute the sample with the provided dilution buffer, and several trials may be necessary. The test sample must be well mixed with the dilution buffer. And also standard curves and sample should be making in pre-experiment. If samples with very high concentrations, dilute samples with PBS first and then dilute the samples with Sample Dilution.

Reagent Preparation and Storage

Bring all reagents and samples to room temperature for 20 minutes before use.

1, Wash Buffer:

If crystals have formed in the concentrate, you can warm it with 40°C water bath (Heating temperature should not exceed 50°C) and mix it gently until the crystals have completely been dissolved. The solution should be cooled to room temperature before use.

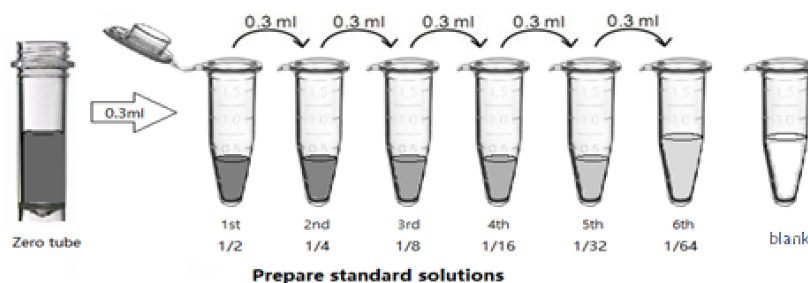
Dilute 30ml (15ml for 48T) Concentrated Wash Buffer into 750ml (375ml for 48T) Wash Buffer with deionized or distilled water. Put unused solution back at 2-8°C.

2, Standards:

1). Add 1 ml Sample Dilution Buffer into one Standard tube (labeled as zero tube), keep the tube at room temperature for 10 minutes and mix them thoroughly.

Note: If the standard tube concentration higher than the range of the kit , please dilute it and labeled as zero tube.

2). Label 7 EP tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Add 0.3ml of the Sample Dilution Buffer into each tube. Add 0.3ml of the above Standard solution (from zero tube) into 1st tube and mix them thoroughly. Transfer 0.3ml from 1st tube to 2nd tube and mix them thoroughly. Transfer 0.3ml from 2nd tube to 3rd tube and mix them thoroughly, and so on. Sample Dilution Buffer was used for the blank control.



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Note: It is best to use Standard Solutions within 2 hours.

3, Preparation of Biotin-labeled Antibody Working Solution:

Prepare it within 1 hour before experiment.

- 1) Calculate required total volume of the working solution: $0.1\text{ml/well} \times \text{quantity of wells}$. (Allow 0.1-0.2ml more than the total volume.)
- 2) Dilute the Biotin-detection antibody with Antibody Dilution Buffer at 1:100 and mix them thoroughly. (i.e. Add 1ul Biotin-labeled antibody into 99ul Antibody Dilution Buffer.)

4, Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution:

Prepare it within 30 minutes before experiment.

- 1) Calculate required total volume of the working solution: $0.1\text{ml/well} \times \text{quantity of wells}$. (Allow 0.1-0.2ml more than the total volume.)
- 2) Dilute the SABC with SABC Dilution Buffer at 1:100 and mix them thoroughly. (i.e. Add 1ul of SABC into 99ul of SABC Dilution Buffer.)

Assay Procedure

When diluting samples and reagents, they must be mixed completely and evenly. Before adding TMB into wells, equilibrate TMB Substrate for 30 minutes at 37°C. It is recommended to plot a standard curve for each test.

1. Set standard, test samples, control (blank) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate.
2. Add 50µL of Assay Diluent to each well.
3. **Prepare Standards:** Aliquot 50ul of zero tube, 1st tube, 2nd tube, 3rd tube, 4th tube, 5th tube, 6th tube and Sample Dilution Buffer (blank) into the standard wells.
4. **Add Samples:** Add 50ul of properly diluted sample into test sample wells , **mix by gently tapping the plate frame for 1 minute.**
5. **Incubate:** Seal the plate with a cover and incubate at 37°C for 90 minutes.
6. **Wash:** Remove the cover and discard the plate content, and wash plate 2 times with Wash Buffer. Do NOT let the wells dry completely at any time.
7. **Biotin-labeled Antibody:** Add 100ul Biotin-labeled antibody working solution into above wells (standard, test sample and blank wells). Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 37°C for 60 minutes.
8. **Wash:** Remove the cover, and wash plate 3 times with Wash Buffer, and let the Wash Buffer stay in the wells for 1-2 minutes each time.
9. **HRP-Streptavidin Conjugate (SABC):** Add 100ul of SABC Working Solution into each well, cover the plate and incubate at 37°C for 30 minutes.
10. **Wash:** Remove the cover and wash plate 5 times with Wash Buffer, and let the wash buffer stay in the wells for 1-2 minutes each time.
11. **TMB Substrate:** Add 90ul TMB Substrate into each well, cover the plate and incubate at 37°C in dark within 10-20 minutes. (**Note:** The reaction time can be shortened or extended according to the actual color change, but not more than 30 minutes. You can terminate the reaction when apparent gradient appeared in standard wells.)
12. **Stop:** Add 50ul Stop Solution into each well. The color will turn yellow immediately. The adding order of Stop Solution should be as the same as the TMB Substrate Solution.

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13. **OD Measurement:** Read the O.D. absorbance at 450nm in Microplate Reader immediately after adding the stop solution. Regarding calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The target concentration of the samples can be interpolated from the standard curve. It is recommended to use some professional software to do this calculation, such as Curve Expert 1.3 or 1.4.

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

Summary

Step1: Add 50µL of Assay Diluent to each well

Step2: Add 50ul standard or sample to each well and incubate for 90 minutes at 37°C

Wash step: Aspirate and wash plates 2 times.

Step3: Add 100ul Biotin-labeled antibody working solution to each well and incubate for 60 minutes at 37°C.

Wash step: Aspirate and wash plates 3 times.

Step4: Add 100ul SABC Working Solution into each well and incubate for 30 minutes at 37°C.

Wash step: Aspirate and wash plates 5 times.

Step5: Add 90ul TMB Substrate. Incubate 10-20 minutes at 37°C.

Step6: Add 50ul Stop Solution. Read at 450nm immediately and calculation.