Rat TNF-α ELISA KIT

Catalog Number	Size
E16RE0003-096	96 Tests
E16RE0003-048	48 Tests

Rat TNF-α ELISA KIT

For the quantitative determination of rat tumor necrosis factor (TNF-α) concentrations in cell culture supernates, serum, and plasma.This package insert must be read in its entirety before using this product. If you have questions or experience problems with this product, please contact our Technical Support staff. Our scientists commit themselves to providing rapid and effective help.

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

INTRODUCTION

The prototype ligand of theTNFsuperfamily, TNF-α/TNFSF1A, is a pleiotropic cytokine that plays a central role in inflammation and apoptosis (1-4).TNF-α is produced by activated macrophages and

other cell types including T and B cells, NK cells, LAK cells, astrocytes, endothelial cells, smooth muscle cells and some tumor cells (5-8).

Rat TNF-α cDNA encodes a 235 amino acid (aa) residue type II membrane protein (9). The 156 aa residue soluble TNF-αis released from the C-terminus of the membrane-anchored TNF-α by TNF-α-converting enzyme (TACE), a matrix metalloprotease (10).

The membrane-anchored form of TNF-α has been shown to have lytic activity and may also have an important role in intercellular communication (11). The biologically active TNF-α has been shown to exist as a trimer (12, 13).TNF-α is reported to promote inflammatory cell infiltration by upregulating leukocyte adhesion molecules on endothelial cells, serve as a chemotactic agent for monocytes, and activate phagocyte killing mechanisms (14). Deficiencies in either TNF-α or its receptors can increase susceptibility to infection by intracellular pathogens (15-16). TNF-α may also play a role in lymphoid tissue development. Knockout mice lack splenic B cell follicles and the ability to form germinal centers (17, 18). Other potential physiological roles for TNF-α and its receptors include regulating the differentiation of hematopoietic stem and progenitor cells (19-21).

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. Following incubation unbound samples are removed during a wash step, and then a detection antibody specific for TNF-α is added to the wells and binds to the combination of capture antibody-TNF-α in sample. Following a wash to remove any unbound combination, and enzyme conjugate is added to the

wells. Following incubation and wash steps a substrate is added. A coloured product is formed in proportion to the amount of TNF-α present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven TNF-α standard dilutions and TNF-α sample concentration determined.

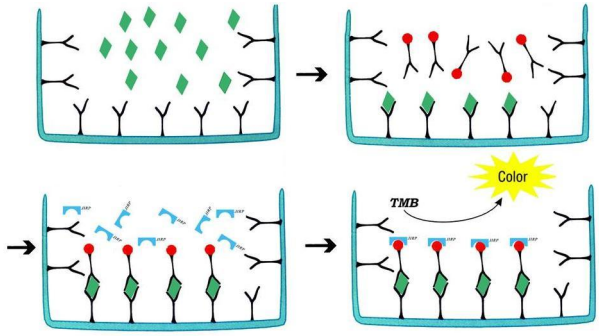


Figure 1:Schematic diagram of the assay

REAGENTS

1. Aluminium pouches with a Microwell Plate coated with monoclonal antibody to rat TNF-α (8×12)
2. 2 vials rat TNF-α Standard lyophilized, 2000 pg/ml upon reconstitution
3. 2 vials concentrated Biotin-Conjugate anti-rat TNF-α antibody
4. 2 vials Streptavidin-HRP solution,
5. 1 bottle Standard /sample Diluent
6. 1 bottle Biotin-Conjugate antibody Diluent
7. 1 bottle Streptavidin-HRP Diluent
8. 1 bottle Wash Buffer Concentrate 20x (PBS with 1% Tween-20)
9. 1 vial Substrate Solution
10. 1 vial Stop Solution
11. 3 pieces Adhesive Films
12. package insert

NOTE: [96 Tests]

STORAGE

Table 1: Storage of the kit

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="9">Opened/ Reconstituted Reagents</td><td>Standard /sample Diluent</td><td rowspan="8">May be stored for up to 1 month at 2 - 8° C.**</td></tr>
<tr><td>Concentrated Biotin-Conjugate</td></tr>
<tr><td>Streptavidin-HRP solution</td></tr>
<tr><td>Biotin-Conjugate antibody Diluent</td></tr>
<tr><td>Streptavidin-HRP Diluent</td></tr>
<tr><td>Wash Buffer Concentrate 20x</td></tr>
<tr><td>Substrate Solution</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Standard</td><td>Aliquot and store for up to 1 month at ≤20°C.
Avoid repeated freeze-thaw cycles. Diluted standard shall not be reused.</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.**</td></tr>
</table>

**Provided this is within the expiration date of the kit.

THE REQUIRED ITEMS (not provided, but can help to buy):

1. Microplate reader (450nm).
2. Micro-pipette and tips: 0.5-10, 2-20, 20-200, 200-1000ul.
3. 37 ℃ incubator, double-distilled water or deionized water, coordinate paper, graduated cylinder.

PRECAUTIONS FOR USE

1. Store kit regents between 2℃and 8℃. After use all reagents should be immediately returned to cold storage(2℃ to 8℃).

2. Please perform simple centrifugation to collect the liquid before use.
3. To avoid cross contamination, please use disposable pipette tips.
4. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material. Avoid contact of skin or mucous membranes with kit reagents or specimens. In the case of contact with skin or eyes wash immediately with water.
5. Use clean, dedicated reagent trays for dispensing the washing liquid, conjugate and substrate reagent. Mix all reagents and samples well before use.
6. After washing microtiter plate should be fully pat dried. Do not use absorbent paper directly into the enzyme reaction wells.
7. Do not mix or substitute reagents with those from other lots or other sources. Do not use kit reagents beyond expiration date on label.
8. Each sample, standard, blank and optional control samples should be assayed in duplicate or triplicate.
9. Adequate mixing is very important for good result. Use a mini-vortexer at the lowest frequency or Shake by hand at 10min interval when there is no vortexer.
10. Avoid microtiter plates drying during the operation.
11. Dilute samples at the appropriate multiple, and make the sample values fall within the standard curve. If samples generate values higher than the highest standard, dilute the samples and repeat the assay.
12. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time and temperature, and kit age can cause variation in binding.
13. This method can effectively eliminate the interference of the soluble receptors, binding proteins and other factors in biological samples.

SAMPLE COLLECTION AND STORAGE

1. **Cell Culture Supernates** - Remove particulates by centrifugation.
2. **Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum, avoid hemolysis and high blood lipid samples.

3. **Plasma** - Recommended EDTA as an anticoagulant in plasma. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection.
4. Assay immediately or aliquot and store samples at -20℃. Avoid repeated freeze-thaw cycles.
5. Dilute samples at the appropriate multiple (recommended to do pre-test to determine the dilution factor).
   Note: The normal rat serum or plasma samples are suggested to make a 1:2 dilution.

REAGENT PREPARATION

1. Bring all reagents to room temperature before use.
2. **Wash Buffer** - Dilute 10mL of Wash Buffer Concentrate into deionized or distilled water to prepare 200mL of Wash Buffer. If crystals have formed in the concentrate Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. **Standard** - Reconstitute the Standard with 1.0mL of Standard /sample Diluent. This reconstitution produces a stock solution of 2000 pg /mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

   Pipette 500μL of Standard/sample Diluent into the 1000 pg/mL tube and the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 2000 pg/mL standard serves as the high standard. The Standard/ sample Diluent serves as the zero standard (0 pg/mL).

   If you do not run out of re-melting standard, store it at -20℃. Diluted standard shall not be reused.
4. Working solution of Biotin-Conjugate anti-rat TNF-α antibody:
   Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with the Biotin-Conjugate antibody Diluent in a clean plastic tube.

   The working solution should be used within one day after dilution.
5. Working solution of Streptavidin-HRP: Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with the Streptavidin-HRP Diluent in a clean plastic tube.

   The working solution should be used within one day after dilution.

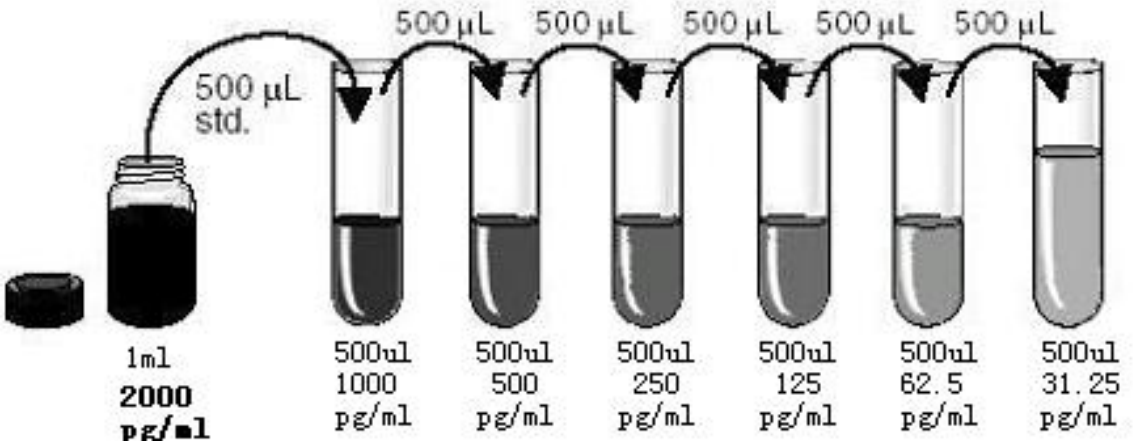


Figure 2: Preparation of TNF-α standard dilutions

GENERAL ELISA PROTOCOL

1. Prepare all reagents and working standards as directed in the previous sections.
2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2-8℃ sealed tightly.
3. Add 100µL of Standard, control, or sample, per well. Cover with the adhesive strip provided. Incubate for 1.5 hours at 37℃.
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (350µL) using a squirt bottle, manifold dispenser or auto-washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µL of the working solution of Biotin-Conjugate to each well. Cover with a new adhesive strip and incubate 1 hours at 37℃.
6. Repeat the aspiration/wash as in step 3.
7. Add 100 µL of the working solution of Streptavidin-HRP to each well. Cover with a new adhesive strip and incubate for 30 minutes at 37℃ Avoid placing the plate in direct light.
8. Repeat the aspiration/wash as in step 3.

9. Add 100 μL of Substrate Solution to each well. Incubate for 10-20 minutes at 37℃. Avoid placing the plate in direct light.
10. Add 100 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well immediately, using a microplate reader set to 450 nm.(optionally 630nm as the reference wave length;610-650nm is acceptable)

ASSAY PROCEDURE SUMMARY

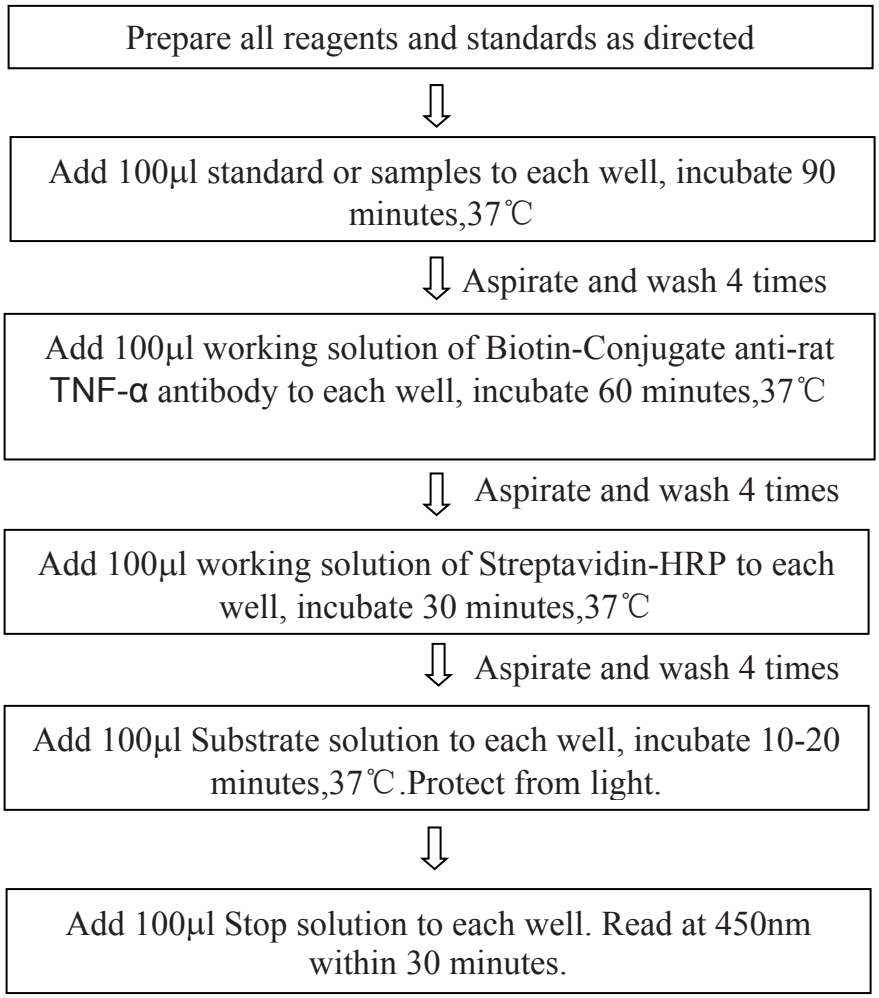


Figure 3: Assay procedure summary

TECHNICAL HINTS

1. When mixing or reconstituting protein solutions, always avoid foaming.
2. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

3. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
4. Substrate Solution should remain colorless until added to the plate. Stop Solution should be added to the plate in the same order as the Substrate Solution. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
5. A standard curve should be generated for each set of samples assayed. According to the content of tested factors in the sample, appropriate diluted or concentrated samples, it is best to do pre-experiment.

CALCULATION OF RESULTS

1. Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.
2. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.
3. The data may be linearized by plotting the log of the TNF-α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
4. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Table 2:Typical data using the TNF-α ELISA (Measuring wavelength: 450nm,Reference wavelength:630nm)

Standared (pg/ml)	OD.	OD.	Average	Corrected
0	0.119	0.114	0.117	——
31.25	0.211	0.205	0.208	0.153
62.5	0.273	0.283	0.278	0.217
125	0.390	0.395	0.393	0.343
250	0.626	0.623	0.625	0.585
500	1.020	1.027	1.024	1.026
1000	1.709	1.706	1.708	1.741
2000	2.510	2.505	2.508	2.501

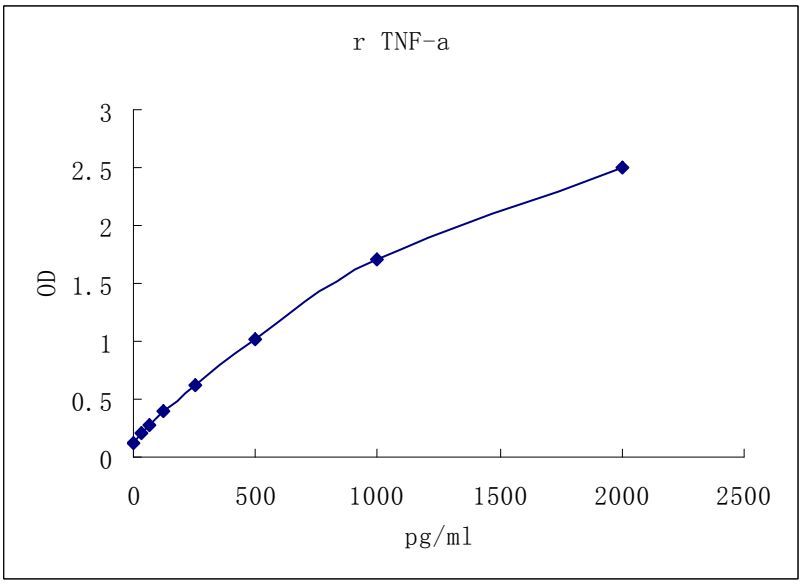


Figure 4:Representative standard curve for TNF-α ELISA.TNF-α was diluted in serial two-fold steps in Sample Diluent.

Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

SENSITIVITY, SPECIFICITY AND REPEATABILITY

1. **REPEATABILITY**: The coefficient of variation of both intra-assay and inter-assay were less than 10%.
2. **SENSITIVITY**: The minimum detectable dose was 15pg/mL.
3. **SPECIFICITY**: This assay recognizes both natural and recombinant rat TNF-α. The factors listed below were prepared at 50ng/ml in Standard /sample Diluent and assayed for cross-reactivity and no significant cross-reactivity or interference was observed.

Table 3: Factors assayed for cross-reactivity

Recombinant human	Recombinant mouse	Recombinant rat
TNF-α	TNF sRI	CINC-1
TNF-β	TNF sRII	GDNF
TNF sRI		β-NGF
TNF sRII		PDGF-BB
		IFN-γ

REFERENCES

1. Kwon, B. et al. (1999) Curr. Opin. Immunol. 11:340.
2. Idriss, H.T. and J.H. Naismith (2000) Microsc. Res. Tech. 50:184.
3. Sedgwick, J.D. et al. (2000) Immunol. Today 21:110.
4. MacEwan, D.J. (2002) Brit. J. Pharmacol. 135:855.
5. Vilcek J. and T.H. Lee (1991) J. Biol. Chem. 266:7313.
6. Ruddle, N.H. (1992) Curr. Opinion Immunol. 4:327.
7. Tumor Necrosis Factor: Structure, Function and Mechanism of Action, Aggarwal, B.B. and J. Vilcek eds. (1991) Marcel Dekker, Inc., New York.
8. Beutler, B. and A. Cerami (1989) Annu. Rev. Immunol. 7:625.
9. Kwon, J. et al. (1993) Gene 132:227.
10. Gearing, A.J.H. et al. (1994) Nature 370:555.
11. Pennica, D. et al. (1985) Proc. Natl. Acad. Sci. USA 82:6060.
12. Gearing, A.J.H. et al. (1994) Nature 370:555.
13. Perez, C. et al. (1990) Cell 63:251.
14. Jones, E.Y. et al. (1989) Nature 338:225.
15. Eck, M.J. and S.R. Sprang (1989) J. Biol. Chem. 264:17595.
16. Wang, C.Y. et al. (1998) Science 281:1680.
17. Peschon, J.J. et al. (1998) J. Immunol. 160:943.
18. Wellmer, A. et al. (2001) Infect. Immun. 69:6881.
19. Trevejo, J.M. et al. (2001) Proc. Natl. Acad. Sci. USA 98:12162.
20. Kasahara, S. et al. (2003) J. Virol. 77:2469.
21. Pasparakis, M. et al. (1996) J. Exp. Med. 184:1397.
22. Taniguchi, T. et al. (1997) Lab. Invest. 77:647.
23. Jacobsen, F.W. et al. (1994) Proc. Natl. Acad. Sci. USA 91:10695.