Product Manual

TNF-α (mouse), ELISA kit

Catalog #: ADI-900-047

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 (mouse) ELISA kit is a complete kit for the quantitative determination of mouse TNF-α in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to mouse TNF-α immobilized on a microtiter plate to bind the mouse TNF-α in the standards or sample. A recombinant mouse TNF-α Standard is provided in the kit. After a short incubation the excess sample or standard is washed out and a polyclonal antibody to mouse TNF-α is added. This antibody binds to the mouse TNF-α captured on the plate. After a short incubation the excess antibody is washed out and donkey anti-rabbit IgG conjugated to Horseradish peroxidase is added, which binds to the polyclonal mouse 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 mouse 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 2 is 1 normal (1N) hydrochloric acid. 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.
- The kit performance has been tested with a variety of sample types, however it is possible that high levels of interfering substances may cause variation in assay results.

Avoid freeze / thaw cycles

- The mouse TNF-α Standard provided, Catalog No. 80-0962, should be handled with care because of the known and unknown effects of TNF-α.
- The mouse TNF-α Standard should be stored at or below -20°C. Do not repeatedly freeze-thaw.

MATERIALS SUPPLIED

1. **mouse TNF-α Microtiter Plate, 1 x 96 Wells, Catalog No. 80-0352**

   A break-apart strip plate coated with monoclonal antibody specific to mouse TNF-α.

2. **mouse TNF-α ELISA Antibody, 5 mL, Catalog No. 80-0353**

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

3. **Assay Buffer 16, 50 mL, Catalog No. 80-1628**

   Tris buffered saline containing protein.

4. **mouse TNF-α ELISA Conjugate, 5 mL, Catalog No. 80-1166**

   A blue solution of donkey anti-rabbit antibody conjugated to Horseradish peroxidase.

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

   Tris buffered saline containing detergents.

6. **mouse TNF-α Standard, 2 vials, Catalog No. 80-0962**

   Two vials containing 1200 picograms each of lyophilized mouse TNF-α.

7. **TMB Substrate, 5 mL, Catalog No. 80-0615**

   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, 10 mL, Catalog No. 80-0377**

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

9. **mouse TNF-α Assay Layout Sheet, 1 each, Catalog No. 30-0126**

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

Reagents require separate storage conditions.

STORAGE

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

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 50 µL and 1,000 µL.
3. Disposable test tubes for dilution of samples and standards.
4. Repeater pipets for dispensing 50 µL.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. A microplate shaker
8. Absorbent 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 TNF-α (mouse) ELISA is compatible with mouse 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 13 for details of suggested dilutions. Culture fluids and serum 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. Culture media used as samples and for standard preparation must be diluted at least 1:2 in Assay Buffer 16. 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 mouse TNF-α in the appropriate matrix.

Samples must be stored frozen to avoid loss of bioactive mouse 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 mouse 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. Mouse TNF-α samples diluted with Assay Buffer 16 should be run with a standard curve diluted in the same buffer. 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 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. mouse TNF-α Standards

Using Assay Buffer 16

Allow the mouse TNF-α standard vial to warm to room temperature. Add 120 µL of deionized water to the lyophilized mouse TNF-α vial and vortex. To this vial, add 480 µL of Assay Buffer 16 and vortex again. Label this vial Standard #1. Label seven 12 x 75 mm glass or polypropylene tubes #2 through #8. Pipet 250 µL of Assay Buffer 16 into tubes #2 through #8. Add 250 µL of Standard #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #8. The concentration of mouse TNF-α in tubes #1 through #8 will be 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 15.63 pg/mL respectively. See mouse TNF-α Assay Layout Sheet for dilution details. **STORE STANDARD AT -20˚C, avoid repeated freeze-thaws.**

Using Tissue Culture Media (Diluted 1:2 in Assay Buffer 16)

Allow the mouse TNF-α standard vial to warm to room temperature. Add 120 µL of deionized water to the lyophilized mouse TNF-α vial and vortex. To this vial, add 480 µL of 1:2 tissue culture media and vortex again. Label this vial Standard #1. Label seven 12 x 75 mm glass or polypropylene tubes #2 through #8. Pipet 250 µL of 1:2 tissue culture media into tubes #2 through #8. Add 250 µL of Standard #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #8. The concentration of mouse TNF-α in tubes #1 through #8 will be 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 15.63 pg/mL respectively. See mouse TNF-α Assay Layout Sheet for dilution details. **STORE STANDARD AT -20°C; avoid repeated freeze thaws.**

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 50 µL of Standards #1 through #8 for Assay Buffer Standards or Standards #1 through #8 for 1:2 Tissue Culture Media into the appropriate wells.
3. Pipet 50 µL of standard diluent (Assay Buffer 16 or Tissue Culture Media) into the S0 (0pg/ml standard) wells.
4. Pipet 50 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 2 hours at ~500 rpm*.
7. Empty the contents of the wells and wash by adding ~300 µL 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 50 µL of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 2 hours at ~500 rpm*.
10. Empty the contents of the wells and wash by adding ~300 µL 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 50 µL of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm*.
13. Empty the contents of the wells and wash by adding ~300 µL 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 50 µL of Substrate Solution into each well.
15. Incubate for 30 minutes at room temperature.
16. Pipet 50 µL Stop Solution to each well.
17. Blank the plate reader against the Blank wells, read the optical density at 450 nm, 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 the readings.

* The plate shaker speed was based on a BellCo Mini Orbital Shaker (mod no. 7744-08096). The actual speed of the plate shaker should be such that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of mouse TNF-α in the samples. We recommend that the data be handled by an immunoassay software package utilizing a four parameter logistic curve fitting program (such as AssayBlaster™, catalog number ADI-28-0002). Such software is often supplied by plate reader manufacturers. If data reduction software is not readily available, the concentration of mouse 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. Using linear graph paper, plot the Average Net OD for each standard versus mouse TNF-α concentration in each standard. Approximate a straight line through the points. The concentration of mouse TNF-α in the unknowns can be determined by interpolation.

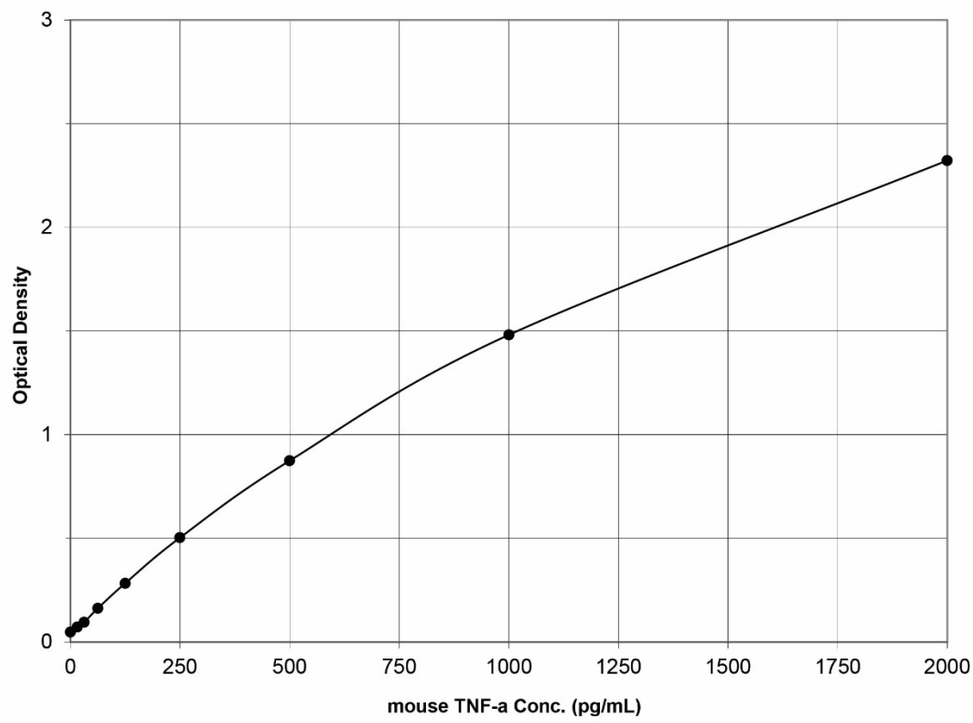
TYPICAL RESULTS

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

Sample	Average OD	Net OD	m TNF-α (pg/mL)
Blank	(0.043)		
S0	0.090	0.047	0
S1	2.365	2.322	2,000
S2	1.524	1.481	1,000
S3	0.917	0.874	500
S4	0.546	0.503	250
S5	0.326	0.283	125
S6	0.205	0.162	62.5
S7	0.138	0.095	31.25
S8	0.115	0.072	15.63
Unknown 1	1.239	1.196	782.3
Unknown 2	0.390	0.347	161.8

TYPICAL STANDARD CURVES

A typical standard curve in Assay Buffer 16 is shown below. This curve must not be used to calculate mouse 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 Protocols8.

Sensitivity

Sensitivity was calculated in Assay Buffer 16 by determining the average optical density bound for sixteen (16) wells run with 0pg/ml Standard, and comparing to the average optical density for sixteen (16) wells run with Standard #8. The detection limit was determined as the concentration of mouse TNF-α measured at two (2) standard deviations from the 0pg/ml Standard along the standard curve.

Mean OD for S0 = 0.055 + 0.003 (4.6%)

Mean OD for Standard #8 = 0.075 + 0.005 (6.3%)

Delta Optical Density = (15.63-0) = 0.075-0.055 = 0.020

2 SD's of 0pg/ml Standard = 0.005

$$\text{Sensitivity} = \frac{0.005}{0.020} \times 15.63 \text{ pg/mL} = 3.9 \text{ pg/mL}$$

Linearity

A sample containing 722.12pg/ml mouse TNF-α was serially diluted 5 times 1:2 in the Assay Buffer 16 supplied in the kit and measured in the assay. The data was plotted graphically as actual mouse TNF-α concentration versus measured mouse TNF-α concentration. The line obtained had a slope of 1.082 with a correlation coefficient of 0.996.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of mouse TNF-α and running these samples multiple times (n=20) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of mouse TNF-α in multiple assays (n=8).

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

	m TNF-α (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	640.6	8.	
Medium	161.8	4.5	
High	36.8	11.0	
Low	47.2		27.3
Medium	159.2		17.7
High	733.5		12.5

Cross Reactivities

The mouse TNF-α ELISA Kit is specific for bioactive mouse TNF-α. It is unaffected by the presence of the following recombinant molecules: human TNF-α, rat TNF-α, mouse IL-1α, mouse IL-1β, mouse IL-2, mouse IL-3, mouse IL-4, mouse IL-5, mouse IL-6, mouse IL-7, mouse IL-10, mouse IFN-γ and mouse GM-CSF.

SAMPLE RECOVERIES

Please refer to pages 5-7 for Sample Handling recommendations and Standard preparation. Mouse TNF-α concentrations were measured in mouse serum and tissue culture media. Mouse 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*
Mouse Serum	102.0	1:8
Tissue Culture Media	92.6	1:2

* See Sample Handling instructions on page 5 for details.

Note: The normal mouse serum samples tested read below the detection limit of the assay.

REFERENCES

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