



TiterZyme[®] EIA

rat TNF- α

Enzyme Immunometric Assay Kit

Catalog No. 900-086

96 Determination Kit

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Description

The Assay Designs' rat TNF- α Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of rat TNF- α in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to rat TNF- α immobilized on a microtiter plate to bind the rat TNF- α in the sample. After a short incubation the excess sample is washed out and a monoclonal antibody to rat TNF- α labeled with the enzyme Horseradish peroxidase is added. This labeled antibody binds to the rat TNF- α captured on the plate. After a short incubation the excess labeled antibody is washed out and substrate is added. The substrate reacts with the labeled antibody bound to the rat TNF- α captured on the plate. The color generated with the substrate is read at 450 nm, and is directly proportional to the concentration of rat TNF- α in the sample. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

The Tumor Necrosis Factor- α (TNF- α) TiterZyme[®] Enzyme Immunoassay Kit is used for quantitative determination of levels of the TNF- α in biological samples. TNF- α is a 17.5 kDalton, 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^{4,5}. TNF- α has been suggested to play a pro-inflammatory role and has been detected in synovial fluid of patients with rheumatoid arthritis^{6,7}. It is the primary mediator of immune regulation. The biosynthesis of TNF- α is tightly controlled, being produced in extremely small quantities in quiescent cells, but is a major secreted factor in activated cells⁸.

Precautions

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1. Stop Solution is a 1 normal (1N) sulfuric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles, such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The rat TNF- α Standard provided, Catalog No. 80684, should be handled with care, because of the known and unknown effects.
5. After reconstitution, the rat TNF- α Standard and Labeled Antibody should be aliquoted and stored at -20°C. Do not repeatedly freeze-thaw.

Materials Supplied

1. **rat TNF- α Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0682**
A strip microtiter plate coated with rabbit antibody specific to rat TNF- α .
2. **rat TNF- α Labeled Antibody , 1 vial, Catalog No. 80-0683**
Mouse antibody to rat TNF- α conjugated to Horseradish peroxidase.
3. **Assay Buffer, 30 mL, Catalog No. 80-0170**
Phosphate buffered saline, containing proteins and detergents.
4. **Labeled Antibody Diluent, 10.5 mL, Catalog No. 80-0182**
Phosphate buffered saline, containing proteins and detergents.
5. **Wash Buffer Concentrate, 50 mL, Catalog No. 80-0171**
Phosphate buffered saline containing detergents.
6. **rat TNF- α Standard, 1 vial, Catalog No. 80-0684**
A vial containing 25,000 pg of recombinant rat TNF- α .
7. **Substrate Buffer, 5 mL, Catalog No. 80-0173**
A solution of phosphate in buffer. Ready to use.
8. **Peroxide Solution, 5.5 mL, Catalog No. 80-0174**
A 0.01% solution of hydrogen peroxide in water. Ready to use.
9. **TMB Tablets, 2 Tablets, Catalog No. 80-0175**
10. **Stop Solution, 11 mL, Catalog No. 80-0176**
A 1N solution of sulfuric acid in water. Keep tightly capped. Caution: **Caustic**.
11. **rat TNF- α Assay Layout Sheet, 1 each, Catalog No. 30-0140**
12. **Plate Sealer, 2 each, Catalog No. 30-0012**

Storage

All components of this kit are stable at 4°C until the kit's expiration date.

Materials Needed but Not Supplied

1. Deionized or distilled water. No difference in assay results are seen with distilled water.
2. Precision pipets for volumes between 100 μ L and 1,000 μ L.
3. Disposable test tubes for dilution of samples and standards.
4. Repeater pipet for dispensing 100 μ L.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. A 37°C incubator.
8. Adsorbent paper for blotting.
9. Microplate reader capable of reading at 450 nm., preferably with correction between 570 nm and 590 nm.
10. Graph paper for plotting the standard curve.

Sample Handling

The Assay Designs' rat TNF- α TiterZyme[®] Enzyme Immunometric Assay (EIA) is compatible with rat TNF- α samples in a wide range of matrices after dilution in the appropriate diluent. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions.

Samples must be stored frozen to avoid loss of bioactive rat TNF- α . If samples are to be run within 24 hours, they may be stored at 4°C. Otherwise, samples must be stored frozen at -70°C to avoid loss of bioactive rat TNF- α . Up to three freeze/thaw cycles of serum has been shown to have no effect on rat TNF- α levels. Nonetheless, excessive freeze/thaw cycles should be avoided. Prior to assay, frozen sera should be brought to room temperature slowly and gently mixed by hand. Do not thaw samples in a 37°C incubator. Do not vortex or sharply agitate samples.

Procedural Notes

1. Do not mix reagents from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses plates with removable strips. Unused strips must be kept desiccated at 4°C in the sealed foil bag. The strips should be used in the frame provided.
8. **Prior to addition of standard, antibody, and substrate, ensure that there is no residual wash buffer in these wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Prepare Wash Buffer by diluting 25 mL of the supplied concentrate with 975 mL of deionized water. This can be stored at 4°C until the kit expiration date, or for 3 months, whichever is earlier.

2. rat TNF- α Standards

Add 500 μ L of deionized water to the rat TNF- α Standard. Let it sit at room temperature for 5 minutes. Mix it gently. This solution contains 50,000 pg/mL rat TNF- α .

Label seven 12 x 75 mm glass tubes #1 through 7. Pipet 220 μ L of Assay Buffer into tubes #1 through #7. Add 220 μ L of the 50,000 pg/mL standard to tube #1. Vortex. Add 220 μ L of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #7.

The concentration of rat TNF- α in tubes #1 through #7 will be 25,000, 12,500, 6,250, 3,125, 1,562.5, 781.25, and 390.63 pg/mL respectively. See rat TNF- α Assay Layout Sheet for dilution details.

3. Preparation of Labeled Antibody Conjugate

Add the entire contents of one (1) bottle of Labeled Antibody Diluent to the vial of rat TNF- α Labeled Antibody. Let it stand at room temperature for 5 minutes and then vortex it gently. After reconstitution, any unused rat TNF- α Labeled Antibody should be aliquoted and stored at -20°C.

4. Preparation of Substrate

Just prior to addition of the substrate solution to the plate, prepare the substrate by adding 1 substrate tablet to 2.5 mL of Substrate Buffer and mix allowing the tablet to completely dissolve before proceeding. Ensure that the tablet has completely dissolved before proceeding. Add 2.75 mL of the peroxide solution to this and mix well. Use within 15 minutes.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100 µL of Assay Buffer into the So (0 pg/mL Standard) wells.
3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents, and seal with the plate sealer.
6. Incubate at 37°C for 1 hour.
7. Wash the plate 7 times by adding 200 µL of wash solution per well with a multichannel pipet. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of the Labeled Antibody into each well, except the Blank.
9. Seal the plate and incubate at 4°C for 30 minutes. Prepare Substrate (See page 5, Section 4).
10. Wash the plate 9 times by adding 200 µL of wash solution per well with a multichannel pipet. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µL of the Substrate Solution to each well.
12. Incubate for 30 minutes at room temperature in the dark.
13. Add 100 µL of Stop Solution to each well.
14. Blank the plate reader against the Blank wells, read the optical density at 450nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of rat TNF- α in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of rat TNF- α can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average Blank OD}$$

2. Plot the Average Net OD for each standard versus rat TNF- α concentration in each standard.
3. Using linear graph paper, plot the Average OD for each standard versus rat TNF- α concentration in each standard. Approximate a straight line through the points. The concentration of rat TNF- α in the unknowns can be determined by interpolation.

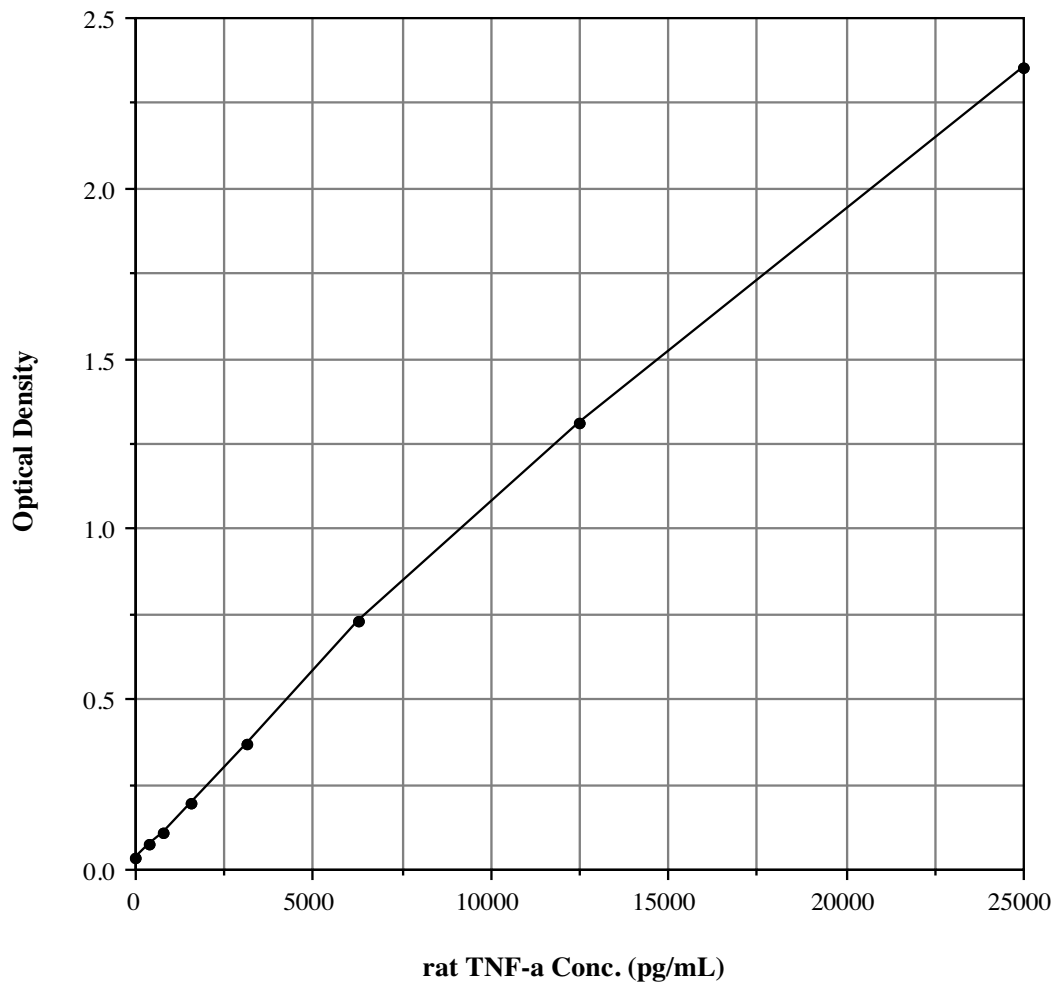
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

<u>Sample</u>	<u>Average OD</u>	<u>Net OD</u>	<u>rat TNF-α (pg/mL)</u>
Blank	(0.062)		
0 standard	0.096	0.034	0
S1	2.413	2.351	25,000
S2	1.371	1.309	12,500
S3	0.789	0.727	6,250
S4	0.430	0.368	3,125
S5	0.255	0.194	1,562.5
S6	0.167	0.106	781.25
S7	0.135	0.073	390.63

Typical Standard Curve

The typical standard curve shown below **must not** be used to calculate rat TNF- α concentrations; each user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols³.

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run at 0 pg/mL rat TNF- α , and comparing to the average optical density for sixteen (16) wells run with Standard #7. The detection limit was determined as the concentration of rat TNF- α measured at two (2) standard deviations from 0 pg/mL Standard along the standard curve.

Average Optical Density for the 0 pg/mL Standard = 0.038 \pm 0.007 (19.32%)

Average Optical Density for Standard #7 = 0.052 \pm 0.006 (11.55%)

Delta Optical Density (390.63-0 pg/mL) = 0.013

2 SD's of the 0 pg/mL Standard = 2 x 0.007 = 0.014

Sensitivity = $\frac{0.014}{0.013}$ x 390.63 pg/mL = **420.7 pg/mL**

Linearity

A sample containing 15,118 pg/mL rat TNF- α was diluted 5 times 1:2 into Assay Buffer and measured in the TiterZyme[®] EIA assay. The data was plotted graphically as actual rat TNF- α concentration versus measured rat TNF- α concentration.

The line obtained had a slope of 0.9084 and a correlation coefficient of 0.99963.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of rat TNF- α and running these samples multiple times (n=7) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of rat TNF- α in multiple assays (n=4).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of rat TNF- α determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	<u>rat TNF-α Concentration</u> (pg/mL)	<u>Intra Assay</u> <u>%CV</u>	<u>Inter Assay</u> <u>%CV</u>
Low	334.4	9.7	
Medium	1681.2	9.7	
High	6695.3	3.9	
Low	357.5		9.0
Medium	1691.4		3.1
High	7033.8		1.0

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant in Assay Buffer. These samples were then measured in the rat TNF- α assay, and the measured rat TNF- α concentration calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

<u>Compound</u>	<u>Cross Reactivity</u>
rat TNF- α	100%
rat IL-1 β	$\leq 0.1\%$
rat IL-6	$\leq 0.1\%$
rat Osteopontin	$\leq 0.1\%$
rat MCP-1	$\leq 0.1\%$
rat Leptin	$\leq 0.1\%$
rat CINC-1	$\leq 0.1\%$
rat CINC-2 α	$\leq 0.1\%$
rat CINC-2 β	$\leq 0.1\%$
rat CINC-3	$\leq 0.1\%$

Sample Recoveries

Rat TNF- α concentrations were measured in Tissue Culture Media (RPMI-1640 plus 10% FBS) and rat serum. For samples in tissue culture media, ensure that the standards have been diluted into the same media (refer to page 4). Rat TNF- α was spiked into the undiluted samples of these media which were then diluted with the kit Assay Buffer and assayed in the kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery*</u>	<u>Recommended Dilution*</u>
Tissue Culture Media	91.6	None
rat Serum	87.4	$\geq 1:5$

* See Sample Handling instructions on page 4 for details.

References

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LIMITED WARRANTY

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For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

Material Safety Data Sheet (MSDS) available on our website or by fax.

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