Product Manual

TNF-α (human), ELISA kit

Catalog #: ADI-900-099

96 Well Kit

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

TABLE OF CONTENTS

DESCRIPTION

The TNF-α (human), ELISA kit is a complete kit for the quantitative determination of human TNF-α in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to human TNF-α immobilized on a microtiter plate to bind the human TNF-α in the standards or sample. A recombinant human TNF-α Standard is provided in the kit. After a short incubation the excess sample or standard is washed out and a biotinylated polyclonal antibody to human TNF-α is added. This antibody binds to the human TNF-α captured on the plate. After a short incubation the excess antibody is washed out and Streptavidin conjugated to Horseradish peroxidase is added, which binds to the biotinylated human TNF-α antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450nm. The measured optical density is directly proportional to the concentration of human TNF-α in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

INTRODUCTION

Tumor Necrosis Factor-α (TNF-α) 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[3,4]. TNF-α has been suggested to play a pro-inflammatory role and has been detected in synovial fluid of patients with rheumatoid arthritis[5,6]. 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 cells[7].

SAFETY WARNINGS & PRECAUTIONS

Handle with care

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- Stop Solution is a 1 normal (1N) hydrochloric acid solution. This solution is caustic; care should be taken in use.
- The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
- 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.
- The human TNF-α Standard provided, Catalog No. 80-0618, should be handled with care because of the known and unknown effects of TNF-α.

Avoid freeze / thaw cycles

MATERIALS SUPPLIED

1. **human TNF-α Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0946**

   A plate using break-apart strips coated with monoclonal antibody specific to human TNF-α.

2. **human TNF-α ELISA Antibody, 10ml, Catalog No. 80-0944**

   A yellow solution of biotinylated rabbit polyclonal antibody to human TNF-α.

3. **Assay Buffer 13, 55ml, Catalog No. 80-1500**

   Tris buffered saline containing proteins and detergents.

4. **human TNF-α ELISA Conjugate, 10ml, Catalog No. 80-0945**

   A blue solution of Streptavidin conjugated to Horseradish peroxidase.

5. **Wash Buffer Concentrate, 100ml, Catalog No. 80-1287**

   Tris buffered saline containing detergents.

6. **human TNF-α Standard, 0.5ml, Catalog No. 80-0618**

   A solution of 10,000 pg/mL human TNF-α.

7. **TMB Substrate, 10ml, Catalog No. 80-0350**

   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. Ready to use**. Protect from prolonged exposure to light.**

8. **Stop Solution 2, 10ml, Catalog No. 80-0377**

   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: Caustic.

9. **human TNF-α Assay Layout Sheet, 1 each, Catalog No. 30-0164**

10. **Plate Sealer, 3 each, Catalog No. 30-0012**

Reagents require separate storage conditions.

STORAGE

All components of this kit, except the Standard, are stable at 4°C until the kit's expiration date. The Standard must be stored at or below -20°C.

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 100µl and 1,000µl.
3. Disposable test tubes for dilution of samples and standards.
4. Repeater pipets for dispensing 100µl.
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 450nm, preferably with correction between 570nm and 590nm.
10. Graph paper for plotting the standard curve.

SAMPLE HANDLING

The TNF-α (human), ELISA kit is compatible with human TNF-α samples in a wide range of matrices. Samples diluted sufficiently into the proper diluent can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 12 for details of suggested dilutions. Culture fluid, serum, and plasma are suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples in the majority of Tissue Culture Media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the Tissue Culture Media instead of Assay Buffer 13. There will be a small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of human TNF-α in the appropriate matrix.

Samples must be stored frozen to avoid loss of bioactive human 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 human TNF-α. 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 components 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 break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.

8. Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.
9. It is important that the matrix for the standards and samples be as similar as possible. Human TNF-α samples diluted with Assay Buffer 13 should be run with a standard curve diluted in the same buffer. Serum and plasma samples should be evaluated against a standard curve run in Assay Buffer 13, while tissue culture samples should be read against a standard curve diluted in the same complete but non-conditioned media. See Reagent Preparation, step #2.

REAGENT PREPARATION

1. Wash Buffer

Prepare the Wash Buffer by diluting 50ml of the supplied concentrate with 950ml of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. human TNF-α Standards

Allow the 10,000 pg/mL human TNF-α standard solution to warm to room temperature. Label seven 12 x 75mm glass tubes #1 through #7. Pipet 900µl of standard diluent (Assay Buffer 13 or tissue culture media) into tube #1. Pipet 500µl of standard diluent into tubes #2 through #7. Add 100µl of the 10,000 pg/mL standard to tube #1. Vortex thoroughly. Add 500µl of tube #1 to tube #2 and vortex thoroughly. Add 500µl of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #7. The concentration of human TNF-α in tubes #1 through #7 will be 1,000, 500, 250, 125, 62.5, 31.25 and 15.63 pg/mL respectively. See human TNF-α Assay Layout Sheet for dilution details.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards, controls 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 pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100µl of standard diluent (Assay Buffer 13 or Tissue Culture Media) into the S0 (0pg/ml standard) wells.
3. Pipet 100µl of Standards #1 through #7 into the appropriate wells.
4. Pipet 100µl of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at 37°C for 2 hours.
7. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. 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 100µl of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at 37°C for 1 hour.
10. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. 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 100µl of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at 37°C for 30 minutes.
13. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. 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.
14. Pipet 100µl of Substrate Solution into each well.
15. Incubate for 30 minutes at room temperature.
16. Pipet 100µl Stop Solution 2 to each well.
17. Blank the plate reader against the Blank wells, read the optical density at 450nm, preferably with correction between 570 and 590nm. 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 the readings.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of human 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 human 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.

   Average Net OD = Average OD - Average Blank OD

2. Plot the Average Net OD for each standard versus human TNF-α concentration in each standard. Approximate a straight line through the points. The concentration of human TNF-α in the unknowns can be determined by interpolation.

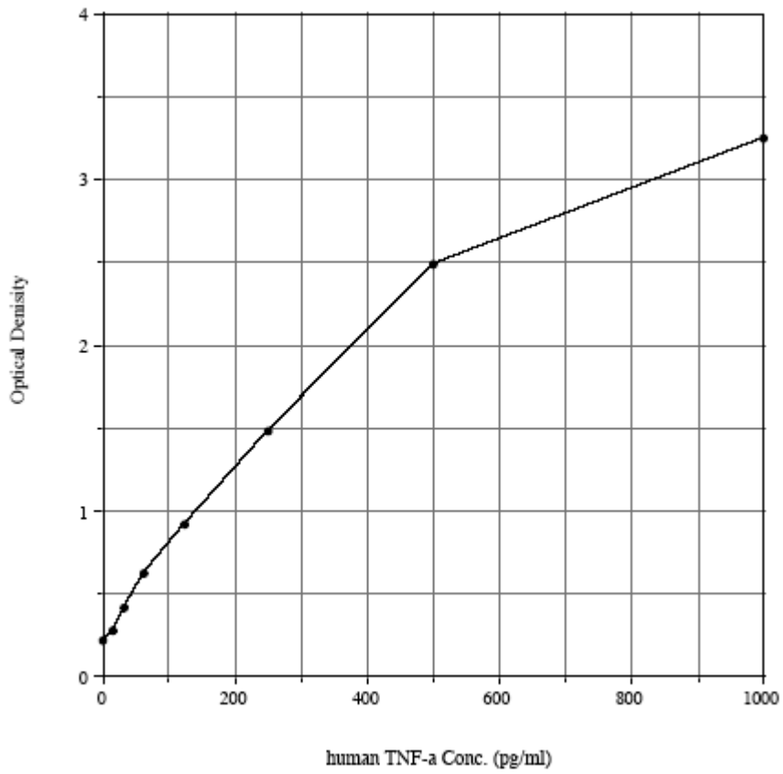
TYPICAL RESULTS

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

Sample	Average OD	Net OD	h TNF-α (pg/mL)
Blank	(0.075)		
S0	0.298	0.223	0
S1	3.323	3.248	1,000
S2	2.564	2.489	500
S3	1.556	1.481	250
S4	0.997	0.922	125
S5	0.695	0.620	62.5
S6	0.490	0.415	31.25
S7	0.350	0.275	15.63
Unknown 1	2.453	2.378	484
Unknown 2	0.818	0.743	93

TYPICAL STANDARD CURVES

A typical standard curve is shown below. This curve must not be used to calculate human 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 Protocols[8].

Sensitivity

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

Mean OD for S0 = 0.152 ± 0.017 (11.5%)

Mean OD for Standard #7 = 0.215 ± 0.015 (6.8%)

Delta Optical Density = (15.63 - 0 pg/mL) = 0.215 - 0.152 = 0.063

2 SD's of 0 pg/mL Standard = 2 x 0.017 = 0.034

Sensitivity = $\frac{0.034}{0.063}$ x 15.63 pg/mL = 8.43 pg/mL

Linearity

A sample containing 755 pg/mL human TNF-α was serially diluted 5 times 1:2 in the Assay Buffer 13 supplied in the kit and measured in the assay. The data was plotted graphically as actual human TNF-α concentration versus measured human TNF-α concentration. The line obtained had a slope of 0.9856 with a correlation coefficient of 0.9967

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of human TNF-α and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring two samples with low and high concentrations of human TNF-α in multiple assays (n=8). The precision numbers listed below represent the percent coefficient of variation for the concentrations of human TNF-α determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	h TNF-α (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	134.5	4.5	
Medium	525.6	4.7	
High	934.4	3.6	
Low	193.0		6.0
High	542.2		11.8

Cross Reactivities

The human TNF-α ELISA Kit is specific for bioactive human TNF-α. It is unaffected by the presence of the following recombinant molecules: human TNF-β, human sTNF RI, human sTNF RII, mouse TNF-α, mouse sTNF RI, mouse sTNF RII, rat TNF-α and porcine TNF-α.

SAMPLE RECOVERIES

Please refer to pages 6 and 7 for Sample Handling recommendations and Standard preparation. Human TNF-α concentrations were measured in human serum, human EDTA plasma and Tissue Culture Media. Human TNF-α was spiked into the undiluted samples of these matrices which were then diluted with the appropriate diluent and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Human Serum	91.2	1:8
Human EDTA Plasma	93.6	1:8
Tissue Culture Media	92.2	None

* See Sample Handling instructions on page 6 for details.

REFERENCES

1. T. Chard, “An Introduction to Radioimmunoassay & Related Techniques, 4th Ed.”, (1990) Amsterdam:Elsevier.
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7. B. Beutler, et. al., Nature, (1985) 316: 552.
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NOTES

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