CHEMICON®
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ChemiKine™

Human TNFα

Sandwich ELISA Kit

Cat. No. CYT226

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

Tumor Necrosis Factor-alpha is a cytokine secreted by activated macrophages, monocytes, T cells, neutrophils, and NK cells. The actions of TNFα are numerous and affect a broad range of cell types. Resting macrophages can be induced by TNFα to synthesize IL-1 and prostaglandin E2. TNFα together with other inflammatory cytokines induces the production of acute phase proteins from hepatocytes during the inflammatory response and is a potent promoter of angiogenesis. It causes cytolysis and cytostasis of many tumor cell lines in vitro and induces hemorrhagic necrosis of transplanted tumors. The factor also enhances phagocytosis and cytotoxicity in neutrophilic granulocytes and modulates the expression of many other proteins, including fos, myc, IL-1 and IL-6. TNF is found in the synovial fluid of patients suffering from arthritis and is responsible for some of the severe effects of Gram-negative sepsis.

Test Principle

***ChemiKine*™** Human Tumor Necrosis Factor-α (TNFα) kit is a sandwich enzyme immunoassay (EIA), which measures the "free" forms of the cytokine TNFα. With the ***ChemiKine*™** assay system, pre-coated mouse monoclonal antibodies generated against human TNFα are used to capture human TNFα in a sample. Simultaneously, TNFα specific rabbit polyclonal antibodies detect TNFα in the sample. The assay is visualized using goat anti-rabbit alkaline phosphatase conjugate and an ensuing chromagenic substrate reaction. The amount of TNFα detected in each sample is compared to an TNFα standard curve which demonstrates a direct relationship between Optical Density (O.D.) and cytokine concentration: i.e. the higher the O.D. the higher the cytokine concentration in the sample

Application

***ChemiKine*™** Human TNFα kit is designed to measure the amount of "free" TNFα in tissue cell culture supernatants and biological fluid (serum, plasma, and serum-free) samples. There are enough reagents included in this kit for one 96-well immunoassay plate. We recommend running duplicate wells for samples and standards.

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Analytical Sensitivity and Detection Limits

Sensitivity:	4.8 pg/mL
Range of Detection:	15.6 pg/mL to 1000 pg/mL
Crossreactivity:	< 0.5% against cytokine standards
Intra-assay Variation:	± 8.3%
Inter-assay Variation:	± 10.8%

Kit Components

1. Wash Buffer: (Catalog No. CYT226a) One 50 mL bottle of 20X Concentrate.
2. Assay Diluent: (Catalog No. CYT226b) One 50 mL bottle (Ready to Use).
3. TNFα Standard: (Catalog No. CYT226c) One vial (Lyophilized).
4. Rabbit anti-human TNFα Polyclonal Antibody: (Catalog No. CYT226d) One vial (Lyophilized).
5. Goat anti-Rabbit Conjugated Alkaline Phosphatase: (Catalog No. CYT226e) One vial (Lyophilized).
6. Color Reagent A: (Catalog No. CYT226f) One 12 mL bottle.
7. Color Reagent B: (Catalog No. CYT226g) One 12 mL bottle.
8. Stop Solution: (Catalog No. CYT226h) One bottle containing 10 mL of (Ready to Use) 0.5M Sulfuric Acid. **(Caution: Caustic material. Eye, hand, face, and clothing protection should be worn when handling this material.)**
9. Break-Away Immunoplate Pre-coated with Mouse Anti-Human TNFα Monoclonal Antibody: (Catalog No. CYT226i) One Pre-coated 96-Well Immunoplate sealed in a foil pouch.
10. Acetate Plate Sealers: Two Acetate Plate Sealers.

    Note: *The 96-well plate in each **ChemiKine™** kit is vacuum sealed to provide additional long term stability. During the shipment of the product the plate bag may lose its seal. The dessicant enclosed in each plate bag will keep the plate stable when stored at* 2-8°C.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Pipettors & tips capable of accurately measuring 10 -1000 µL
3. Graduated serological pipettes, 25 mL and/or 10 mL
4. 96-well microtiter Plate Reader with 490 nm filter
5. Graph paper for manual plotting of data
6. 12x75 and 13x100 test tubes
7. 15 mL and 25 mL centrifuge tubes
8. Mechanical vortex
9. One 1 liter container
10. Deionized water for dilution of the Wash Buffer

Color Generation System

The ***ChemiKine*™** Sandwich assay uses a two step color generating system. In this system, alkaline phosphatase dephosphorylates NADPH (Substrate) to NADH. The NADH then serves as the cofactor, which activates a cycling redox reaction driven by alcohol dehydrogenase and diaphorase. The latter reaction forms a deep red colored product (formazan), which absorbs light at 490 nm.

Precautions

- Wash Buffer, Assay Diluent, Color Reagent A, and Color Reagent B contain sodium azide. Sodium azide may react with copper and lead plumbing to form highly explosive metal azides. Upon disposal, flush with large amounts of water to prevent azide build-up. Avoid contact with skin.
- *Caustic Material:* Stop Solution (0.5M Sulfuric Acid). **Caution: Eye, hand, face, and clothing protection should be worn when handling this material.**
- *Human Source Material*: The human source material supplied has been tested by a method currently acceptable to FDA and found to be non-reactive for HIV-1/2 Antibody, HCV Antibody, a Serologic Test for Syphilis (STS), and Hepatitis B Surface Antigen (HBsAg). However, all materials should be handled carefully in accordance with good laboratory practices.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.

Technical Hints

- Store Kit and kit reagents at 2-8°C. Do not freeze.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values.
- **Recommended Method for Plate Washing:**
  1. Remove existing fluid from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels.
  2. Forcefully pipet 250 µL of diluted Wash Buffer into each well with a multi-channel pipet.
  3. Remove fluid from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels.
  4. Repeat washing and flicking 4 times.
  5. Dispense 250 µL of diluted Wash Buffer a fifth time and let soak for 10 minutes. After soaking for 10 minutes, blot and aspirate each well to remove any excess fluid.

- All samples and standards should be run in duplicate.
- All samples and kit reagents should be at room temperature (20-25°C) prior to use.
- Thaw samples at room temperature. Do not use a water bath to thaw samples.
- If using a multi-channel pipettor, always use clean disposable reagent reservoirs for the addition of the Biotinylated Antibody, Streptavidin Alkaline Phosphatase solution, the Color Reagent solution, and Stop Solution.
- Use clean pipet tips for each transfer to avoid cross contamination.
- Do not mix reagents from different kit lots.
- Avoid microbial contamination of kit reagents.
- Avoid exposure of kit reagents to excessive heat or light during storage and incubation.
- Do not freeze kit reagents.
- If using samples that are clotted, hemolyzed, or microbially contaminated, or if there is any question about the integrity of a sample, make a notation regarding the sample and its location on the plate and interpret the results accordingly.
- Individual components of the assay contain preservatives. Gloves should be worn and good laboratory practices should be followed while performing the assay to avoid skin contact.
- Do not mix the Color Reagents in advance. Care must be taken not to contaminate the Color Reagent solution. If the solution turns red immediately after mixing, do not use.

Sample Collection & Storage

Serum Samples

a) If using serum separator tube (SST) - allow samples to clot for 30 minutes at room temperature. Centrifuge for 10 minutes at 5000 rpm. Remove serum and assay promptly or aliquot and store the samples at -20°C. Avoid multiple freeze-thaw cycles.

b) If not using serum separator tube (SST) - allow samples to clot overnight at 4°C. Centrifuge for 10 minutes at 5000 rpm. Remove serum and assay promptly or aliquot and store the samples at -20°C. Avoid multiple freeze-thaw cycles.

Plasma Samples

Collect plasma using EDTA or heparin (pyrogen free) as an anticoagulant. Within 30 minutes of collection, centrifuge at 5000 rpm. Assay promptly or aliquot and store the samples at -20°C. Avoid multiple freeze-thaw cycles.

Cell Culture Supernatant Samples

Centrifuge to remove particulate matter and assay immediately or aliquot and store samples at -20°C. Avoid multiple freeze-thaw cycles.

Serum Free Biological Samples

(CSF, synovial fluid, saliva, etc.) Upon sample collection, assay promptly or aliquot and store the samples at -20°C. Avoid multiple freeze-thaw cycles.

Preparation of Reagents

1. **Rabbit anti-Human TNFα Antibody**

   Vortex the bottle of Assay Diluent and reconstitute the lyophilized rabbit anti-Human TNFα Antibody with 3.5 mL of Assay Diluent and vortex.

2. **Recombinant TNFα Standard**

   a) Use of Diluents:

      i. For tissue culture samples, use your tissue culture media to dilute the standards.

      ii. For serum/plasma and serum-free samples (e.g. CSF, urine samples, synovial fluid, etc.), use the Assay Diluent provided to dilute the standards.

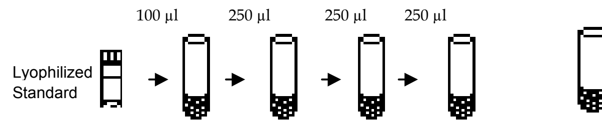
   Note: *In every case, the lyophilized Standard should be reconstituted with the Assay Diluent provided.*

   b) Label 5 12x75 test tubes #0-4. Add 900 μL of the appropriate diluent to Standard tube #1, depending on the sample matrix being used. To the remaining Standard tubes, #2-4 and "0 Dose", add 750 μL of the same diluent.

   c) Reconstitute the lyophilized TNFα Standard with 1000 μL of Assay Diluent and vortex. This solution has a concentration of 10,000 pg/mL.

   d) Remove 100 μL of the reconstituted TNFα Standard from the vial and add it to Standard tube #1 and vortex. This has a concentration of 1,000 pg/mL.

   e) Standards #2-4 are then prepared by performing a 1:4 dilution of the preceding standard. Refer to Fig. 1. For example, to make Standard #2, remove 250 μL of Standard #1 and add it to tube #2 and vortex and so on. Do not add any TNFα Standard to the "0 Dose" Standard tube.



Initial Volume (µL):					
1,000	**900**	**750**	**750**	**750**	**750**
Concentration (pg/mL):					
10,000	**1,000**	**250**	**62.5**	**15.6**	**0.0**
Standard Number:					
	#1	**#2**	**#3**	**#4**	**"0 Dose"**

Figure 1: Serial Dilution of TNFα Standard

3. **Diluting Wash Buffer**

   Dilute entire contents of concentrated Wash Buffer to 1.0 Liter with deionized water. Stir to homogeneity.

4. **Goat Anti-Rabbit Conjugated Alkaline Phosphatase**

   Reconstitute the lyophilized Goat anti-Rabbit Conjugated Alkaline Phosphatase with 6 mL of Assay Diluent and vortex.

5. **Color Reagents**: *Prepare After Washing Plate in Step 7 of Assay Procedure*

   a) Allow Color Reagent A and Color Reagent B to come to room temperature. **Do not mix reagents in advance.**

   b) Approximately 20 mL of the Color Reagent Solution is needed for an entire plate. If only a portion of the immunoplate is being used at one sitting, use the table below for the appropriate volumes of each to use.

   c) Mix appropriate volumes of each reagent in a clean 15 mL or 25 mL screw-cap centrifuge tube and vortex just prior to use.

TABLE 1:

	Volume of Color Reagent A Required	Volume of Color Reagent B Required
100% of Plate Used	12 mL	12 mL
75% of Plate Used	9 mL	9 mL
50% of Plate Used	6 mL	6 mL
25% of Plate Used	3 mL	3 mL

Mixing & Storage of Color Reagents

- The cycling reaction is temperature sensitive, therefore *these reagents must be at room temperature prior to use*.
- **Do not mix reagents in advance**. Allow Color Reagent A and Color Reagent B to come to room temperature and mix appropriate volumes (*refer to Table 1*) of room temperature Color Reagents in a clean screw-top tube just prior to use and vortex.
- Discard any remaining mixed Color Reagent that was not used.
- Store unmixed/unused Color Reagent A and Color Reagent B at 2-8° C for a maximum of 6 months.

Storage of Other Kit Components

- Unused reconstituted kit reagents should be stored at 2-8°C for a maximum of 14 days.
- Unused plate strips should be placed back in the foil pouch with the desiccant and stored at 2-8°C for a maximum of 14 days.

Assay Instructions

1. In duplicate, dispense 100 µL of the "0 Dose" and Standards #1-4 into their designated wells.

   NOTE: A STANDARD CURVE MUST BE RUN AT EACH SITTING.

2. Unknown Samples:

   a) **Serum/Plasma Samples:** In duplicate, to each of the designated wells, add 50 µL of each serum/plasma sample and 50 µL of Assay Diluent.

   b) **Tissue Culture and Serum-free Samples:** In duplicate, to each of the designated wells, add 100 µL of each sample.

   *(*See Note in Calculation of Results Section on page 13 for data analysis)*

3. Dispense 25 µL of diluted Rabbit anti-Human TNFα into each well. Seal the plate with Acetate Plate Sealer to prevent evaporation and incubate the plate at room temperature for 3 hours.

   Note: At this point you should allow Color Reagent A and Color Reagent B solutions to come to room temperature.

4. **IMPORTANT WASH STEP:**

   Gently remove the plate sealer and wash the plate 5 times. A thorough washing of the plate is extremely important to reduce background. We recommend using a multi-channel pipette to fill each well with 250 µL of diluted Wash Buffer. Fluid removal from the wells is best accomplished by inverting the plate over a sink and flicking the fluid out of the wells and then blotting the plate on clean paper towels. Using the multichannel pipet add 250 µL of Wash Buffer to each well; flick and blot the plate. Repeat this procedure for a total of 4 times. Dispense 250 µL of diluted Wash Buffer a fifth time and let plate soak for 10 minutes. <u>After the 10 minute soak, blot and aspirate each well to remove any excess fluid.</u>

   For users of automatic plate washers: It is important to ensure that the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. The result may be poor precision and an unsuitable standard curve. For best results, we recommend 2 cycles of 5 washes each, dispensing 250 µL of 1X Wash Buffer per well with a 10 minute soak interval between the 2 cycles.

5. Allow Color Reagent A and Color Reagent B to come to room temperature, refer to pages 9 and 10 for reagent preparation. Dispense 50 µL of the diluted Goat anti-Rabbit Conjugated Alkaline Phosphatase into each well. Reseal the plate and incubate at room temperature for 45 minutes.

6. Gently remove the plate sealer. Wash the plate 5 times using the wash method described above being sure to soak plate with wash buffer for 10 minutes on wash cycle #5 before final fluid removal and aspiration of each well.

7. Dispense 200 µL of the prepared Color Reagent Solution into each well. Incubate at room temperature for 15 minutes or until Standard #1 reaches an O.D. of 1.6 - 1.8 O.D. units.

8. The Color Reagent incubation time may vary according to laboratory conditions. Therefore, we recommend that you read the plate at 490 nm during the 15 minute incubation to monitor the speed at which color is generated. If the plate reader being used has a shake mode, set the shaker for 3.0 seconds. If no shaker is available, simply hold the plate firmly and tap the edges to insure good mixing of the color reagent fluid in the wells.

   It is very important that the color generation is monitored very closely, as the time for development may vary. When the O.D. for Standard #1 has reached 1.6, take a reading and save it. Dispense 50 µL of Stop Solution at this time into each well **IN THE SAME ORDER** that the Color Reagent Solution was added. The addition of the Stop Solution is an optional step, which you may choose to omit.

9. Read the plate a final time at 490 nm.

Calculation of Results

Manual Plotting: Plot the standard curve on linear graph paper. Known concentrations of TNFα are plotted on the X-axis and the corresponding OD on the Y-axis. The standard curve should result in a straight line that shows a direct relationship between TNFα concentrations and the corresponding ODs (absorbances). In other words, the greater the concentration of TNFα in the sample, the higher the OD. The concentration of TNFα in unknown samples may be determined by plotting the sample OD on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of TNFα in the unknown sample.

**Note: If analyzing serum/plasma samples, which were diluted 1:2 in the Assay Procedure, the resulting potency estimates will need to be multiplied by a dilution factor of 2.*

Plate Reader/PC Interface: An alternative approach is to enter the data into a computer program curve fitting software. The resultant data will best fit a linear curve fit. Currently existing spreadsheet software can also perform some simple plotting.

* EUROPEAN PATENTS: 0060123, 0027036, 0132948

US PATENTS: 446231, 4595655, 4668639

Warranty

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