TNF-α (Human)
ELISA Kit Protocol

(Cat. No.: EK-072-28)
# TNF-α (Human) ELISA KIT PROTOCOL

## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction and Protocol Overview</td>
<td>1</td>
</tr>
<tr>
<td>List of Components</td>
<td>3</td>
</tr>
<tr>
<td>Material Required but Not Supplied</td>
<td>4</td>
</tr>
<tr>
<td>Reagent Preparation</td>
<td>4</td>
</tr>
<tr>
<td>Human TNF-α Standard Preparation</td>
<td>5</td>
</tr>
<tr>
<td>Human TNF-α ELISA Protocol</td>
<td>5</td>
</tr>
<tr>
<td>Additional Recommended Procedural Note</td>
<td>7</td>
</tr>
<tr>
<td>Summary of Assay Protocol</td>
<td>8</td>
</tr>
<tr>
<td>Calculation of Results</td>
<td>10</td>
</tr>
<tr>
<td>Storage</td>
<td>11</td>
</tr>
<tr>
<td>Note</td>
<td>11</td>
</tr>
<tr>
<td>References</td>
<td>11</td>
</tr>
</tbody>
</table>
INTRODUCTION AND PROTOCOL OVERVIEW

Tumor Necrosis Factor-alpha (TNF-alpha) is a 17.5kDa, 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-α ELISA Kit is designed to measure the concentration of Human TNF-α from Human serum/plasma, or conditioned medium.

The immunoplate in this kit is pre-coated with Anti-Human TNF-α Capture Antibody and the non-specific binding sites are blocked. The Human TNF-α 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-α Detection Antibody which can bind to the Human TNF-α trapped in the wells is added. The enzyme-substrate reaction is terminated by the addition of a stop solution. The intensity of the color is directly proportional to the amount of Human TNF-α in the standard solutions or samples. A standard curve of Human TNF-α with known concentration can be established accordingly. The Human TNF-α with unknown concentration in samples can be determined by extrapolation to this standard curve.

ASSAY CONDITIONS:

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 for the sensitivity of the kit to detect it.
CAUTION: Phoenix Pharmaceuticals guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for their particular needs and establish optimum sample concentrations.

Assay Principle

Human TNF-α Standards or samples

Anti-TNF-α (H) capture Antibody

Biotinylated Anti-TNF-α (H) detection antibody

Streptavidin-HRP

Substrate (TMB)

Color Development
LIST OF COMPONENTS
Store all components at 4C. DO NOT FREEZE.

1. 20x Assay Buffer concentrate (50ml).................................Catalog no. EK-BUF

2. 96 Well Anti-Human TNF-α Capture Antibody-Coated Plate (1Plate)

3. Human TNF-α Standard (5ng/ml)

4. Biotinylated Anti-Human TNF-α Detection Antibody (1 vial)

5. Human TNF-α Positive Control (2 vials)

6. Streptavidin-horseradish peroxidase (SA-HPR) (30µl)

7. Substrate Solution (TMB) (12ml)

8. Stop Solution 2N HCl (15ml)

9. Acetate plate sealer (APS) (3 pieces)

10. Assay Diagram (1 sheet)
MATERIALS REQUIRED BUT NOT SUPPLIED

- Micropipettor(s) and disposable pipette tips
- Multi-channel pipette capable of dispensing 50-100µl
- Solution reservoir (recommended)
- Microtiter plate washer (recommended)
- Orbital plate shaker capable of 300-500rpm (recommended)
- Microtiter plate reader capable of absorbance measurement between 450 nm-650nm.
- Well-closed containers (15ml tubs 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 assays. 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 the 1x assay buffer used to wash the plate and to 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. **Streptavidin-horseradish peroxidase (SA-HRP):** Centrifuge the SA-HRP vial (30µl) provided in this kit (3,000-5,000rpm, 5 seconds) and dilute SA-HRP with 1x assay buffer to 1:2000 before use. Vortex thoroughly.
TNF-α (Human) ELISA Kit Protocol

HUMAN TNF-α ELISA PROTOCOL

Thoroughly read this protocol before performing an assay and please allo all kit components to return to room temperature (20-23°C) before use (25-45 minutes).

1. Centrifuge (3,000-5,000r.p.m., 5 seconds) and dilute the recombinant Human TNF-α standard with 900µl 1x assay buffer, vortex. Allow the solution to sit at least 10 minutes at room temperature (20-23°C) to completely dissolve in solution. The concentration of this stock solution is 5000pg/ml. Vortex and centrifuge before use.

Prepare Human TNF-α standard solutions with 1x assay buffer as follows:

<table>
<thead>
<tr>
<th>Standard No.</th>
<th>Std. volume</th>
<th>Assay Buffer</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock</td>
<td>100µl (liquid)</td>
<td>900µl</td>
<td>5000pg/ml</td>
</tr>
<tr>
<td>#1</td>
<td>100µl of stock</td>
<td>900µl</td>
<td>500pg/ml</td>
</tr>
<tr>
<td>#2</td>
<td>600µl of #1</td>
<td>300µl</td>
<td>333pg/ml</td>
</tr>
<tr>
<td>#3</td>
<td>600µl of #2</td>
<td>300µl</td>
<td>222pg/ml</td>
</tr>
<tr>
<td>#4</td>
<td>600µl of #3</td>
<td>300µl</td>
<td>148pg/ml</td>
</tr>
<tr>
<td>#5</td>
<td>600µl of #4</td>
<td>300µl</td>
<td>98pg/ml</td>
</tr>
<tr>
<td>#6</td>
<td>600µl of #5</td>
<td>300µl</td>
<td>65pg/ml</td>
</tr>
<tr>
<td>#7</td>
<td>600µl of #6</td>
<td>300µl</td>
<td>43pg/ml</td>
</tr>
</tbody>
</table>
2. Remove Capture Antibody-Coated Plate from its zip-lock foil pouch. Remove any unused strips from the plate frame, reseal them in the foil pouch 4°C.
3. Leave wells A-1 and A-2 empty as Blank.
4. Add 100µl of the prepared Human TNF-α Standard solutions from #7 to #1 (in reverse order of serial dilution) in duplicate to each well.
5. Centrifuge (3,000-5,000 r.p.m., 5 seconds) and dilute the TNF-α positive control with 200µl 1x assay buffer, vortex. Allow the solutions to sit at least 10 minutes at room temperature (20-23°C) to completely dissolve in solution. Add 100µl of Human TNF-α Positive Control solution in duplicate.
6. Add 100µl samples in duplicate into their designated wells.
7. Seal the immunoplate with acetate plate sealer (APS). Incubate for 2 hours at room temperature (20-23°C) on a plate shaker (300-400rpm).
8. Before washing the plate, remove the plate sealer carefully. Completely discard the liquid from wells. Wash each well with 300-350µl assay buffer four times. At the end of the wash, discard the buffer, invert the plate and tap on a clean absorbent towel.
9. Centrifuge the biotinylated anti-Human TNF-α Detection Antibody vial provided in this kit (3,000-5,000 r.p.m., 5 seconds) and dilute the biotinylated detection Antibody to 1:75 with 1x assay buffer. (For example, to make detection antibody working solution that is sufficient for 96 wells, pipette 133ul from the vial into 9.86ml of 1x assay buffer.). Vortex thoroughly before use.
10. Add 100µl biotinylated anti-Human TNF-α 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 8.
12. Add 100µl 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 a plate shaker (300-400 rpm).
13. Wash 4 times with the 1x assay buffer as described in step 8.
14. Add 100µl 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 (300-400rpm).
15. Add 100µl 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 through mixing. Fo to the next step within 20 minutes.
16. Read absorbance O.D. at 450nm using a Microtiter Plate Reader.
ADDITIONAL RECOMMENDED PROCEDURAL NOTES:

- 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 back in the foil pouch with a dessicant 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.
- A multi-channel pipette is NOT recommended to load the biotinylated detection antibody or standard because variations in results may occur.
- 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.
TNF-α (Human) ELISA Kit Protocol

SUMMARY OF ASSAY PROTOCOL

Add 100µl/well of Human TNF-α Standard, Positive Control, or diluted Samples

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

Wash immunoplate 4 times with 300-350µl/well of 1x assay buffer

Add 100µl/well Biotinylated anti-Human TNF-α Detection Antibody

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

Wash immunoplate 4 times with 300-350µl/well of 1x assay buffer

Add 100µl/well of SA-HRP solution

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

Wash immunoplate 4 times with 300-350µl/well of 1x assay buffer

Add 100µl/well of substrate solution (TMB)

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

Terminate the reaction with 100µl/well of Stop Solution

Read absorbance O.D. at 450nm and calculate results
CALCULATION OF RESULTS

Plot the standard curve on log-log graph paper. Known concentrations of Human TNF-α Standard and its corresponding 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-α concentrations and the corresponding O.D. absorbance. As the standard concentration increases, the intensity of the blue color increases, and in turn the O.D. absorbance, increases.

The concentration of Human TNF-α in 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 in the unknown sample.

Refer to QC Data sheet for acceptable values of the Positive Control.
TNF-α (Human) ELISA Kit Protocol

STORAGE

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

NOTE:

1. It is recommended that the solutions be used on the same day of rehydration.
2. After adding stop buffer, read the plate within 20 minutes.

REFERENCES
