KAMIYA BIOMEDICAL COMPANY

TNF-α ELISA, Mouse

For the quantitative determination of TNF-α in mouse serum and culture media

Cat. No. KT-129

For research use only.

PRODUCT INFORMATION

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INTENDED USE
The TNF-α ELISA, Mouse is for the quantitative determination of TNF-α in mouse serum and culture media. For research use only.

DESCRIPTION
The TNF-α ELISA, Mouse is a complete kit for the quantitative determination of mouse Tumor Necrosis Factor-α (TNF-α) in biological fluids. Please read the complete kit package 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 calibrators or samples. A recombinant Mouse TNF-α calibrator is provided in the kit. After a short incubation the excess sample or calibrator 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 (HRP) is added, which binds to the polyclonal rabbit anti-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 450 nm. The measured optical density is directly proportional to the concentration of mouse TNF-α in either calibrators or samples.

PRINCIPLE
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. TNF-α has been suggested to play a pro-inflammatory role and has been detected in synovial fluid of patients with rheumatoid arthritis. 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.

PRECAUTIONS
FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Stop Solution is a 1 normal (1N) hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the HRP 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 Mouse TNF-α Calibrator provided should be handled with care because of the known and unknown effects of TNF-α.
5. The Mouse TNF-α Calibrator should be stored at or below -20℃. Do not repeatedly freeze-thaw.

COMPONENTS
1. Mouse TNF-α Microtiter Plate, One Plate of 96 Wells
   A plate using break-apart strips coated with monoclonal antibody specific to mouse TNF-α.
2. Mouse TNF-α Antibody, 5 mL
   A yellow solution of rabbit polyclonal antibody to mouse TNF-α.
3. Assay Buffer, 55 mL
   Tris buffered saline containing proteins.
4. HRP Conjugate, 5 mL
   A blue solution of donkey anti-rabbit antibody conjugated to HRP.
5. Wash Buffer Concentrate, 100 mL
   Tris buffered saline containing detergents.
6. Mouse TNF-α Calibrator, 2 vials
   1200 pg of mouse TNF-α, lyophilized
   Avoid repeated freeze/thaw cycles.
7. TMB Substrate, 5 mL
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. Ready to use.

Protect from prolonged exposure to light.

8. Stop Solution, 10 mL
   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: Caustic.
9. Mouse TNF-α Assay Layout Sheet
10. Plate Sealer, 3 each

STORAGE

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

MATERIALS REQUIRED BUT NOT PROVIDED

- De-ionized or distilled water.
- Precision pipettes for volumes between 50 µL and 1,000 µL.
- Disposable test tubes for dilution of samples and calibrators.
- Repeater pipettes for dispensing 50 µL.
- Disposable beakers for diluting buffer concentrates.
- Graduated cylinders.
- A microplate shaker.
- Adsorbent paper for blotting.
- Microplate reader capable of reading at 450 nm, preferably with reference wavelength between 570 nm and 590 nm.
- Graph paper for plotting the calibration curve.

SAMPLE HANDLING

The TNF-α ELISA, Mouse 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 calibration curve. Please refer to the Sample Recovery recommendations on page 6 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 calibrators have been diluted into the tissue culture media instead of Assay Buffer. Culture media used as samples and for calibrator preparation must be diluted at least 1:2 in Assay Buffer. There will be a small change in binding associated with running the calibrators and samples in media. Users should only use calibration 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 (RT) slowly and gently mixed by hand. Do not thaw samples in a 37°C incubator. Do not vortex or sharply agitate samples.

PRECAUTIONS

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to RT for at least 30 minutes before opening.
3. Calibrators can be made up in either glass or plastic tubes.
4. Pre-rinse the pipette tip with reagent, use fresh pipette tips for each sample, calibrator and reagent.
5. Pipette calibrators 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 foil bag. 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 calibrators and samples be as similar as possible. Mouse TNF-α samples diluted with Assay Buffer should be run with calibrators diluted in the same buffer. Tissue culture samples should be read against a calibration curve obtained with calibrators 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 de-ionized water. This can be stored at RT until the kit expiration, or for 3 months, whichever is earlier.

2. Mouse TNF-α Calibrators

   Assay Buffer
   Allow the 1200 pg mouse TNF-α Calibrator vial to warm to RT. Label eight 12 x 75 mm glass tubes #1 through #8. Pipette 400 µL of Assay Buffer into tube #1. Pipette 250 µL of Assay Buffer into tubes #2 through #8. Add 120 µL of de-ionized water to the lyophilized mouse TNF-α vial and vortex. Add 100 µL of reconstituted calibrator to tube #1. Vortex thoroughly. Transfer 250 µL of tube #1 to tube #2 and vortex thoroughly. Transfer 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. STORE CALIBRATOR AT -20℃, avoid repeated freeze-thaws.

   Tissue Culture Media (Diluted 1:2 in Assay Buffer)
   Allow the 1200 pg Mouse TNF-α Calibrator vial to warm to RT. Label eight 12 x 75 mm glass tubes #1 through #8. Pipette 400 µL of 1:2 tissue culture media into tube #1. Pipette 250 µL of 1:2 tissue culture media into tubes #2 through #8. Add 120 µL of de-ionized water to the lyophilized mouse TNF-α vial and vortex. Add 100 µL of reconstituted calibrator to tube #1. Vortex thoroughly. Transfer 250 µL of tube #1 to tube #2 and vortex thoroughly. Transfer 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. STORE CALIBRATOR AT -20℃, avoid repeated freeze thaws.

ASSAY PROCEDURE

Bring all reagents to RT for at least 30 minutes prior to opening.
All calibrators, 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 foil pouch and seal the ziploc. Store unused wells at 4℃.
2. Pipette 50 µL of Calibrators #1 through #8 for Assay Buffer Calibrators or Calibrators #1 through #8 for 1:2 Tissue Culture Media into the appropriate wells.
3. Pipette 50 µL of calibrator diluent (Assay Buffer or Tissue Culture Media) into the S0 (0 pg/mL calibrator) wells.
4. Pipette 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 RT on a plate shaker for 2 hours at ~500 rpm.
7. Empty the contents of the wells and wash by adding 400 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. Pipette 50 µL of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at RT on a plate shaker for 2 hours at ~500 rpm.
10. Empty the contents of the wells and wash as in step 7 by adding 400 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 HRP Conjugate to each well, except the Blank.
12. Seal the plate and incubate at RT on a plate shaker for 30 minutes at ~500 rpm.
13. Empty the contents of the wells and wash as in step 7 by adding 400 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. Pipette 50 µL of Substrate Solution into each well.
15. Incubate for 30 minutes at RT.
16. Pipette 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 reference wavelength 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.

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 4 parameter logistic curve-fitting program. 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 calibrator and sample by subtracting the average Blank OD from the average OD for each calibrator and sample.
   Average Net OD = Average OD - Average Blank OD
2. Using linear graph paper, plot the Average Net OD for each calibrator versus mouse TNF-α concentration in each calibrator. 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	mouse 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	782.284
Unknown 2	0.390	0.347	161.833

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.

Sensitivity

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

Mean OD for S0 = 0.055 $\pm$ 0.003 (4.6%)
Mean OD for Calibrator #8 = 0.075 $\pm$ 0.005 (6.3%)
Delta Optical Density (15.63-0) = 0.075-0.055 = 0.020
2 SD's of 0 pg/mL Calibrator = 0.005
Sensitivity = 0.005/ 0.020 x 15.63 pg/mL = 3.9 pg/mL

Linearity

A sample containing 722.12 pg/mL mouse TNF-α was serially diluted 5 times 1:2 in the Assay Buffer 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.

	mouse 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	159.200		17.7
High	733.500		12.5

Cross-Reactivities

The TNF-α ELISA, Mouse 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 3 and 4 for Sample Handling recommendations and Calibrator 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 with the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Mouse Serum	102.0	1:8
Tissue Culture Media	92.6	1:2

*Note: The normal mouse serum samples tested read below the detection limit of the assay.
*See Sample Handling instructions on page 3 for details.

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