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# Rat Tumor necrosis factor $\alpha$ , TNF- $\alpha$ ELISA kit

Catalog No: E0133r  
96 Tests  
Operating instruction

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**FOR RESEARCH USE ONLY; NOT FOR THERAPEUTIC OR DIAGNOSTIC APPLICATIONS!  
PLEASE READ THROUGH ENTIRE PROCEDURE BEFORE BEGINNING!**

## Intended use

This immunoassay kit allows for the in vitro quantitative determination of rat Tumor necrosis factor  $\alpha$ , TNF- $\alpha$  concentrations in serum, plasma and other biological fluids.

## Introduction

The prototype ligand of the TNF superfamily, TNF- $\alpha$ /TNFSF1A, is a pleiotropic cytokine that plays a central role in inflammation and apoptosis. It is synthesized as a 26 kDa, type II transmembrane protein that is 233 amino acids in length. It contains a 30 amino acid (aa) cytoplasmic domain, a 26 aa transmembrane segment, and a 177 aa extracellular region. TNF- $\alpha$  is assembled intracellularly to form a transmembrane, non-covalently-linked homotrimeric protein. The 157 aa residue soluble form of TNF- $\alpha$  (sTNF- $\alpha$ ) is released from the C-terminus of the transmembrane protein through the activity of TNF- $\alpha$ -converting enzyme (TACE), a membrane-bound disintegrin metalloproteinase. Rat cells known to express TNF- $\alpha$  include B cells, colonic columnar epithelial cells, NK and CD3+CD56+ hepatic natural T cells, macrophages, monocytes and monocyte-derived dendritic cells, CD4+ and CD8+ T cells, mast cells, neutrophils, keratinocytes, plasma cells, and adipocytes.

## Test principle

The microtiter plate provided in this kit has been pre-coated with an antibody specific to TNF- $\alpha$ . Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for TNF- $\alpha$  and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain TNF- $\alpha$ , biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm  $\pm$  2 nm. The concentration of TNF- $\alpha$  in the samples is then determined by comparing the O.D. of the samples to the standard curve.

## Materials and components

Reagent	Quantity
Assay plate	1

Standard	2
Sample Diluent	1 × 20ml
Assay Diluent A	1 × 10ml
Assay Diluent B	1 × 10ml
Detection Reagent A	1 × 120µl
Detection Reagent B	1 × 120µl
Wash Buffer(25 x concentrate)	1 × 30ml
Substrate	1 × 10ml
Stop Solution	1 × 10ml
Plate sealer for 96 wells	5
Instruction	1

### Other supplies required

Luminometer.

Pipettes and pipette tips.

EP tube

Deionized or distilled water.

### Sample collection and storage

**Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 × g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C.

**Plasma** - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 × g at 2 - 8°C within 30 minutes of collection. Store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

**Other biological fluids** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

**Note:** Serum and plasma to be used within 7 days may be stored at 2-8 °C, otherwise samples must stored at -20°C (≤ 1 months) or -80°C (≤ 2 months) to avoid loss of bioactivity and contamination. Avoid freeze-thaw cycles. When performing the assay slowly bring samples to room temperature.

DO NOT USE HEAT-TREATED SPECIMENS.

### Limitations of the procedure

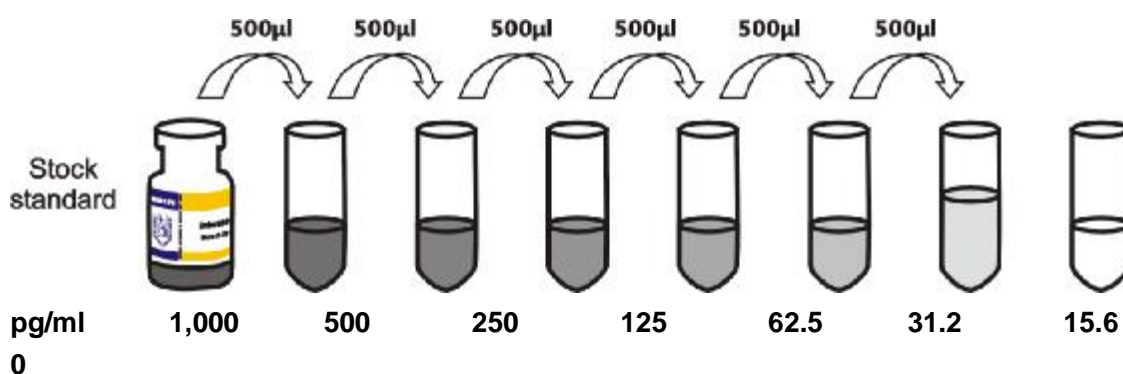
1. The kit should not be used beyond the expiration date on the kit label.
2. Do not mix or substitute reagents with those from other lots or sources.
3. If samples generate values higher than the highest standard, further dilute the samples and repeat the assay. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
4. This assay is designed to eliminate interference by soluble receptors, ligands, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

### Reagent preparation

Bring all reagents to room temperature before use.

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 750 mL of Wash Buffer.

**Standard** - Reconstitute the **Standard** with 1.0 mL of **Sample Diluent**. This reconstitution produces a stock solution of 1,000 pg/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions (Making serial dilution in the wells directly is not permitted). The undiluted standard serves as the high standard (1,000 pg/ml). The **Sample Diluent** serves as the zero standard (0 pg/ml).



**Detection Reagent A and B** - Dilute to the working concentration using **Assay Diluent A and B** (1:100), respectively.

### Assay procedure

Allow all reagents to reach room temperature (Please do not dissolve the reagents at 37°C directly.). **All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming.** Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be resealed and stored at 4°C until the kits expiry date. Prepare all reagents, working standards and samples as directed in the previous sections. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

1. Add 100 µl of **Standard**, Blank, or Sample per well. Cover with the Plate sealer. Incubate for 2 hours at 37°C.
2. Remove the liquid of each well, don't wash.
3. Add 100 µl of **Detection Reagent A** working solution to each well. Cover with the Plate sealer. Incubate for 1 hour at 37°C. **Detection Reagent A** working solution may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.
4. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (approximately 400 µl) using a squirt bottle, multi-channel pipette, manifold dispenser 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.

5. Add 100  $\mu$ l of **Detection Reagent B** working solution to each well. Cover with a new Plate sealer. Incubate for 1 hours at 37°C.
6. Repeat the aspiration/wash five times as in step 4.
7. Add 90  $\mu$ l of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate within 30 minutes at 37°C. Protect from light.
8. Add 50  $\mu$ l of **Stop Solution** to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well at once, using a microplate reader set to 450 nm.

#### **Important Note:**

1. Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents should be freshly prepared prior to use and all required strip-wells are secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.
2. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. The reconstituted Standards can be used only once. This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated. It is recommended to suck more than 10 $\mu$ l for once pipetting.
3. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay.
4. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.
5. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
6. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
7. Duplication of all standards and specimens, although not required, is recommended.
8. Substrate Solution is easily contaminated. Please protect it from light.

#### **Specificity**

This assay recognizes recombinant and natural rat TNF- $\alpha$ . No significant cross-reactivity or interference was observed.

#### **Sensitivity**

The minimum detectable dose of rat TNF- $\alpha$  is typically less than 7.8 pg/ml.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero.

### **Detection Range**

15.6-1,000 pg/mL. The standard curve concentrations used for the ELISA's were 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL.

### **Calculation of results**

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the TNF- $\alpha$  concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. It is recommended to use some related software to do this calculation, such as curve expert 13.0. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

### **Storage of test kits and instrumentation**

1. The **Standard**, **Detection Reagent A** and **Detection Reagent B** should be stored at -20°C upon being received. Other reagents are kept according to the labels on vials. But for long term storage, please keep the whole kit at -20°C. The unused strips should be kept in a sealed bag and stored at 2-8°C in their pouch with the desiccant provided to minimize exposure to damp air. The test kit may be used throughout the expiration date of the kit (six months from the date of manufacture). Opened test kits will remain stable until the expiring date shown, provided it is stored as prescribed above.
2. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results.
3. Do not remove microtiter plate from the storage bag until needed.
4. A microtiter plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 OD or greater at 450nm wavelength is acceptable for use in absorbance measurement.
5. Use fresh disposable pipette tips for each transfer to avoid contamination.
6. Do not substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
7. Valid period: six months.

### **Precaution**

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

