KAMIYA BIOMEDICAL COMPANY

Rat TNF-α ELISA

For the quantitative determination of TNF-α in rat serum

Cat. No. KT-670

For Research Use Only.

PRODUCT INFORMATION

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PRODUCT
The **K-*ASSAY*®** Rat TNF-α (Tumor Necrosis Factor-Alpha) ELISA is an enzyme immunoassay for the quantitative determination of TNF-α in rat serum. For research use only.

INTRODUCTION
Tumor necrosis factor-alpha (TNF-α) is one of several cytokines, including IL-6 and IL-1β, that stimulate the acute phase response. It is produced by numerous cells, including macrophages, endothelial cells and cardiomyocytes. Its release induces the hepatic expression positive acute phase proteins including C-reactive protein, serum amyloid-A and haptoglobin. TNF-α levels peak rapidly after an inflammatory stimulus and quickly fall to base line levels. TNF-α is a good early stage biomarker of inflammation.

PRINCIPLE
The rat TNF-α ELISA uses anti-rat TNF-α antibodies for solid phase (microtiter wells) immobilization and biotinylated anti-rat TNF-α antibodies for detection. Test samples, diluted as necessary, are incubated in the microtiter wells for 2 hours. The microtiter wells are subsequently washed, and biotinylated anti-rat TNF-α is added and incubated for 1 hour. This results in rat TNF-α molecules being sandwiched between the immobilization and detection antibodies. After washing the wells, horseradish peroxidase (HRP)-labeled avidin is added and incubated for 30 minutes. The HRP-avidin tightly binds to the biotinylated antibody. The wells are then washed to remove unbound HRP-labeled avidin, and TMB Reagent (an HRP substrate) is added and incubated for 20 minutes. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of rat TNF-α is proportional to the optical density of the test sample and is derived from a calibration curve.

COMPONENTS
- Anti-rat TNF-α coated microtiter plate (96 wells provided as 12 detachable strips of 8)
- Reference TNF-α stock (lyophilized), 2 vials
- 20X Wash Buffer, 50 mL
- Diluent, 1 x 50 mL
- TMB Reagent, 11 mL
- Stop Solution (1N HCl), 11 mL
- 2X Biotinylated rat TNF-α detection antibody, 6 mL
- 2X Avidin-HRP conjugate, 6 mL

MATERIALS REQUIRED BUT NOT PROVIDED
- Precision pipettes and tips
- Distilled or de-ionized water
- Vortex mixer
- Absorbent paper or paper towels
- Graph paper (PC graphing software is preferable)
- Polypropylene or glass tubes
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an OD range of 0-4 OD
- Microplate incubator/shaker with an approximate mixing speed of 150 rpm
- Microplate washer

WASH SOLUTION PREPARATION
The wash solution is provided as a 20X stock. Prior to use dilute the contents of the bottle (50 mL) with 950 mL of distilled or de-ionized water.

CALIBRATOR PREPARATION

1. The rat TNF-α calibrator is provided as a lyophilized stock. Add the volume of diluent indicated on the vial label and mix gently until dissolved ***(the reconstituted calibrator should be aliquoted and frozen at -20°C after reconstitution if further use is intended).***
2. Label 8 polypropylene or glass tubes as 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, and 0 pg/mL.
3. In the tube labeled 250 pg/mL, prepare the 250 pg/mL calibrator as described on the stock vial label.
4. Pipette 250 µL of diluent into the tubes labeled 125, 62.5, 31.25, 15.63, 7.81, 3.91, and 0 pg/mL.
5. Prepare the 125 pg/mL calibrator by diluting and mixing 250 µL of the 250 pg/mL calibrator with the 250 µL of diluent in the tube labeled 125 pg/mL.
6. Similarly prepare the 62.5, 31.25, 15.63, 7.81, and 3.91 pg/mL calibrators by serial dilution.

SAMPLE PREPARATION

In our studies, TNF-α levels ranged from below the level of detection to more than 7,000 pg/mL. Optimal dilutions should be determined empirically. However, in order to avoid matrix effects, samples should be diluted a minimum of 20-fold. The diluent provided with the kit should be used for dilution. If samples are not to be tested immediately, we recommend that they be aliquoted and frozen at -80°C. Avoid repeated freeze-thaws.

BIOTINYLATED DETECTION ANTIBODY PREPARATION

For each 8-well strip used in the assay, mix 0.5 mL of 2X rat TNF-α detection antibody with 0.5 mL of diluent. **Prepare 5-10 minutes prior to use.**

AVIDIN-HRP CONJUGATE PREPARATION

For each 8-well strip used in the assay, mix 0.5 mL of 2X avidin-HRP conjugate with 0.5 mL of diluent. **Prepare 5-10 minutes prior to use.**

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µL of calibrators and diluted samples into the wells (we recommend that calibrators and samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at 25°C for 2 hours.
4. Using a plate washer, wash the microtiter wells 4 times with 1x wash solution (350 µL per well per wash). The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto paper towels to remove all residual droplets.
6. Add 100 µL of diluted biotinylated detection antibody into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at 25°C for 1 hour.
8. Wash as detailed in 4 and 5 above.
9. Add 100 µL of diluted avidin-HRP conjugate into each well.
10. Incubate on an orbital micro-plate shaker at 100-150 rpm at 25°C for 30 minutes
11. Wash the wells 6 times as detailed in 4-5 above.
12. Dispense 100 µL of TMB Reagent into each well.
13. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
14. Stop the reaction by adding 100 µL of Stop Solution to each well.
15. Gently mix if necessary. It is important to make sure that all the blue color changes to yellow.
16. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

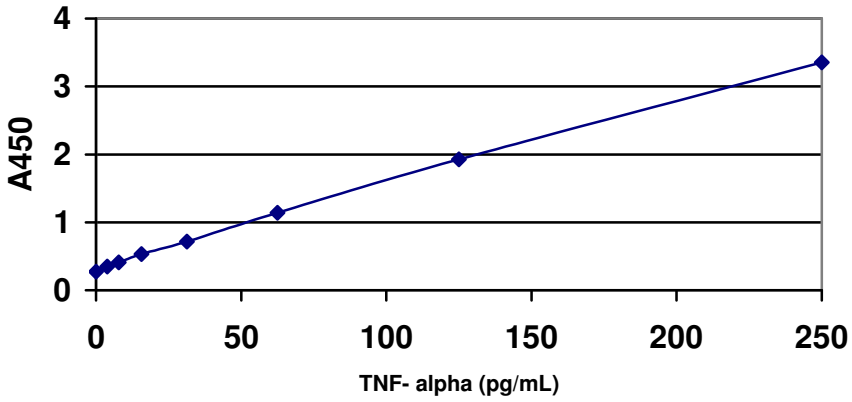
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference calibrators and samples.
2. Construct a calibration curve by plotting the mean absorbance obtained from each reference calibrator against its concentration in pg/mL on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of rat TNF-α in pg/mL from the calibration curve.
4. Multiply the derived concentration by the dilution factor, if applicable, to determine the actual concentration of rat TNF-α in the original sample.
5. If available, PC graphing software should be used for the above steps. We find that a second order polynomial model usually provides a good fit for the calibration curve.
6. If the OD_{450} values of the sample fall outside the calibration curve, samples should be diluted appropriately and re-tested.

TYPICAL CALIBRATION CURVE

A typical calibration curve with optical density readings at 450 nm on the Y axis against rat TNF-α concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and calibration curve in each experiment.

Rat TNF-α (pg/mL)	Absorbance (450 nm)
250.0	3.353
125.0	1.925
62.5	1.14
31.3	0.717
15.6	0.534
7.8	0.411
3.9	0.348
0.0	0.271



STORAGE

Upon receipt, the lyophilized TNF-α stock should be stored at or below -20℃. The remainder of the kit should be stored at 4℃ and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable until the expiration date provided that the components are stored as described above.

GENERAL INSTRUCTIONS

All reagents should be allowed to reach room temperature (18-25℃) before use.

LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed in this insert.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
3. ELISAs are validated at Kamiya Biomedical Company using shaking incubators set to a temperature of 25℃. If the incubation steps are performed at lower or higher temperatures, lower or higher absorbance values may be obtained.

FOR RESEARCH USE ONLY

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