

# **Human Tumor Necrosis Factor Alpha (TNF- $\alpha$ ) ELISA Assay Pair (Enzyme-Linked Immunosorbent Assay)**

**Catalog Number: CRS-D002**

**Pack Size: 5 plates / 20 plates**

**IMPORTANT: Please carefully read this manual before performing your experiment.**

***For Research Use Only. Not For Use in Diagnostic or Therapeutic Procedure***

## **INTENDED USE**

The kit is developed for quantitative detection of TNF- $\alpha$  in human serum and cell culture supernates. It is intended for research use only (RUO).

## **BACKGROUND**

Tumor Necrosis Factor alpha (TNF- $\alpha$ ), also known as cachectin, is the prototypic ligand of the TNF superfamily. It is produced primarily by activated macrophages, also secreted by other cells such as CD4+ lymphocyte, NK cell, Neutrophils, mast cells, eosinophils and neurons. It is a pleiotropic molecule and plays a central role in inflammation, immune system development, apoptosis and lipid metabolism. Its overexpression is related to a series of pathological states including Cachexia, septic shock, and autoimmune disorders.

To support the development of CAR-T drugs, ACROBiosystems independently developed human Tumor Necrosis Factor Alpha (TNF- $\alpha$ ) ELISA Assay Pair, which is used for detection and evaluation of stimulatory effects of T cell activating agents for evaluation of the efficacy and function of CAR-T products in drug development and CMC quality control stages.

## **PRINCIPLE OF THE ASSAY**

This assay kit is used to measure the levels of human Tumor Necrosis Factor Alpha (TNF- $\alpha$ ) by employing a standard sandwich-ELISA format. Firstly, attach the Human TNF- $\alpha$  Capture Antibody to the microplate, add the standard samples provided in kit and your samples to the plate, incubate and wash the wells. Then add the Human TNF- $\alpha$  Detection Antibody to the plate and form Antibody-antigen-biotinylated antibody complex, incubate and wash the wells. Next add Streptavidin-HRP to the plate, incubate and wash the wells. At last, load the substrate into the wells and monitor solution color from blue to yellow. The reaction is stopped by the addition of a stop solution and the intensity of the absorbance can be measured at 450 nm and 630 nm. The OD Value reflects the amount of TNF- $\alpha$  bound.

## **PRECAUTIONS**

1. This kit is for research use only and is not for use in diagnostic or therapeutic applications.
2. The kit is suitable for cell supernatant, serum and plasma samples.
3. Do not use reagents past their expiration date.
4. Do not mix or substitute reagents with those from other kits or other lot number kits.

5. If samples generate values higher than the highest standard, dilute the samples with the appropriate calibrator diluent and repeat the assay. If cell supernatant samples need step dilution, except for the final dilution with diluent, other intermediate dilutions can be in cell culture medium.

6. Differences in test results can be caused by a variety of factors, including laboratory operator, pipette usage, plate washing technique, reaction time or temperature, and kit storage.

7. This kit is designed to remove or reduce some endogenous interference factors in biological samples, and not all possible influencing factors have been removed.

**MATERIALS PROVIDED**

**Table1. Materials provided (5 plates)**

Catalog	Components	Size (5 plates)	Format	Storage	
				Unopened	Opened
CRD002-C01	Human TNF- $\alpha$ Capture Antibody	60 $\mu$ g	Powder	2-8°C	-70°C
CRD002-C02	Human TNF- $\alpha$ Standard	30 $\mu$ g	Powder	2-8°C	-70°C
CRD002-C03	Human TNF- $\alpha$ Detection Antibody	30 $\mu$ g	Powder	2-8°C	-70°C
CRD002-C04	Streptavidin-HRP	100 $\mu$ L	Liquid	2-8°C, avoid light	2-8°C, avoid light

**Table2. Materials provided (20 plates)**

Catalog	Components	Size (20 plates)	Format	Storage	
				Unopened	Opened
CRD002-C01	Human TNF- $\alpha$ Capture Antibody	210 $\mu$ g	Powder	2-8°C	-70°C
CRD002-C02	Human TNF- $\alpha$ Standard	60 $\mu$ g	Powder	2-8°C	-70°C
CRD002-C03	Human TNF- $\alpha$ Detection Antibody	110 $\mu$ g	Powder	2-8°C	-70°C
CRD002-C04	Streptavidin-HRP	120 $\mu$ L	Liquid	2-8°C, avoid light	2-8°C, avoid light

**SRORAGE**

1. The unopened kit is stable for 12 months from the date of manufacture if stored at 2°C to 8°C.

2. The opened kit should be stored per Table 1. The shelf life is 30 days from the date of opening.

*Note: a. Do not use reagents past their expiration date.*

*b. Find the expiration date on the outside packaging.*

**REAGENTS/EQUIPMENT NEEDED BUT NOT SUPPLIED**

1. 96 well microplates: Corning , Catalog# 42592
2. Coating Buffer (1×PBS): Solarbio, Catalog # P1020 (1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, pH 7.2-7.4, 0.2 μm filtered)
3. 1xWashing Buffer(1×PBST): Solarbio, Catalog # P1033 (0.05% Tween-20 in PBS, pH 7.2-7.4)
4. Blocking Buffer: 2% BSA(Yancheng Saibao, Catalog # N/A ) in 1×Washing Buffer
5. Dilution Buffer: 0.5% BSA(Yancheng Saibao, Catalog # N/A ) in 1×Washing Buffer
6. Substrate Solution: InnoReagents, Catalog # TMB-S-004
7. Stop Solution: 2 N H<sub>2</sub>SO<sub>4</sub>

**REAGENT PREPARATION**

Bring all reagents and samples to room temperature (20°C-25°C) before use. If crystals have formed in buffer solution, place the sample in an 37°C incubator until the crystals have completely dissolved and bring the solution back to room temperature before use.

According to Table 3(5 plates) or Table 4(20 plates), prepare the provided lyophilized product into a storage solution with ultrapure water, dissolve at room temperature for 15 to 30 minutes, and mix by gently pipetting, avoiding vigorous shaking or vortexing. The reconstituted storage solution should be stored at -70°C. It is recommended that the number of freezing and thawing should not exceed 1 time, and the size of the aliquot should not be less than 10 μg.

**Table 3. Preparation method (5 plates)**

ID	Components	Size (5 plates)	Storage solution concentration.	Reconstituted water Vol.
CRD002-C01	Human TNF-α Capture Antibody	60 μg	200 μg/mL	300 μL
CRD002-C02	Human TNF-α Standard	30 μg	150 μg/mL	200 μL
CRD002-C03	Human TNF-α Detection Antibody	30 μg	200 μg/mL	150 μL

**Table 4. Preparation method (20 plates)**

ID	Components	Size (5 plates)	Storage solution concentration.	Reconstituted water Vol.
CRD002-C01	Human TNF- $\alpha$ Capture Antibody	210 $\mu$ g	400 $\mu$ g/mL	525 $\mu$ L
CRD002-C02	Human TNF- $\alpha$ Standard	60 $\mu$ g	150 $\mu$ g/mL	400 $\mu$ L
CRD002-C03	Human TNF- $\alpha$ Detection Antibody	110 $\mu$ g	400 $\mu$ g/mL	275 $\mu$ L

## **RECOMMENDED SAMPLE PREPARATION**

### **1. Coating**

Dilute Human TNF- $\alpha$  Capture Antibody stock solution to 1.0  $\mu$ g/mL with Coating Buffer to make Human TNF- $\alpha$  Capture Antibody working solution. Add 100  $\mu$ L of Human TNF- $\alpha$  Capture Antibody working solution (1.0  $\mu$ g/mL) to each well, seal the plate with microplate sealing film and incubate overnight (or 16 hours) at 4°C.

### **2. Washing**

Remove the remaining solution by aspiration, add 300  $\mu$ L of 1 $\times$ Washing Buffer to each well, gently tap the plate for 1 minute, remove any remaining 1 $\times$ Washing Buffer by aspirating or decanting, invert the plate and blot it against paper towels. Repeat the wash step above for three times.

### **3. Blocking**

Add 300  $\mu$ L Blocking Buffer to each well, seal the plate with microplate sealing film and incubate at room temperature for 2.0 hours.

### **4. Washing**

Repeat step 2.

### **5. Add Standard and Samples**

#### **5.1 Preparation of Standard curve**

The concentration of the reconstituted human TNF- $\alpha$  Calibrator (CRD002-C02) is 150  $\mu$ g/mL, prepare (Std.-0) by diluting 5  $\mu$ L the reconstituted human TNF- $\alpha$  Calibrator into 370  $\mu$ L Sample Dilution Buffer, mix gently well. Then prepare Std.-1' by diluting 5  $\mu$ L Std.-0 into 495  $\mu$ L Sample Dilution Buffer. At last, prepare the highest concentration of standard curve, Std.-1 (625 pg/mL), by diluting 20  $\mu$ L Std.-1' into 620  $\mu$ L Sample Dilution Buffer. Prepare 1:1 serial dilutions for the standard curve as follows: Pipette 300  $\mu$ L of Sample Dilution Buffer into each tube. Make sure to mix

well every time. Sample Dilution Buffer serves as blank.

Tubes/ Solution Code	Human TNF- $\alpha$ Standard stock solution	Std.-0	Std.-1'	Std.-1	Std.-2	Std.-3	Std.-4	Std.-5	Std.-6	Std.-7
Operating	5 $\mu$ L	5 $\mu$ L	20 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L
Solution Con.	150 $\mu$ g/mL	2000 ng/mL	20 ng/mL	625 pg/mL	312.5 pg/mL	156.3 pg/mL	78.1 pg/mL	39.1 pg/mL	19.5 pg/mL	9.8 pg/mL
Dilution Buffer Vol.		370 $\mu$ L	495 $\mu$ L	620 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L	300 $\mu$ L

## 5.2 Preparation of Samples

- If the sample to be tested is the serum or plasma, dilute test sample at 1:2 with Dilution Buffer. The volume ratio of sample to diluent is 1:1.
- If the sample to be tested is the cell supernatant, dilute test sample at 1:2 with Dilution Buffer. The volume ratio of sample to diluent is 1:1.

## 5.3 Add Samples

Add 100  $\mu$ L Standard (Std.-1 ~ Std.-7) and Samples to each well. For blank Control wells, please add 100  $\mu$ L Dilution Buffer.

*Note: It is recommended to set double holes for samples and standard curves to be tested.*

## 6. Incubation

Seal the plate with microplate sealing film and incubate at room temperature for 1 hour.

## 7. Washing

Repeat step 2.

## 8. Add Human TNF- $\alpha$ Detection Antibody

Dilute Biotinylated-Human TNF- $\alpha$  Detection Antibody stock solution to 0.5  $\mu$ g/mL with Dilution Buffer to make Biotinylated-Human TNF- $\alpha$  Detection Antibody working solution. For all wells, add 100  $\mu$ L Biotinylated-Human TNF- $\alpha$  Detection Antibody (0.5  $\mu$ g/mL) working solution. Please prepare it for one-time use only.

## 9. Incubation

Seal the plate with microplate sealing film and incubate at room temperature for 1 hour.

## 10. Washing

Repeat step 2.

## 11. Add Streptavidin-HRP

For all wells, add 100  $\mu$ L Streptavidin-HRP (dilute at 1:2000) working solution. Please prepare it for one-time use only, avoid light.

## 12. Incubation

Seal the plate with microplate sealing film and incubate at room temperature for 30 min.

## 13. Washing

Repeat step 2.

## 14. Substrate Reaction

Add 100  $\mu$ L Substrate Solution to each well. Seal the plate with microplate sealing film and incubate at room temperature for 20 min, avoid light.

## 15. Termination

Add 50  $\mu$ L Stop Solution to each well, and tap the plate gently to allow thorough mixing.

*Note: The color in the wells should change from blue to yellow.*

## 16. Data Recording

Read the absorbance at 450 nm and 630 nm using UV/Vis microplate spectrophotometer within 10 minutes.

*Note: To reduce the background noise, subtract the value read at  $OD_{450\text{ nm}}$  with the value read at  $OD_{630\text{ nm}}$ .*

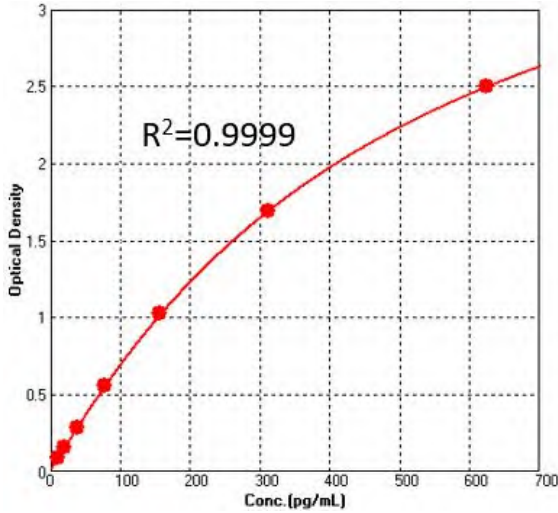
## CALCULATION OF RESULTS

1. Calculate the mean absorbance for each standard, control and sample and subtract average zero standard optical density (O.D.).
2. The standard curve is plotted with the standard concentration as x-axis and the calibrated absorbance value as y-axis. Four parameters logistic are used to draw the standard curve and calculate the sample concentration.
3. Normal range of Standard curve:  $R^2 \geq 0.9900$ .
4. Detection range: 9.8 pg/mL-625 pg/mL. If the OD value of the sample to be tested is higher than 625 pg/mL, the

sample shall be diluted with dilution buffer and assay repeated. If the OD value of the sample to be tested is lower than 9.8 pg/mL, the sample should be reported.

**TYPICAL DATA**

The following data is for reference only. The sample concentration was calculated based on the results of the standard curve.



Conc.(pg/mL)	O.D. -1	O.D. -2	Average	Corrected
625	2.532	2.620	2.576	2.502
312.5	1.710	1.824	1.767	1.693
156.25	1.052	1.150	1.101	1.027
78.125	0.610	0.659	0.635	0.560
39.0625	0.353	0.370	0.362	0.287
19.53125	0.226	0.237	0.232	0.157
9.765625	0.169	0.154	0.162	0.087
0	0.073	0.076	0.075	/

**SPECIFICITY**

This assay recognizes natural and recombinant human TNF-α. No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines.

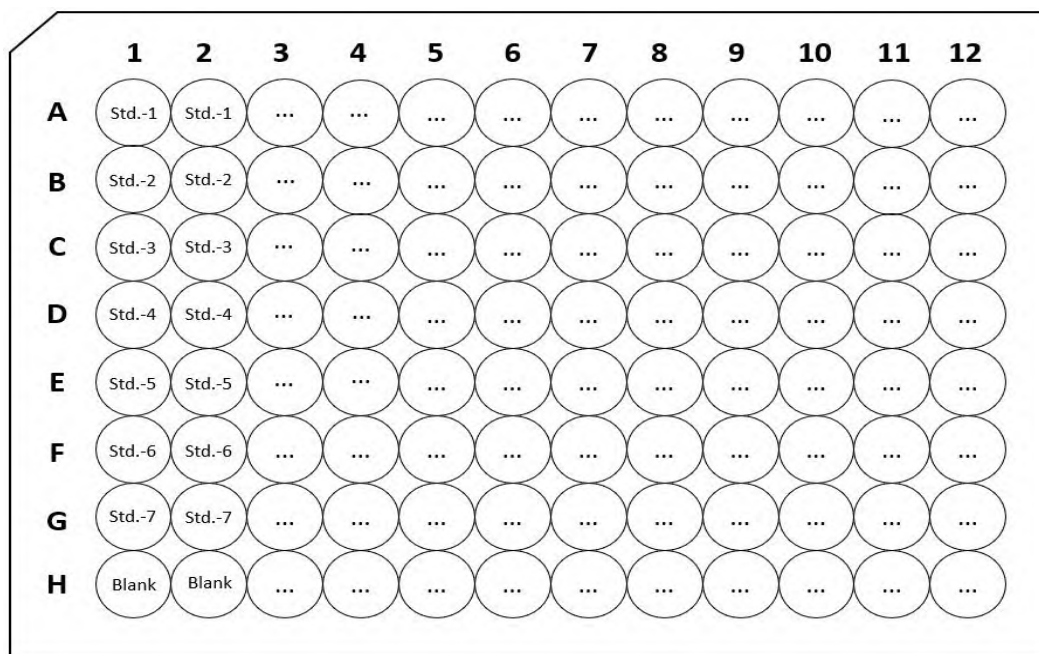
Human
IL-2
IL-4
IL-6
IL-10
GM-CSF
IFN-gamma

**CALIBRATION**

This immunoassay is calibrated against a highly purified E. coli-expressed recombinant human TNF-α (17/232). Reference Reagent is calibrated by NIBSC/WHO in June 2020.



## PLATE LAYOUT



*Note: Blank is a Blank Dilution Buffer hole.*

## TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Poor standard curve	* Inaccurate pipetting	* Check pipettes
Large CV	Inaccurate pipetting Air bubbles in wells	Check pipettes Remove bubbles in wells
High background	Plate is insufficiently washed Contaminated wash buffer	Review the manual for proper wash. Make fresh wash buffer
Very low readings across the plate	Incorrect wavelengths Insufficient development time	Check filters/reader Increase development time
Samples are reading too high, but standard curve looks fine	* Samples contain cytokine levels above assay range	* Dilute samples and run again
Drift	Interrupted assay set-up Reagents not at room temperature	Assay set-up should be continuous - have all standards and samples prepared appropriately before commencement of the assay Ensure that all reagents are at room temperature before pipetting into the wells unless otherwise instructed in the antibody inserts