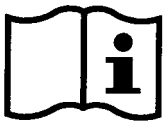
Product information



Userś Manual

Rat TNFα ELISA

Sandwich Enzyme-Linked Immunosorbent Assay for detection of Rat TNF-α

REF **IB99566**

96

Storage: 2-8°C

RUO **For research use only, not for use in diagnostic procedures.**

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1. Introduction

Tumor necrosis factor (TNF)-α is an important cytokine in the innate immune response produced by macrophages, neutrophils, fibroblasts, keratinocytes, NK cells, T and B cells, and tumour cells. TNF-α mediates host responses in acute and chronic inflammatory conditions, and is a mediator of protection from infection and malignancy. TNF-α causes apoptotic cell death, cellular proliferation, differentiation, inflammation, tumorigenesis, and viral replication. TNF-α is initially produced as a biologically active 26 kDa transmembrane protein, which is subsequently cleaved, principally by TNF-α-converting enzyme (TACE), to release the 17kDa free protein. These proteins are arranged in biologically active homotrimers that act on the ubiquitously expressed TNF-α receptors 1 and 2 (TNFR1 and TNFR2). These 17 kDa TNF protomers are composed of two antiparallel β-pleated sheets with antiparallel β-strands, forming a 'jelly roll' β-structure, typical for the TNF family. TNF-R1 is constitutively expressed in most tissues, and can be fully activated by both the membrane-bound and soluble trimeric forms of TNF, while TNF-R2 is only found in cells of the immune system and respond to the membrane-bound form of the TNF homotrimer. Although their extracellular domains share structural and functional homology, their intracellular domains are distinct. Tumor necrosis factor promotes the inflammatory response, which in turn causes many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis and refractory asthma. These disorders are sometimes treated by using TNF inhibitors such as infliximab (Remicade) or adalimumab (Humira).

2. Principles of Method

The design of this assay is based on a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to rat TNF-α. Samples are pipetted into these wells. Nonbound rat TNF-α and other components of the sample should be removed by washing, then monoclonal antibody specific to rat TNF-α added. In order to quantitatively determine the amount of rat TNF-α present in the sample, Streptavidin Horseradish Peroxidase (HRP) should be added to each microplate well. The final step, a TMB-substrate solution added to each well. Finally, a sulfuric acid solution is added and the resulting yellow colored product is measured at 450nm. Since the increases in absorbency is directly proportional to the amount of captured rat TNF-α.

3. Intended Use

The IBL-AMERICA Rat TNF-α ELISA kit is to be used for the determination of Rat TNF-α in Rat serum, Rat plasma, cell lysate, culture supernatants and buffered solution. The assay will recognize native and recombinant Rat TNF-α.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

4. Storage and Stability

All kit components of this kit are stable at 2 to 8°C. Any unused reconstituted standard should be discarded or frozen at -70°C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

5. Chemical Hazard

- Stop solution: This reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. Wear suitable protective clothing, gloves and eye protection. In the event of contact with eyes or skin, wash immediately with plenty of water.
- Standard protein and 2nd Antibody containing Sodium Azide as a preservative.

6. Kit Contents

Contents	Number	Volume
96 Well Plate	1 (in aluminum foil bag with desiccant)	
Washing Buffer	2	(20X) 25 ml
Standard Protein	1 Glass vial (lyophilized)	
Standard/Sample Dilution Buffer	1	25 ml
Secondary Antibody	1 Glass vial (lyophilized)	
Streptavidin HRP(X100)	1	150 µl
Secondary antibody/ Streptavidin HRP Dilution Buffer	1	25 ml
Substrate (TMB)	1	15 ml
Stop Solution	1	15 ml
Protocol booklet	1	
Plate sealers	2	

① 96 Well Plate
: Rat TNF-α microtiter plate, one plate of 96 wells (8 well strips x 12).
A plate using break-apart strips coated with a monoclonal antibody specific to Rat TNF-α.

② Standard Protein
: Recombinant Rat TNF-α.

③ Secondary Antibody
: Biotinylated anti Rat TNF-α antibody.

④ Streptavidin HRP
: Streptavidin Horseradish Peroxidase (HRP, enzyme)

⑤ Substrate (Stabilized chromogen)
: Tetramethylbenzidine (TMB) solution

⑥ Stop Solution
: 1N solution of sulfuric acid (H_2SO_4).

⑦ Plate sealer
: Adhesive sheet.

- Do not mix or interchange different reagents from various kit lots.

7. Materials Required But Not Provided

① Microtiter plate reader capable of measurement at or near 450nm.
② Calibrated, adjustable precision pipettes, preferably with disposable plastic tips (A manifold multi-channel pipette is desirable for large assays.)
③ Distilled or deionized water
④ Data analysis and graphing software
⑤ Vortex mixer
⑥ Polypropylene tubes for diluting and aliquoting standard
⑦ Absorbent paper towels
⑧ Calibrated beakers and graduated cylinders of various sizes

8. Reagent Preparation

1) Rat TNF-α standard

1. Reconstitute the lyophilized rat TNF-α standard by adding 1 ml of *Standard/Sample Dilution Buffer* to make the 5 ng/ml standard stock solution. Allow solution to sit at RT for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting

2. Prepare 1 ml of 800 pg/ml top standard by adding 160 µl of the above stock solution in 840 µl of *Standard/Sample Dilution Buffer*. Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay (12.5 pg/ml ~ 800 pg/ml) as below. *Standard/Sample Dilution Buffer* serves as the zero standard (0 pg/ml).

Standard	Add	Into
800 pg/ml	*160.0 µl of the std.(5 ng/ml)*	*840.0 µl of the Standard/Sample Dilution Buffer*
400 pg/ml	*500 µl of the std.(800 pg/ml)*	*500.0 µl of the Standard/Sample Dilution Buffer*
200 pg/ml	*500 µl of the std.(400 pg/ml)*	*500.0 µl of the Standard/Sample Dilution Buffer*
100 pg/ml	*500 µl of the std.(200 pg/ml)*	*500.0 µl of the Standard/Sample Dilution Buffer*
50 pg/ml	*500 µl of the std.(100 pg/ml)*	*500.0 µl of the Standard/Sample Dilution Buffer*
25 pg/ml	*500 µlof the std(50 pg/ml)*	*500.0 µl of the Standard/Sample Dilution Buffer*
12.5 pg/ml	*500µl of the std.(25 pg/ml)*	*500.0 µl of the Standard/Sample Dilution Buffer*
0 pg/ml	*1.0 ml of the Standard/Sample Dilution Buffer*	

2) Secondary Antibody

100X secondary antibody solution can be made by adding 150 µl *Secondary antibody*/*Streptavidin HRP dilution buffer* in the vial.

1. Equilibrate to room temperature, mix gently.

2. Mix 20 µl *Secondary Antibody concentrated solution* (100X) + 1.98 ml *Secondary antibody*/*Streptavidin HRP dilution buffer*. (Sufficient for one 16-well strip, prepare more if necessary)
   Label as "Working Secondary antibody Solution".
3. Return the unused *Secondary Antibody concentrated solution* to the refrigerator.

3) Streptavidin HRP(X100)

1. Equilibrate to room temperature, mix gently.
2. Mix 20 µl *Streptavidin HRP concentrated solution* (100X) + 1.98 ml *Secondary antibody*/*Streptavidin HRP dilution buffer*. (Sufficient for one 16-well strip, prepare more if needed)
   Label as "Working Streptavidin HRP Solution".
3. Return the unused *Streptavidin HRP concentrated solution* to the refrigerator.

4) Washing buffer

1. Equilibrate to room temperature, mix to re-dissolve any precipitated salt.
2. Mix 0.5 volume *Wash buffer concentrate solution* (20X) + 9.5 volumes of deionized water. Label as "Working Washing Solution".
3. Store both the concentrated and the Working Washing Solution in the refrigerator.

*** Directions for washing**

1. Fill the wells with 300 µl of "Working Washing Buffer".
   Let soak for 1 to 3 minutes and then all residual wash-liquid must be drained from the wells by aspiration (taking care not to scratch the inside of the well) or decantation, followed by forceful tapping of the plate on absorbent paper. Never insert absorbent paper directly into the wells.
   If using an automated washer, the operating instructions for washing equipment should be carefully followed.
2. Incomplete washing will adversary affects the assay and renders false results.
3. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing to avoid strips coming free of the frame.

5) Sample preparation

Blood should be collected by venipuncture. For plasma samples, blood may be drawn into tubes containing sodium citrate or heparin, EDTA. The serum or plasma should be separated from the coagulated or packed cells by centrifugation. Samples may be shipped at room temperature and then stored refrigerated at 2-8℃ if testing is to take place within one week after collection. If testing is to take place later than one week, samples should be stored at -20℃. Avoid repeated freeze/thawing.

9. Assay Procedure

- Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.
- All standards, controls and samples should be run in duplicate for confirmation of reproducibility.
- A standard curve must be run with each assay.
- If particulate matter is present in the analyte, centrifuge or filter prior to analysis.
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.

1) Determine the number of 16-well strips needed for assay. Insert these in the flame(s) for current use (Re-bag extra strips and frame. Refrigerate for further use).
2) For the standard curve, add 100 µl of the standard to the appropriate microtiter wells. Add 100 µl of the *Standard/Sample/secondary antibody Dilution Buffer* to zero wells.
3) Serum and plasma require **at least 20 fold dilution** in the *Standard/Sample Dilution Buffer.* And add 100 µl of samples to each well.
4) Cover the plate with the plate cover and incubate for 2 hours at 37℃.
5) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").
6) Pipette 100 µl of "Working Secondary Antibody Solution" into each well.
7) Cover the plate with the plate cover and incubate for 1 hour at 37℃.
8) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").
9) Add 100 µl "Working Streptavidin HRP Solution" to each well.
10) Cover the plate with the plate cover and incubate for 30 minutes at 37℃.
11) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").
12) Add 100 µl of *Substrate* to each well. The liquid in the wells should begin to turn blue.
13) Incubate the plate at room temperature.

- Do not cover the plate with aluminum foil, or color may develop.
  The incubation time for chromogen substrate is often determined by the microtiter plate reader used. O.D. values should be monitored and the substrate reaction stopped before O.D. of the positive wells exceeds the limits of the instrument. O.D. values at 450nm can only be read after the Stop Solution has been added to each well.
- Because the *Substrate* is light sensitive, avoid the remained *Substrate* solution prolonged exposure to light.

- Typically, reaction is stopped 15~20 minutes after treatment of Substrate, but this time can be adjusted as the user desires.

14) Add 100 µl of *Stop Solution* to each well. The solution in the wells should change from blue to yellow.

15) Read the absorbance of each well at 450nm. Read the plate within 20 minutes of adding the *Stop Solution*.

16) Plot on graph paper the absorbance of the standard against the standard concentration (Optimally, the background absorbance can be subtracted from all data points, including standards, unknowns and controls, prior to plotting.). Draw a smooth curve through these points to construct the standard curve.

17) Read the Rat TNF-α concentrations for the unknown samples and controls from the standard curve plotted in step 18. Multiply value(s) obtained for the unknown sample by the dilution factor (Samples producing signals greater than that of the highest standard should be further diluted in the S*tandard/Sample Dilution Buffer*).

10. Characteristics

1) Typical result

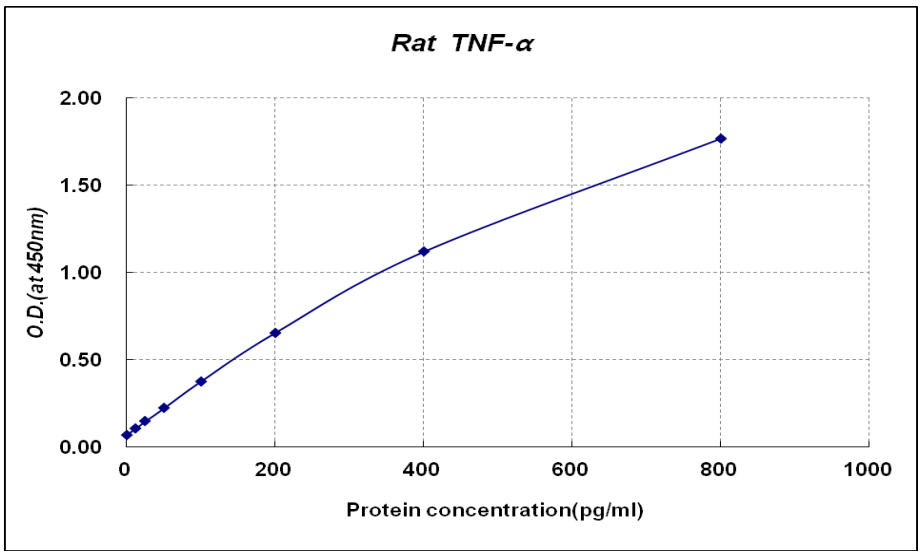
The standard curve below is for illustration only and **should not be used** to calculate results in your assay.

A standard curve must be run with each assay.

Standard Rat TNF-α (pg/ml)	Optical Density (at 450nm)
0	**0.070**
12.5	**0.107**
25	**0.147**
50	**0.222**
100	**0.376**
200	**0.654**
400	**1.119**
800	**1.767**

< Limitations >

- Do not extrapolate the standard curve beyond the 800 pg/ml standard point.
- Other buffers and matrices have not been investigated.
- The rate of degradation of native Rat TNF-α in various matrices has not been investigated.



(TMB reaction incubate at room temperature for 15~20 min)

2) Sensitivity

The minimal detectable dose of Rat TNF-α was calculated to be 3.159 pg/ml, by subtracting three standard deviations from the mean of 12 zero standard replicates (ELISA buffer, S0) and intersecting this value with the standard curve obtained in the same calculation.

N	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
ZERO	**0.071**	**0.07**	**0.069**	**0.075**	**0.077**	**0.066**	**0.067**	**0.07**	**0.069**	**0.069**	**0.073**	**0.074**

Average	**SD**	**LLD**	**LLD mean(pg/ml)**
0.07	**0.003**	**0.08**	**3.159**

3) Specificity

The following substances were tested and found to have no cross-reactivity : human TNF-α, mouse TNF-α, other cytokines.

4) Precision

① Within-Run (Intra-Assay)

(n=12)

Mean (pg/ml)	SD (pg/ml)	CV (%)
100.347	3.890	3.877
199.770	11.000	5.506
400.264	13.507	3.374
801.531	57.209	7.137

② Between-Run (Inter-Assay)

(n=12)

Mean (pg/ml)	SD (pg/ml)	CV (%)
101.089	2.507	2.480
198.171	7.392	3.730
401.396	15.309	3.814
800.534	41.468	5.180

5) Recovery

Recovery on addition is 94.103~100.7% (mean 98.293%)

Added Analyte (pg/ml)	Serum(1/20)+added analyte (450nm)	Serum(1/20)(450nm) +added analyte(450nm)	Recovery (%)
99.123	99.197	99.891	100.7
202.727	185.944	185.448	99.733
398.473	372.182	367.109	98.637
800.683	728.563	685.603	94.103

11. Troubleshooting

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase time of soaking between in wash
	• Too much Streptavidin-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order, or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents allows to come to 20~30℃ before performing assay
Too much signal – whole plate turned uniformly blue	• Insufficient washing – unbound SAV-HRP remaining	• Increase number of washes carefully
	• Too much Streptavidin -HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in presence of residual Streptavidin -HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make new standard curve
No signal when a signal is expected, but standard curve looks fine	• Sample matrix is masking detection	• More diluted sample recommended
Samples are reading too high, but standard curve is fine	• Samples contain protein levels above assay range	• Dilute samples and run again
Edge effect	• Uneven temperature around work surface	• Avoid incubating plate in areas where environmental conditions vary • Use plate sealer

12. Reference

1) Berry M, Brightling C, Pavord I, and Wardlaw A., 2007, Curr Opin Pharmacol. 7(3):279-282

2) Anderson GM, Nakada MT, and DeWitte M, 2004, Curr Opin Pharmacol. 4(4):314-320 3) Palladino MA, Bahjat FR, Theodorakis EA, and Moldawer LL., 2003, Nature Reviews Drug Discovery. 2:736-746

◈ Ordering Information

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