

RayBio® Human TNF-alpha ELISA Kit

**User Manual
(Revised Mar 1, 2012)**

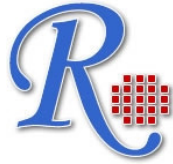
**RayBio® Human TNF-alpha
ELISA Kit Protocol**

(Cat#: ELH-TNFalpha-001)



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RayBiotech, Inc.

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I. INTRODUCTION

TNF- α (tumor necrosis factor- α) is secreted by macrophages, monocytes, neutrophils, T-cells, NK-cells following their stimulation by bacterial lipopolysaccharides. Human TNF- α is a non-glycosylated protein of 17.5 kDa and a length of 157 amino acid. TNF- α shows a wide spectrum of biological activities. It causes cytolysis and cytostasis of many tumor cell lines in vitro. Within hours after injection TNF- α leads to the destruction of small blood vessels within malignant tumors. TNF- α also enhances phagocytosis and cytotoxicity in neutrophilic granulocytes and also modulates the expression of many other proteins. In general, TNF- α and TNF- β display similar spectra of biological activities in vitro systems, although TNF- β is often less potent or displays apparent partial agonist activity.

The RayBio® Human TNF-alpha ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TNF-alpha in serum (human TNF-alpha concentration is pretty low in normal serum and plasma, it may not be detected in this assay), plasma, cell culture supernatants and urine. This assay employs an antibody specific for human TNF-alpha coated on a 96-well plate. Standards and samples are pipetted into the wells and TNF-alpha present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human TNF-alpha antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TNF-alpha bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. REAGENTS

1. TNF-alpha Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human TNF-alpha.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Standards (Item C): 2 vials, recombinant human TNF-alpha.
4. Assay Diluent A (Item D): 30 ml of animal serum with 0.09% sodium azide as preservative. For Standard/Sample (serum/plasma) diluent.
5. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (cell culture medium/urine) diluent.
6. Detection Antibody TNF-alpha (Item F): 2 vial of biotinylated anti-human TNF-alpha (each vial is enough to assay half microplate).
7. HRP-Streptavidin Concentrate (Item G): 200 μ l of 600x concentrated HRP-conjugated streptavidin.
8. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
9. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.

III. STORAGE

May be stored for up to 6 months at 2° to 8°C from the date of shipment. Standard (recombinant protein) should be stored at -20°C or -80°C (recommended at -80°C) after reconstitution. Opened Microplate Wells or reagents may be store for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20°C. Avoid repeated freeze-thaw cycles.

IV. ADDITIONAL MATERIALS REQUIRED

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- 2 Precision pipettes to deliver 2 μ l to 1 ml volumes.
- 3 Adjustable 1-25 ml pipettes for reagent preparation.
- 4 100 ml and 1 liter graduated cylinders.
- 5 Absorbent paper.
- 6 Distilled or deionized water.
- 7 Log-log graph paper or computer and software for ELISA data analysis.
- 8 Tubes to prepare standard or sample dilutions.

V. REAGENT PREPARATION

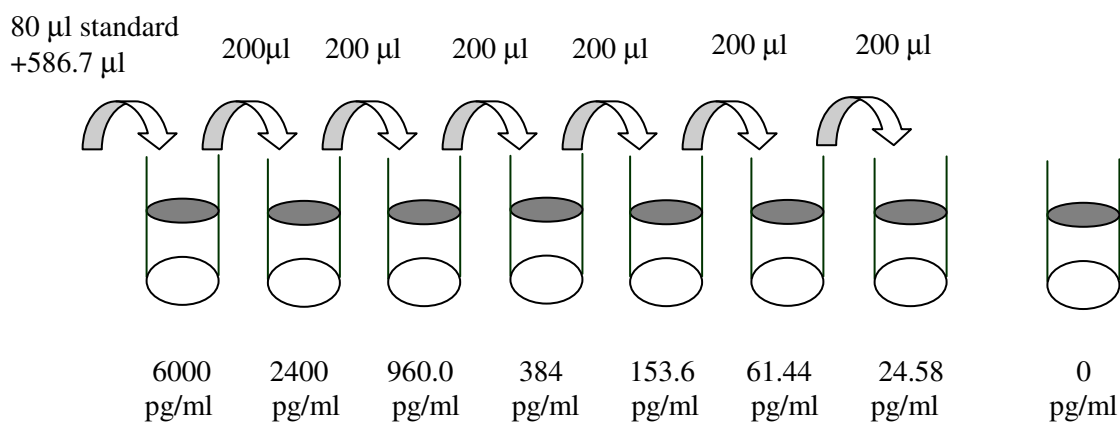
1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Sample dilution: If your samples need to be diluted, Assay Diluent A (Item D) should be used for dilution of serum/plasma samples, and 1x Assay Diluent B (Item E) should be used for dilution of culture supernatants and urine.

Suggested dilution for normal serum/plasma: 2 fold*.

* Please note that levels of the target protein may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.

3. Assay Diluent B should be diluted 5-fold with deionized or distilled water.
4. Preparation of standard: **Briefly spin the vial of Item C** and then add 160 μ l Assay Diluent A (for serum/plasma samples) or 1x Assay Diluent B (for cell culture medium and urine) into Item C vial to prepare a 50

ng/ml standard. **Dissolve the powder thoroughly by a gentle mix.** Add 80 μl TNF-alpha standard from the vial of Item C, into a tube with 586.7 μl Assay Diluent A or 1x Assay Diluent B to prepare a 6000 pg/ml stock standard solution. Pipette 300 μl Assay Diluent A or 1x Assay Diluent B into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Assay Diluent A or 1x Assay Diluent B serves as the zero standard (0 pg/ml).



5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.
6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 μl of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent B and used in step 4 of Part VI Assay Procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP- Streptavidin concentrate should be diluted 600-fold with 1x Assay Diluent B.






For example: Briefly spin the vial (Item G) and pipette up and down to mix gently . Add 20 μ l of HRP-Streptavidin concentrate into a tube with 12 ml 1x Assay Diluent B to prepare a 600-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day Use). Mix well.

VI. ASSAY PROCEDURE:

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 μ l of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C with gentle shaking.
3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 μ l) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 μ l of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
5. Discard the solution. Repeat the wash as in step 3.
6. Add 100 μ l of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking.

7. Discard the solution. Repeat the wash as in step 3.
8. Add 100 μ l of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
9. Add 50 μ l of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VII. ASSAY PROCEDURE SUMMARY

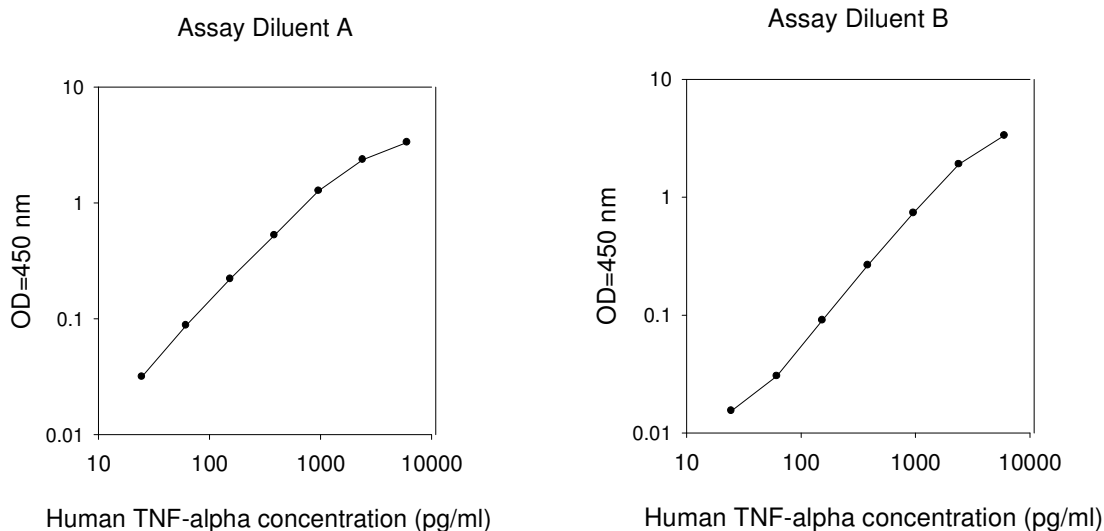
1. Prepare all reagents, samples and standards as instructed.

2. Add 100 μ l standard or sample to each well.
Incubate 2.5 hours at room temperature or over night at 4°C.

3. Add 100 μ l prepared biotin antibody to each well.
Incubate 1 hour at room temperature.

4. Add 100 μ l prepared Streptavidin solution.
Incubate 45 minutes at room temperature.

5. Add 100 μ l TMB One-Step Substrate Reagent to each well.
Incubate 30 minutes at room temperature.

6. Add 50 μ l Stop Solution to each well.
Read at 450 nm immediately.

VIII. CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

A. TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum detectable dose of TNF-alpha is typically less than 30 pg/ml.

C. RECOVERY

Recovery was determined by spiking various levels of human TNF-alpha into human serum, plasma and cell culture media. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Serum	94.53	82-103
Plasma	91.92	80-102
Cell culture media	96.43	84-104

D. LINEARITY

Sample Type		Serum	Plasma	Cell culture media
1:2	Average % of Expected Range (%)	94 83-102	93 82-103	92 81-102
1:4	Average % of Expected Range (%)	94 83-104	92 82-103	99 85-107

E. REPRODUCIBILITY

Intra-Assay: CV<10%
 Inter-Assay: CV<12%

IX. SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested (*e.g.*, human ANG, CD23, Eotaxin, GCSF, GM-CSF, GRO- α , GRO- β , GRO- γ , I-309, IFN- γ , IL-1 α , IL-1 β , IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-15, IL-16, IP-10, MCP-1, MCP-2, MCP-3, MCP-4, MCSF, MIG, MIP-1 α , MIP-1 β , NAP-2, PDGF, PF-4,

PARC, SCF, SDF-1 α , TIMP-1, TIMP-2, TNF β , TGF β 1, TGF β 2, TGF β 3, VEGF).

X. REFERENCES

- 1.** Bonavida B, Immunomodulatory effect of tumor necrosis factor. *Biotherapy* 3: 127-33 (1991).
- 2.** Brouckaert P et al, Tumor necrosis factor, its receptors and the connection with interleukin 1 and interleukin 6. *Immunobiology* 187: 317-29 (1993).
- 3.** Blankenstein T et al, Tumor suppression after tumor cell-targeted tumor necrosis factor alpha gene transfer. *Journal of Experimental Medicine* 173: 1047-52 (1991).

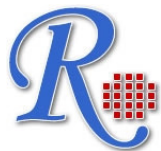
XI. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure a brief spin of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol style="list-style-type: none"> 1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution 	<ol style="list-style-type: none"> 1. Ensure sufficient incubation time; assay procedure step 2 may change to over night 2. Check pipettes and ensure correct preparation
3. Large CV	<ol style="list-style-type: none"> 1. Inaccurate pipetting 	<ol style="list-style-type: none"> 1. Check pipettes
4. High background	<ol style="list-style-type: none"> 1. Plate is insufficiently washed 2. Contaminated wash buffer 	<ol style="list-style-type: none"> 1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed. 2. Make fresh wash buffer
5. Low sensitivity	<ol style="list-style-type: none"> 1. Improper storage of the ELISA kit 2. Stop solution 	<ol style="list-style-type: none"> 1. Store your standard at $<-20^{\circ}\text{C}$ after reconstitution, others at 4°C. Keep substrate solution protected from light 2. Stop solution should be added to each well before measure

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