Version 13-08/24

User's Manual

Swine TNF-α ELISA Kit

REF **DEIA-T6108**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

For the quantitative determination of swine 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.

General Description

Tumor necrosis factor alpha (TNF-α), also known as cachectin, is a member of the TNF ligand superfamily and has been designated TNFSF1A. It binds to the same cell surface receptors, and shares some biological functions with TNF-β /TNFSF1B. TNF-α inhibits the growth of certain tumors. It also plays a critical role in normal host resistance to infection, serving as an immunomodulator and as a mediator of inflammatory responses. Over-production of TNF has been implicated in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders (1-4). TNF-α is produced primarily by activated macrophages (1-6). Various other porcine cell types, including NK cells (6), keratinocytes (7), vascular smooth muscle cells (8), and granulosa lutein cells (9) are also known to produce TNF-α.

The porcine TNF-α gene product is a 232 amino acid (aa) residue type II membrane glycoprotein containing a 35 aa cytoplasmic domain, a 21 aa transmembrane domain and a 178 aa extracellular domain (10-13). The 156 aa residue soluble TNF-α is released from the C-terminus of the membrane protein by TNF-α converting enzyme (TACE, ADAM17), a member of the ADAM (a disintegrin and metalloprotease domain) family of metalloproteases(10, 11, 14). The biologically active TNF-α has been shown to exist as a trimer (1-4). Porcine TNF-α is active on mouse cells and shares 89% and 79% aa sequence identity with human and mouse TNF-α, respectively (10, 14).Two distinct TNF receptors, referred to as type I (type B, p55, or TNFRSF1A) and type II (type A, p75, or TNFRSF1B), that specifically bind TNF-α and TNF-β with equal affinities are known (15-17). The two TNF receptors share aa sequence homology in their extracellular but not their cytoplasmic domains, suggesting that the two receptors employ different signal transduction pathways. Soluble forms of both types of receptors have been found in human and mouse serum (18-20).

These soluble receptors are capable of neutralizing the biological activities of the TNFs and may serve to modulate the activities of TNF. Porcine TNF RI shares 79% and 72% aa homology with the human and mouse TNF RI, respectively (21-23).

Principles of Testing

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standard, control, or sample and the working solution of Biotin-Conjugate are pipetted into the wells. Following incubation and wash steps, any TNF-α present is bound by the immobilized antibody and the detection antibody specific for TNF-α is 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.

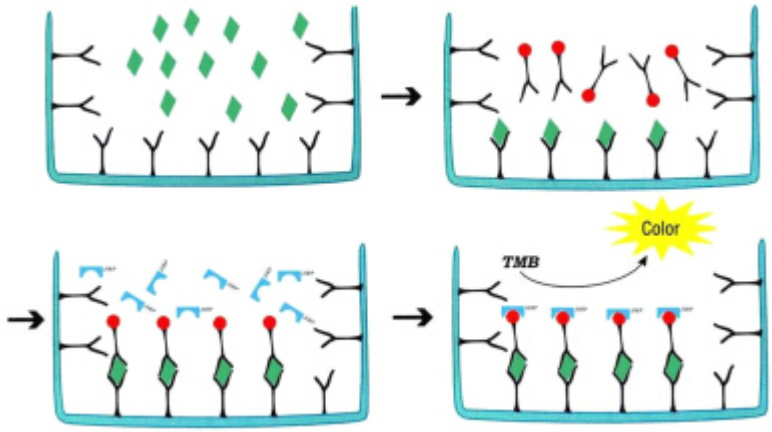


Figure 1: Schematic diagram of the assay

Reagents And Materials Provided

1. Aluminium pouches with a Microwell Plate coated with antibody to swine TNF-α (8×12)
2. 2 vials swine TNF-α Standard lyophilized, 1000 pg/ml upon reconstitution
3. 2 vials concentrated Biotin-Conjugate anti-swine 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. 4 pieces Adhesive Films
12. package insert

NOTE: [96 Tests]

Materials Required But Not Supplied

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

Storage

For more detailed information, please download the following document on our website.

Specimen Collection And Preparation

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°C. 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 swine serum or plasma samples are suggested to make a 1:2 dilution.

Plate Preparation

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°C sealed tightly.
3. Add 100µL of Standard, control, or sample, per well, then add 50 µL of the working solution of Biotin-Conjugate to each well. Cover with the adhesive strip provided and incubate 2 hours at RT. Adequate mixing is very important for good result. Use a mini-vortexer at the lowest frequency.
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 Streptavidin-HRP to each well. Cover with a new adhesive strip and incubate for 30 minutes at RT Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 4.
7. Add 100 µL of Substrate Solution to each well. Incubate for 10-20 minutes at RT. Avoid placing the plate in direct light.
8. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. 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)

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 0.5mL 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 250µL 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°C. Diluted standard shall not be reused.

4. Working solution of Biotin-Conjugate anti-swine 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.

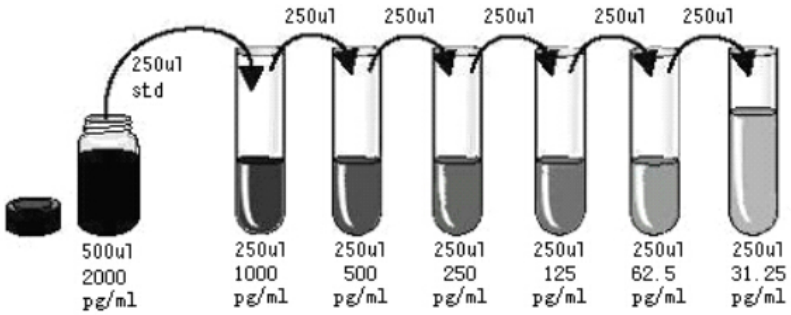


Figure 2: Preparation of TNF-α standard dilutions

Assay Procedure

Assay procedure summary

1. Prepare all reagents and standards as directed.
2. Add 100µL standard or samples to each well , and add 50µL working solution of Biotin-Conjugate antibody to each well, incubate120 minutes, RT.
3. Aspirate and wash 4 times.
4. Add 100µL working solution of Streptavidin-HRP to each well, incubate 30 minutes, RT.
5. Aspirate and wash 4 times.
6. Add 100µL Substrate solution to each well, incubate 10-20 minutes, RT.Protect from light.
7. Add 100µL Stop solution to each well. Read at 450nm within 30 minutes.

Calculation

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

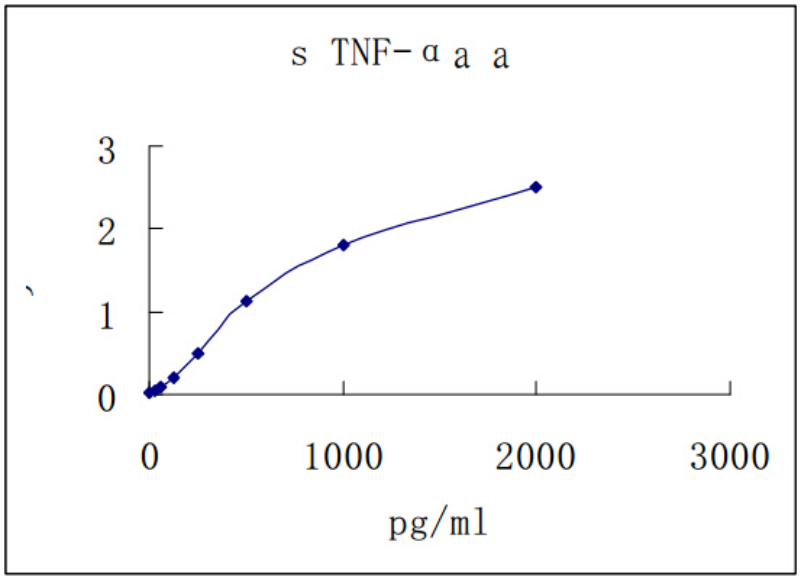
the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

4. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Typical Standard Curve

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

Standared (pg/ml)	OD.	OD.	Average	Corrected
0	0.026	0.024	0.025	——
31.25	0.052	0.047	0.050	0.047
62.5	0.100	0.098	0.099	0.097
125	0.212	0.209	0.211	0.225
250	0.494	0.495	0.495	0.521
500	1.136	1.133	1.135	1.085
1000	1.812	1.808	1.810	1.846
2000	2.517	2.512	2.515	2.504



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.

Performance Characteristics

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.

1. REPEATABILITY: The coefficient of variation of both intra-assay and inter-assay were less than 10%.
2. SENSITIVITY: The minimum detectable dose was 7pg/mL.
3. SPECIFICITY: This assay recognizes both recombinant and natural porcine TNF-α. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD6-33 and assayed for cross-reactivity. Preparations of the following factors at the same concentrations in a mid-range porcine TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Factors assayed for cross-reactivity

Recombinant human	Recombinant mouse	Recombinant porcine
TNF-β	TNF-α	IL-2
TNF RI	TNF RI	IL-4
	TNF RII	IL-10
		IFN-γ

Precautions

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

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