For Research Use Only

January 6, 2012 Ver. 4

[Rat TNF-α ELISA Kit]

(Code No.:AKRTN-010)

Please, read this instruction carefully before use.

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit! For the detailed assay procedure, refer to Key points for ELISA by movie on our website:
http://www.shibayagi.co.jp/index-E.htm

1. Intended use

Rat TNF-α ELISA Kit is a sandwich ELISA system for quantitative measurement of rat TNF-α. This is intended for research use only.

2. Storage and expiration

When the intact kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Reagents, once opened, should be used as soon as possible to avoid losing its optimal assay performance by storage environment.

3. Introduction

TNF-α and TNF-β belong to TNF (tumor necrosis factor) family though TNF-α is more popular. TNF-α is one of lymphokines (cytokines derived from lymphocytes) produced by activated macrophages when stimulated by bacteria (especially by lipopolysaccharides), viruses and parasites. TNF-a was first found as a factor which causes hemorrhagic necrosis in solid tumors, however, it is also inflammatory cytokine as it is involved in inflammatory response caused by bacterial infection, tumor and tissue injury. It is also an adipokines as it is expressed in adipocytes. Inflammatory cytokines act on the liver, and enhance the production and secretion of acute phase proteins such as CRP and SAA. TNF-α promotes adherence of neutrophils to endothelial cells and also induces chemotactic factor to gather phagocytes to the area to cause inflammatory reaction.

TNF-α is secreted from hypertrophic adipocytes and is noted as a factor which increases insulin resistance in the field of glucose metabolism. Upraised TNF-α in blood suppressed tyrosine-kinase activity of insulin receptors in adipose and other tissues, and inhibits expression of GLUT4, and insulin-dependent cellular glucose uptake. TNF-α also induces hyperlipemia by inhibiting the production of LPL (lipoprotein lipase).

4. Assay principle

In Shibayagi's Rat TNF-α ELISA Kit, standards or samples are incubated in monoclonal anti-TNF-α antibody-coated wells to capture TNF-α. After 2 hours incubation and washing, biotin-labeled anti- TNF-α antibody is added and incubated further for 1 hour to bind captured TNF-α. After washing, HRP (horse radish peroxidase)-labeled avidin is added, and incubated for 30 minutes. After washing, HRP-complex remaining in wells is reacted with a chromogenic substrate (TMB) for 30 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is nearly proportional to TNF-α concentration. The standard curve is prepared by plotting absorbance against standard TNF-α concentrations. TNF-α concentrations in unknown samples are determined using this standard curve.

5. Precautions

- For professional use only, beginners are advised to use this kit under the guidance of experienced person.
- Wear gloves and laboratory coats when handling assay materials.
- Do not drink, eat or smoke in the areas where assays are carried out.
- In treating assay samples of animal origin, be careful for possible biohazards.
- This kit contains components of animal origin. These materials should be handled as

potentially infectious.

- Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucus membranes. Especially be careful for the reaction stopper because it is 1 M sulfuric acid. The reaction stopper and the substrate solution may cause skin/eyes irritation. In case of contact with these wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- The materials must not be pipetted by mouth.
- Residual samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
- Dispose consumable materials and unused contents in accordance with applicable regional/national regulatory requirements.
- Use clean laboratory glassware.
- In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate cover supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. (including wind from air conditioner)(*①), and humidity less than 30%. For more details, watch our web movie [Assay circumstance].

6. Reagents supplied

Components	State	Amount
(A) Antibody-coated 96 well-plate (Dried-plate)	Use after washing	96 wells/1 plate
(B) Rat standard TNF-α (30 ng/ml) (recombinant)	Concentrated. Use after dilution	100 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-labeled anti- TNF-α antibody	Concentrated. Use after dilution.	100 µl/1 vial
(E) ） HRP-avidin conjugate	Concentrated. Use after dilution.	100 µl/1 vial
(F) Chromogenic substrate (TMB) solution	Ready for use.	12 ml/1 bottle
(H) Reaction stopper (1M H_2SO_4) Be careful!	Ready for use.	12 ml/1 bottle
(Ⅰ) Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate cover	—	1 plate
Instruction Manual	—	1 copy

7. Equipments required but not supplied ☐Use as a check box

☐Purified water (distilled water)
☐Test tubes for preparation of standard solution series.
☐Glassware for dilution of washing buffer (a graduated cylinder, a bottle)
☐Pipettes (disposable tip type). One should be able to deliver 10-50 µl precisely, and another for 100-400 µl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 µl.
☐Paper towel to remove washing buffer remaining in wells.
☐A vortex-type mixer.
☐A shaker for 96 well-plate (600-1200rpm)
☐An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle (refer to our web movie [Washing of microplate].
☐A 96 well-plate reader (450nm ±10nm, 620nm: 600-650nm)
Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).

8. Preparation of reagents

◆Bring all reagents of the kit to room temperature (20-25 °C) before use.

◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

【Concentrated reagents】

[(B) Rat standard TNF-α (30 ng/ml)]

Make a serial dilution of original standard solution to prepare each standard solution. Example is shown below.

Volume of standard solution	Buffer solution	Concentration(pg/ml)
Original solution : 40 µl	360 µl	3000
3000 pg/ml solution : 100 µl	185 µl	1053
1053 pg/ml solution : 100 µl	185 µl	369
369 pg/ml solution : 100 µl	185 µl	129
129 pg/ml solution : 100 µl	185 µl	45.3
45.3 pg/ml solution : 100 µl	185 µl	15.9
0 (Blank)	185 µl	0

[(D) Biotin-labeled anti- TNF-α antibody]

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.

[(E) HRP-avidin conjugate]

Prepare working solution by dilution of (E) with the buffer solution (C) to 1:100.

[(I) Concentrated washing buffer (10x)]

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with deionized water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of deionized water.

【Storage and stability】

[(A) Antibody-coated well-plate]

If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

[(B) Rat standard TNF-α (30 ng/ml)]

Standard solutions prepared above should be used as soon as possible, and should not be stored.

[(C) Buffer solution] and [(F) Chromogenic substrate solution]

If not opened, store at 2-8 °C. It maintains stability until expiration date. Once opened, we recommend using them as soon as possible to avoid influence by environmental condition.

[(D) Biotin-labeled anti- TNF-α antibody] and [(E) HRP-avidin conjugate]

Unused working solution (already diluted) should be disposed.

[(H) Reaction stopper (1 M H_2SO_4)]

Close the stopper tightly and store at 2-8 ° C. It maintains stability until expiration date.

[(I) Concentrated washing buffer (10x)]

The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date. Dispose any unused diluted buffer.

9. Technical tips

- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best not to mix the reagents with those that have been preserved for some period.
- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips, and 1 tip for 1 well.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution. Otherwise, store them in a dark place at 2-8 °C.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.

- The chromogenic substrate (TMB) solution should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- As the antibody-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity of over 0.4 m/sec. and the humidity of less than 30%, completely close each well in addition to cover the well plate with a plate cover in each step of incubation.
  Ex.) Cover the well plate with parafilm, and put the plate cover on it. Or place the well plate with the plate cover in an incubator, or in a styrofoam box. Take the best way depending on situation of each laboratory. For more details, watch our web movie **[Assay circumstance]**.

10. Preparation of samples

This kit is intended to measure TNF-α in rat serum or plasma. The necessary sample volume for the standard procedure is 10 µl.

Samples should be immediately assayed or stored below –35 °C for several days. Defrosted samples should be mixed thoroughly for best results. Hemolytic and hyperlipemic serum samples are not suitable. If presence of interfering substance is suspected, examine by dilution test at more than 2 points. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.

Sample dilution should be carried out with the buffer solution of the kit using small test tubes such as PP, PE or glass, before assay.

Storage and stability

If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Avoid repeated freezing and thawing cycles.

11. Assay procedure

Remove the cover sheet of the 96 well-plate after bringing up to room temperature.

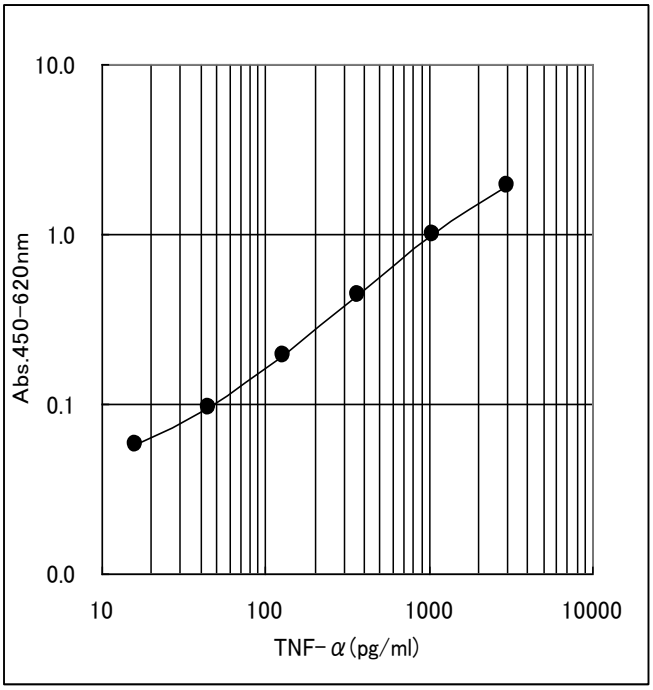
(1) Wash the antibody coated plate (A) by filling the well with washing buffer and discard 4 times (*②), then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
(2) Pipette 40µl of buffer solution (C) and 10µl of samples to the designated sample wells. Sample volume can be adjusted within 10-50µl, but total volume to add to each well should be 50µl.
(3) Pipette 50 µl of standard solution to the wells designated for standards.
(4) Shake the plate gently on a plate shaker (*③).
(5) Put a plate cover on the plate and incubate for 2 hours at 20-25°C.
(6) Discard the reaction mixture and rinse wells as step (1).
(7) Pipette 50 µl of biotin-labeled anti- TNF-α solution (D) to all wells, and shake as step (4).
(8) Put a plate cover on the plate and incubate the plate for 1 hour at 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 50 µl of HRP-conjugated avidin solution (E) to all wells, and shake as step (4).
(11) Put a plate cover on the plate and incubate the plate for 30 minutes at 20-25°C.
(12) Discard the reaction mixture and rinse wells as step (1).
(13) Pipette 50 µl of chromogenic substrate solution (F) to wells, and shake as step (4).
(14) Put a plate cover on the plate and incubate the plate for 30 minutes at 20-25°C.
(15) Add 50 µl of the reaction stopper (H) to all wells and shake as step (4).
(16) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

*Refer to the page 7-8 for notes of *② and *③.

12. Calculations

(1) Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against TNF-α concentration (pg/ml) on X-axis. Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.

(2) Using the standard curve, read the TNF-α concentration of a sample at its absorbance*, and multiply the assay value by dilution factor. Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, please repeat the assay after proper dilution of samples with the buffer solution. * We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.



Rat TNF-α assay standard curve (an example)
Absorbance may change due to assay situation.

13. Performance characteristics

- Assay range

The assay range of the kit is 15.9 ~ 3,000 pg/ml. (For 5x dilution, 79.5 ~ 15,000 pg/ml)

- Specificity

The kit uses a monoclonal antibody which reacts with rat TNF-α.
rTNF-α = recombinant TNF-α *Cross reaction at 3,000 pg/ml.

Samples	Cross reaction	Samples	Cross reaction
Rat rTNF-α	100%	Mouse r TNF-α	+
Rat TNF-β	< 0.2%	+: Cross reaction	

No cross reaction at 3,000 pg/ml: Rat IFNγ, rat GM-CSF, porcine rTNF-α, human rTNF-α, human rTNF-β

- Precision of assay

Within assay variation (2 samples, 5 replicates assay,) Mean CV was less than 5 %.

- Reproducibility

Between assay variation (3 samples, 4 days, 4 replicates assay) Mean CV was less than 5 %

- Recovery test

rTNF-α was added in 3 concentrations to 2 serum samples and was assayed.
The recoveries were 93.1 ~ 106%

- Dilution test

2 serum samples were serially diluted by 3 steps.
The dilution curves showed excellent linearity. (R^2= 0.9974 ~ 1)

14. Reference assay data

Rat TNF-α mean assay value: 95.4 pg/ml, SD: 11.7 pg/ml
Strain: CD, 11 males, 8 week-old, serum
These data should be considered as guidance only. Each laboratory should establish its own normal and pathological reference ranges for TNF-α levels independently.

15. Trouble shooting

- Low absorbance in all wells
  Possible explanations:
  1) The standard or samples might not be added.
  2) Reagents necessary for coloration such as Biotin-labeled antibody, HRP-conjugated avidin, or TMB might not be added.
  3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-labeled antibody or HRP-avidin conjugate.
  4) Contamination of enzyme inhibitor(s).
  5) Influence of the temperature under which the kits had been stored.
  6) Excessive hard washing of the well plate.
  7) Addition of TMB solution soon after taking out from a refrigerator might cause poor coloration owing to low temperature.
- Blank OD was higher than that of the lowest standard concentration (15.9 pg/ml).
  Possible explanations:
  Improper or inadequate washing. (Change washing frequency from 4 times to 5-8 times at the constant stroke after the reaction with HRP-conjugated anti-TNF-α antibody.)
- High coefficient of variation (CV)
  Possible explanation:
  1) Improper or inadequate washing.
  2) Improper mixing of standard or samples.
  3) Pipetting at irregular intervals.
- Q-1: Can I divide the plate to use it for the other testing?
  A-1: Yes, cut off the clear seal on the plate with cutter along strip. Put the residual plate, which is still the seal on, in a refrigerator soon
- Q-2: I found 96 well-plate is empty when I opened the box.
  A-2: As this kit is dried type, not preservation stabilizer is added.

For detailed FAQS and explanations, refer to **"Trouble shooting and Important Points in Shibayagi's ELISA kits"** on our website (http://www.shibayagi.co.jp/en/tech_004.html).

Summary of assay procedure ☐ : Use as a check box

***First, read this instruction manual carefully and start your assay after confirmation of details.**

For more details, watch our web movie **[ELISA by MOVIE]** on our website.

☐Bring the well-plate and all reagents to 20-25°C for 2 hours.

☐Concentrated washing buffer must be diluted to 10 times by purified water.

☐Standard TNF-α solution dilution example 1:

Concentration (pg/ml)	3000		1053		369		129		45.3		15.9	0
Std. TNF-α solution (µl) Orig.sol.	40	→	100*	→	100*	→	100*	→	100*	→	100*	0
Buffer solution (µl)	360		185		185		185		185		185	185

*One rank higher standard.

Standard TNF-α solution dilution example 2:

Concentration (pg/ml)	3000		1500		600		300		120		40		16	0
Std. TNF-α solution (µl) Orig.sol.	40	→	200*	→	200*	→	200*	→	200*	→	200*	→	200*	0
Buffer solution (µl)	360		200		300		200		300		400		300	300

*One rank higher standard.

		Precautions & related info
☐	Antibody-coated 96 well-plate (Dried-plate)	
☐	↓ Washing 4 times(*②)	*⑥
☐	Diluted samples (e.g. buffer (C) 40µl + sample 10µl), or Standards 50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
☐	Meanwhile, dilute biotin-labeled anti-TNF-α antibody (D) to 100x with buffer (C) returned to 20-25°C.	This should be prepared during incubation.
☐	↓ Washing 4 times(*②)	*⑥
☐	Biotin-labeled anti-TNF-α antibody 50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
☐	Meanwhile, dilute HRP-conjugated avidin (E) to 100x with Buffer (C) returned to 20-25°C.	This should be prepared during incubation.
☐	↓ Washing 4 times(*②)	*⑥
☐	HRP-conjugated avidin 50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 30 min at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
☐	↓ Washing 4 times(*②)	*⑥
☐	Chromogenic substrate (TMB) 50 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 30 min at 20-25°C.	*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4) 50 µl	After dispense, the color turns to yellow depending on the concentration.
☐	↓ Shaking(*③)	Immediately shake.
☐	Measurement of absorbance (450nm, Ref 620nm(*⑤))	Ref. wave cancels the dirt in the back of plate.

*②Guideline of washing volume: 300µl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change washing frequency from 4 times to 5-8 times at the constant stroke after the reaction with HRP conjugated streptavidin.
Standard of plate-washing pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie [Washing of microplate].

*③Guideline of shaking: 600-1,200rpm for 10 seconds x 3 times.

*④Put a plate cover on the plate during the reaction after shaking.

*⑤600-650 nm can be used as reference wavelength.

*⑥After removal of wash buffer, immediately dispense the next reagent.

*⑦Refer to our web movie [Handling of pipetting].

*⑧Refer to our web movie [Assay circumstance].

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	**3000 pg/ml**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
B	**1053 pg/ml**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
C	**369 pg/ml**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
D	**129 pg/ml**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
E	**45.3 pg/ml**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
F	**15.9 pg/ml**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
G	**0**	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40
H	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41

Assay worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

[Storage condition] Store the kit at 2-8°C (Do not freeze).
[Term of validity] 6 months from production (Expiration date is indicated on the container.)

This kit is manufactured by **Shibayagi Co., Ltd.**
1062-1 Ishihara, Shibukawa, Gunma, Japan
377-0007
TEL.+81-279-25-0279, FAX.+81-279-23-0313
URL:http://www.shibayagi.co.jp/
E-mail: syc-info@shibayagi.co.jp

Distributed by:
Xceltis GmbH
Pirnaer Str. 24
68309 Mannheim / Germany
Tel.: +49-(0)621-872096-0
Fax: +49-(0)621-872096-29
E-mail: info@xceltis.de