

# TNF- $\alpha$ (Human)

## ELISA KIT PROTOCOL

(Catalog No.: EK-072-28)



PHOENIX PHARMACEUTICALS, INC.

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## INTRODUCTION

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Tumor Necrosis Factor-alpha (TNF-alpha) is a 17.5 kDa, 157 amino acid protein that is a potent lymphoid factor, which exerts cytotoxic effects on a wide range of tumor cells and other target cells. TNF alpha has been suggested to play a pro-inflammatory role and has been detected in synovial fluid of patients with rheumatoid arthritis. TNF alpha is the primary mediator of immune regulation. The biosynthesis of TNF alpha is tightly controlled being produced in extremely small quantities in quiescent cells, but is a major secreted factor in activated cells.

Phoenix Pharmaceutical's Human TNF- $\alpha$  ELISA Kit is designed to measure the concentration of Human Interleukin-6 from human serum/plasma, or conditioned medium.

## PROTOCOL OVERVIEW

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The immunoplate in this kit is precoated with Anti-Human TNF- $\alpha$  Capture Antibody and the nonspecific binding sites are blocked. The Human TNF- $\alpha$  in the sample or in the standard solution can bind to the capture antibody immobilized in the wells. After washing procedure, the biotinylated anti-human TNF- $\alpha$  Detection Antibody which can bind to the Human TNF- $\alpha$  trapped in the wells is added. After washing, the Streptavidin-Horseradish Peroxidase (SA-HRP) which catalyzes the substrate solution (TMB) is added. The enzyme-substrate reaction is terminated by the addition of a stop solution. The intensity of the the color is directly proportional to the amount of Human TNF- $\alpha$  in the standard solutions or samples. A standard curve of Human TNF- $\alpha$  with known concentration can be established accordingly. The Human TNF- $\alpha$  with unknown concentration in samples can be determined by extrapolation to this standard curve.

## ASSAY CONDITIONS

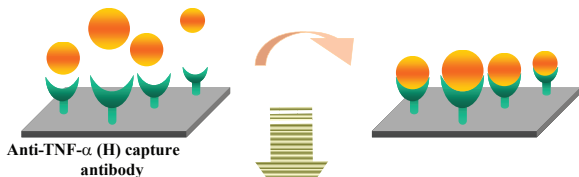
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Plasma, serum, culture media, tissue homogenate, CSF, urine or any biological fluid can be assayed as long as the level of the sample is high enough the for the sensitivity of the kit to detect it.

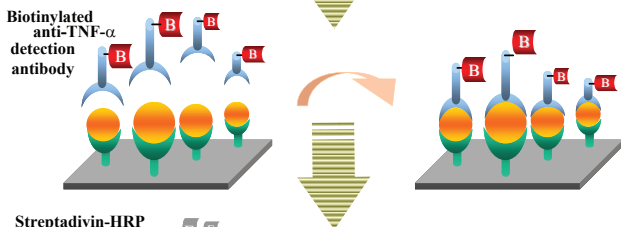
**Phoenix Pharmaceuticals guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for its particular use and establish optimum sample concentrations.**

ASSAY PRINCIPLE

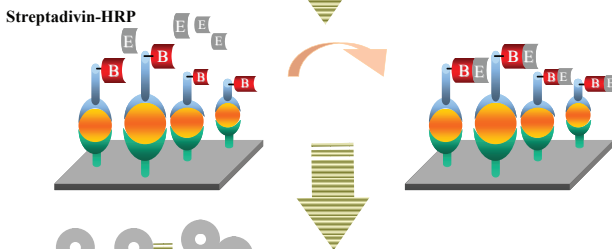
Human TNF- $\alpha$  Standards or samples



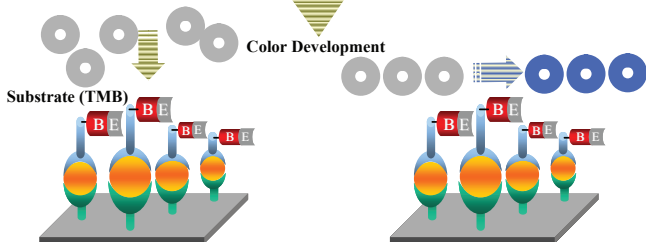
Anti-TNF- $\alpha$  (H) capture antibody



Biotinylated anti-TNF- $\alpha$  detection antibody



Streptavidin-HRP



Substrate (TMB)

Color Development

## LIST OF COMPONENTS

*Store all components at 4°C. DO NOT FREEZE.*

1. 20x Assay Buffer Concentrate (50ml).....**Catalog No. EK-BUF**
2. 96 Well anti-TNF- $\alpha$  .....**Catalog No. EK-Plate-072-28**  
Capture Antibody-Coated Plate (1 plate)
3. Human TNF- $\alpha$  Standard.....**Catalog No. EK-S-072-28**  
(5000pg/vial)
4. Biotinylated anti-Human TNF- $\alpha$ .....**Catalog No. EK-D-072-28**  
Detection Antibody (1 vial, 100 $\mu$ l)
5. Human TNF- $\alpha$  Positive Control.....**Catalog No. EK-PC-072-28**  
(2 vials)
6. Streptavidin-Horseradish..... **Catalog No. EK-SA-HRP**  
Peroxidase (SA-HRP) (30 $\mu$ l)
7. Substrate Solution (TMB) (12ml).....**Catalog No. EK-SS**
8. Stop Solution 2N HCl (15ml) .....**Catalog No. EK-HCL**
9. Acetate Plate Sealer (APS) (3 pieces).....**Catalog No. EK-APS**
10. Assay Diagram (1 sheet)

## MATERIALS REQUIRED BUT NOT SUPPLIED

- Micropipettor(s) and disposable pipette tips
- Multi-channel pipette capable of dispensing 50-100 $\mu$ l
- Solution Reservoir (*recommended*)
- Microtiter plate washer (*recommended*)
- Orbital plate shaker capable of 300-500 rpm (*recommended*)
- Microtiter plate reader capable of absorbance measurement 450nm
- Well-closed containers (15ml tubes or more in capacity)
- Absorbent material for blotting

## REAGENT PREPARATION

Note: *The kit should be equilibrated to room temperature (20-23°C) before opening any vials and starting the assay. It is highly recommended that the solutions be used as soon as possible after rehydration.*

1. **1x Assay Buffer:** Dilute the **20x** Assay Buffer Concentrate with 950ml of distilled water. This assay buffer will be used to wash the plate and reconstitute all of the other components in this kit. If crystals are observed in the **20x** Assay Buffer warm the bottle in a 37°C water bath for approximately 30 minutes or until the crystals disappear. After preparation, store **1x** Assay Buffer at 4°C.
2. **Biotinylated anti-Human TNF- $\alpha$  Detection Antibody:** Dilute Biotinylated anti-Human TNF- $\alpha$  Detection Antibody to 1:125 and mix thoroughly before use.
3. **Streptavidin-Horseradish Peroxidase (SA-HRP):** Centrifuge the SA-HRP vial (30 $\mu$ l) provided in this kit (3,000-5,000 rpm, 5 seconds) and dilute SA-HRP with **1x** Assay Buffer to 1:2000 before use. Vortex thoroughly.
4. **Human TNF- $\alpha$  Positive Control:** For Human TNF- $\alpha$  Positive Control, add 200 $\mu$ l of **1x** Assay Buffer (centrifuge the tube to dislodge powder from cap or walls). Vortex thoroughly.

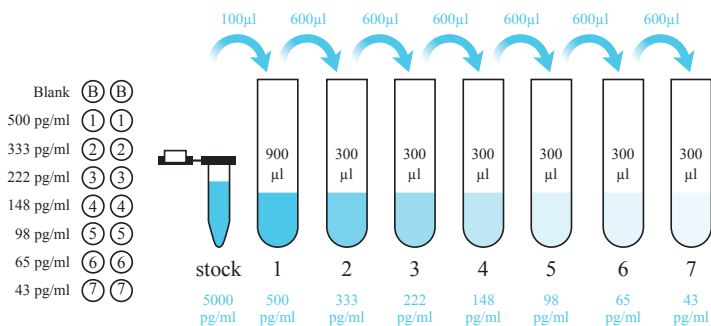
## HUMAN TNF- $\alpha$ STANDARD PREPARATION

1. For the recombinant Human TNF- $\alpha$  Standard, add 900 $\mu$ l **1x** Assay Buffer, vortex. Allow the solution to sit at least 10 minutes at room temperature (20-23°C). Vortex and centrifuge before use. The concentration of this stock solution is 5000pg/ml.

**2. Prepare Standard solutions as follows:**

Standard No.	Standard Protein Volume	Assay Buffer Volume	Concentration
Stock	100 $\mu$ l (Liquid)	900 $\mu$ l	5000pg/ml
#1	100 $\mu$ l of Stock	900 $\mu$ l	500pg/ml
#2	600 $\mu$ l of #1	300 $\mu$ l	333pg/ml
#3	600 $\mu$ l of #2	300 $\mu$ l	222pg/ml
#4	600 $\mu$ l of #3	300 $\mu$ l	148pg/ml
#5	600 $\mu$ l of #4	300 $\mu$ l	98pg/ml
#6	600 $\mu$ l of #5	300 $\mu$ l	65pg/ml
#7	600 $\mu$ l of #6	300 $\mu$ l	43pg/ml

Table of Standard Dilutions



Immunoplate loading map

Visual Guide of the Standard Dilutions



## HUMAN TNF- $\alpha$ ELISA PROTOCOL

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1. Thoroughly read this protocol before performing an assay. Allow all reagents to come to room temperature (20-23°C) prior to the start of the assay.
2. Remove Capture Antibody-Coated Plate from its zip-lock foil pouch. Remove unneeded strips from the plate frame, reseal them in the foil pouch, and return the foil pouch to 4°C.
3. Wash each well with 350 $\mu$ l of **1x** Assay Buffer. Allow it to sit for at least five minutes. Discard the buffer, invert and blot dry plate. Do not let wells dry before proceeding to the next step.
4. Leave wells A-1 and A-2 empty as **Blank**.
5. Add 100 $\mu$ l of the prepared Human TNF- $\alpha$  Standard solutions from #7 to #1 (reverse order of serial dilution) in duplicate to each well.
6. Add 100 $\mu$ l of Human TNF- $\alpha$  Positive Control solution in duplicate.
7. Add 100 $\mu$ l diluted samples in duplicate into their designated wells.
8. Seal the immunoplate with Acetate Plate Sealer (APS). Incubate for 2 hours at room temperature (20-23°C) on a plate shaker (300-400 rpm).
9. Before washing the plate, remove the plate sealer carefully. Completely discard the liquid from wells. Wash each well with 300-350 $\mu$ l assay buffer four times. At the end of each wash, discard the buffer, invert the plate, and tap on a clean absorbent towel.
10. Add 100 $\mu$ l Biotinylated anti-Human TNF- $\alpha$  Detection Antibody into each well. Reseal the immunoplate with plate sealer and incubate for 2 hours at room temperature (20-23°C) on a plate shaker (300-400rpm).
11. Wash 4 times with the **1x** Assay Buffer as described in step 9.
12. Add 100 $\mu$ l SA-HRP solution into each well. Reseal the immunoplate with plate sealer and incubate the plate for 30 minutes at room temperature (20-23°C) on plate shaker (300-400rpm).

13. Wash 4 times with the **1x** Assay Buffer as described in step 9.
14. Add 100 $\mu$ l Substrate Solution (TMB) provided in this kit into each well. Reseal the plate with plate sealer to protect from light and incubate the plate for 20-30 minutes at room temperature (20-23°C) on a plate shaker (300-400 rpm).
15. Add 100 $\mu$ l Stop Solution (2N Hydrochloric Acid) into each well to stop the reaction. The color in the well should change from blue to yellow. If the color change does not appear to be uniform, gently tap the plate to ensure thorough mixing. Proceed to the next step within 20 minutes.
16. Read Absorbance O.D. at 450nm using a Microtiter Plate Reader.

**ADDITIONAL RECOMMENDED PROCEDURAL NOTES:**

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- Reagents of different lot numbers should not be mixed.
- Recheck the reagent labels when loading the plate to ensure that everything is added correctly.
- Unused microplate strips should be placed in the foil pouch with a desiccant and stored at 4°C. Do not allow moisture to enter the wells.
- When handling the plate, avoid touching the bottom.
- Manual washing may cause high duplicate coefficient variations. To reduce this factor, liquid from the plate should be removed by inverting and blotting the plate on an absorbent material.
- If the room temperature is not within the suggested range (20-23°C), variations in results may occur.
- The same reservoir for the reagents may be reused if the reservoir is washed well with distilled water before each use.
- Each laboratory must determine the appropriate dilution factors for the samples to be measured to ensure that the samples are within the dynamic range of the standard curve.
- High levels of interfering proteins may cause variations within the sample results. Therefore, it is imperative to select the appropriate sample preparation procedure to obtain the optimal results.
- Each time a new tip is used, make sure the tip is secure and free of air bubbles. For better intra-assay variation, aspirate and expel a reagent or sample back into the container a few times prior to loading.
- Avoid submerging the whole tip into reagents because droplets can accumulate at the end of the tip causing an excess of reagent to be loaded into the well. This can lead to poor results.
- For optimal results, an orbital plate shaker capable of 300-500 rpm is recommended for all incubations.
- Modification of the existing protocol (i.e. standard dilutions, pipetting technique, washing technique, incubation time or temperature, storage conditions, and kit expiration) may affect the sensitivity and specificity of the test.

## SUMMARY OF ASSAY PROTOCOL

Add 100 $\mu$ l/well of TNF- $\alpha$  standard, sample,  
or positive control **except the Blank wells**

Incubate at room temperature (20-23°C) for 2 hours

Wash immunoplate 4 times with 350 $\mu$ l/well of 1x assay buffer

Add 100 $\mu$ l/well of Biotinylated anti-TNF- $\alpha$  Detection Antibody

Incubate at room temperature (20-23°C) for 2 hours

Wash immunoplate 4 times with 350 $\mu$ l/well of **1x** assay buffer

Add 100 $\mu$ l/well of SA-HRP solution

Incubate at room temperature (20-23°C) for 30 minutes

Wash immunoplate 4 times with 350 $\mu$ l/well of **1x** assay buffer

Add 100 $\mu$ l/well of Substrate Solution (TMB)

Incubate at room temperature (20-23°C) for 20-30 minutes

Terminate reaction with 100 $\mu$ l/well of 2N HCL

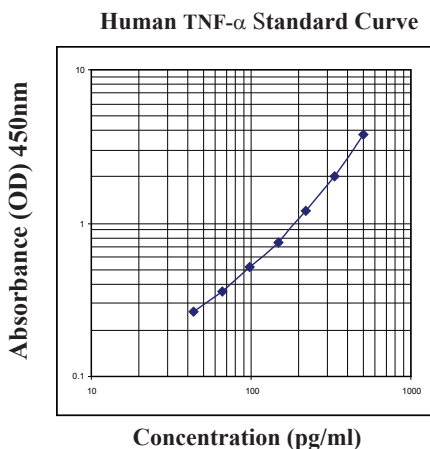
Read absorbance O.D. at **450nm** and calculate results

## CALCULATION OF RESULTS

Plot the standard curve on log-log graph paper. Known concentration of Human Interleukin-6 Standard and its corresponding O.D. reading is plotted on the log scale (X-axis) and the log scale (Y-axis), respectively. The standard curve shows a correlated relationship between Human TNF- $\alpha$  concentrations and the corresponding O.D. absorbance. As the standard concentration increases, the intensity of the yellow color, and in turn the O.D. absorbance, increases.

The concentration of Human Interleukin-6 within a sample is determined by plotting the sample's O.D. on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point will intersect the X-axis at a coordinate corresponding to the Human TNF- $\alpha$  concentration in the unknown sample.

Refer to QC Data sheet for acceptable values of the positive control.



## STORAGE

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1. Store the kit at 4°C upon receipt. The kit should be equilibrated to room temperature (20-23°C) before assay.
2. Store 1x Assay Buffer at 4°C.
3. Remove any unneeded strips from Human TNF- $\alpha$  antibody-Coated plate, reseal them in zip-lock foil and keep at 4°C.
4. Keep rehydrated solution of Human TNF- $\alpha$  Standard, Biotinylated anti-Human TNF- $\alpha$  Detection Antibody and SA-HRP at 4°C. Prepare only the required amount.

## NOTE:

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1. It is recommended that the solutions be used on the same day of rehydration.
2. Unextracted serum samples of normal subjects are to be diluted with 1x Assay buffer.
3. After adding Stop Solution, read the plate within 20 minutes.

## REFERENCES

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