



Instructions for Use

Human sTNF-RII (p75/80) ELISA

Q- sTNF-RII

Enzyme Linked Immunoassay (ELISA) for the Quantification of Human Soluble TNF-RII (p75/80 kDa) from Cell Culture Supernatant, Serum, Plasma or Other Body Fluids

REF Q-TNFRII



2-8°C

*For research use only.
Not for use in diagnostic procedures*

MATRIKS BIOTEK LABORATORIES

Contents

Page

1. INTRODUCTION	3
2. PRINCIPLE OF THE ASSAY	4
3. WARNINGS AND PRECAUTIONS	4
4. STORAGE AND STABILITY OF THE KIT	4
5. SAMPLE COLLECTION AND STORAGE	5
6. MATERIALS SUPPLIED	5
7. MATERIALS REQUIRED BUT NOT SUPPLIED	6
8. PROCEDURE NOTES	6
9. PRE-TEST SETUP INSTRUCTION	6
9.1. PREPARATION OF WASH BUFFER (1X)	6
9.2. PREPARATION OF sTNF-RII STANDARDS	6
10. TEST PROCEDURE	7
11. QUALITY CONTROL	7
12. CALCULATION & INTERPRETATION OF RESULTS	8
13. ASSAY CHARACTERISTICS	8
13.1. SPECIFICITY	8
13.2. SENSITIVITY	8
13.3. PRECISION OF KIT	8
13.4. RECOVERY	8
14. REFERENCES	9

1. INTRODUCTION

Tumor necrosis factor (TNF) is a pleiotropic inflammatory cytokine and it was originally discovered in sera of animals and was found to cause hemorrhagic necrosis of some transplantable mouse and human tumors. Most organs of the body appear to be affected by TNF, and the cytokine serves a variety of functions. The TNF family consists of two forms of protein designated as TNF- α (or cachectin) and TNF- β (or lymphotoxin). Both forms share nearly 30% sequence homology and compete for binding to the same receptors. The actions of both TNFs are produced by interacting with a cell through specific high-affinity cell surface receptors.

Two distinct TNF receptors have been identified and cloned. Except for red blood cells and platelets, virtually all cell types studied show the presence of one or both of these receptor types. One receptor type is termed as TNF-RI (or p55/60 kDa) and the other is termed as TNF-RII (or p75/80 kDa). The gene for TNF-RI encodes a transmembrane protein of 426 amino acid residues (182 amino acids for the extracellular domain and 223 amino acids for the intracellular domain with a single membrane span). The gene for TNF-RII encodes a transmembrane protein of 439 amino acid residues (235 amino acids for the extracellular domain and 178 amino acids for the intracellular domain with a single membrane span). They share nearly 28 % similarities on their extracellular domain, but their intracellular parts are totally different from each other, suggesting the possibility that the two receptor types employ different signal transduction pathways.

Soluble TNF binding proteins have been identified in human serum and urine and demonstrated that they can neutralize the biological activities of TNF. Two types have been identified and designated as sTNF-RI and sTNF-RII. These soluble forms have now been shown to represent truncated forms of the two types of TNF receptors described above. The sTNF-RI and sTNF-RII apparently arise as a result of shedding of the extracellular domains of TNF-RI and TNF-RII respectively, and at a concentrations of about 1-2 ng/mL in the serum and urine of healthy subjects.

Elevated levels of sTNF-Rs have been found in serum or plasma in association with pathological conditions such as endotoxemia, meningococemia, and HIV infection and in plasma and ascites of patients in association with infections and malignancies.

The Matriks Biotek, Human sTNF-RII Immunoassay is a solid phase ELISA designed to measure human soluble TNF-RII (p75/80) in cell culture supernatants, serum, plasma, urine, or other body fluids. This assay will help to decipher the possible diagnostic and prognostic value of circulating sTNF-RII (p75/80 kDa) in various neoplastic and inflammatory diseases.

This immunoassay has been designed to accurately quantitate the recombinant as well as native human soluble TNF-RII (p75/80). Since the measurement of human sTNF-RII by the Matriks Biotek, Human sTNF-RII Immunoassay is insensitive to added TNF, it is obvious that this measurement corresponds to the total amount of the sTNF-RII present in samples, i.e., the total amount of free sTNF-RII plus the total amount of sTNF-RII bound to TNF.

2. PRINCIPLE OF THE ASSAY

The Matriks Biotek, Human sTNF-RII Enzyme-Linked Immunosorbent Assay (ELISA) kit is a solid phase assay based on the sandwich principle and designed for the quantitative measurement of human sTNF-RII in serum, plasma, cell culture supernatants and urine. Standards and samples are incubated in the wells of microtitre plate coated with a monoclonal antibody specific for human TNF-RII with the presence of a second horse radish peroxidase (HRP) conjugated anti-human TNF-RII monoclonal antibody. The capture and the HRP conjugated monoclonal antibodies bind to different epitopes on hTNF RII. Following incubation, unbound HRP conjugated anti-human TNF-RII is removed during a wash step and a substrate-chromogen solution reactive with HRP is added to the wells. The color developed is proportional to the amount of sTNF-RII in standards or samples. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. Results of samples can be determined directly using the standard curve.

3. WARNINGS AND PRECAUTIONS

3.1. For research and professional use only.

3.2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information please refer to the local distributor.

3.3. In case of severe damage of the kit package please contact Matriks Biotek Laboratories, or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.

3.4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.

3.5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.

3.6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.

3.7. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.

3.8. Avoid contact with Stop solution. It may cause skin irritations and burns.

4. STORAGE AND STABILITY OF THE KIT

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The strips of microtiter plate is stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

5. SAMPLE COLLECTION AND STORAGE

Serum, Plasma (EDTA, Heparin, Citrate), Urine and Cell culture supernatant were tested with this assay. Other biological samples might be suitable for use.

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	-20°C	Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human sTNF-RII (p75/80 kDa). If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Keep away from heat or direct sun light Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.
Stability:	1 d	6 mon	

6. MATERIALS SUPPLIED

1 x 12 x 8	MTP	Microtiter Plate Aluminium pouch with a microtiter plate pre-coated with monoclonal antibody specific for human TNF-RII (p75/80).
2 vial (5 ng/vial)	STND LYOPHILIZED	Recombinant human TNF-RII Recombinant hTNF-RII in a buffered protein base with preservatives; lyophilized.
1 x 12 mL	ASSAY BUF	Assay Buffer Blue colored. Ready to use. Contains protein and stabilizers.
1 x 12 mL	HRP CONJUG	Peroxidase Conjugate Red colored. Ready to use. Contains HRP conjugated monoclonal antibody specific for human TNF-RII (p75/80).
1 x 12 mL	TMB SUBS	TMB Substrate Solution Ready to use. Contains TMB
1 x 12 mL	TMB STOP	TMB Stop Solution Ready to use. 1N HCl.
1 x 50 mL	WASH BUF CONC	Wash Buffer, Concentrate (20x) Contains Buffer with Tween 20.
2 x1	FILM	Adhesive Film For covering of Microtiter Plate during incubation.

7. MATERIALS REQUIRED BUT NOT SUPPLIED

- 7.1. Micropipettes (Multipette Eppendorf or similar devices, < 3% CV).
- 7.2. Calibrated measures.
- 7.3. Tubes for dilution.
- 7.4. Wash bottle, automated or semi-automated microtiter plate washing system
- 7.5. Microtiter plate reader capable of reading absorbance at 450 nm.
- 7.6. Bidistilled or deionised water, paper towels, pipette tips and timer.

8. PROCEDURE NOTES

- 8.1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
- 8.2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25°C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
- 8.3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or already used reagents.
- 8.4. Use a pipetting scheme to verify an appropriate plate layout.
- 8.5. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel micropipettor for pipetting of solutions in all wells.
- 8.6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
- 8.7. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

9. PRE-TEST SETUP INSTRUCTIONS

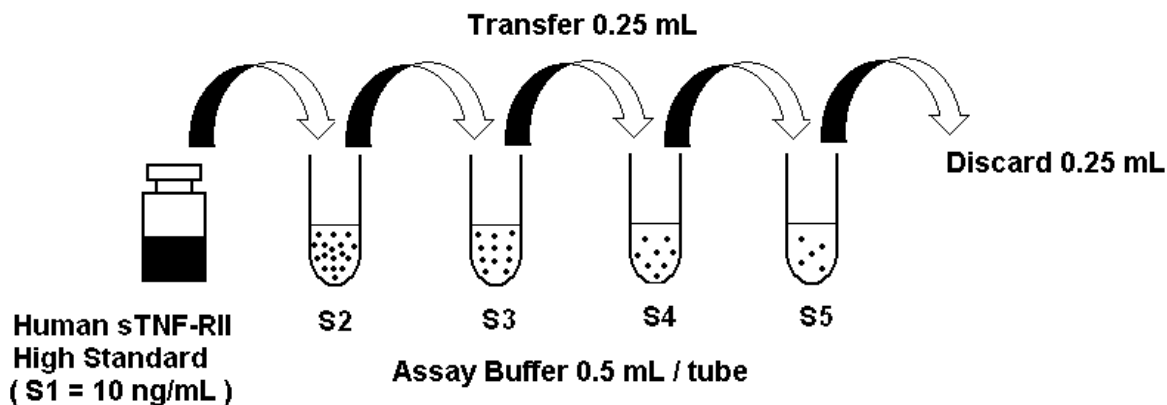
Bring all reagents to room temperature before use.

9.1. Preparation of Wash Buffer (1X): Prepare Wash Buffer before starting assay procedure. If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 50 mL of 20X Wash Buffer Concentrate into deionized or distilled water to prepare 1000 mL of Wash Buffer. Mix gently to avoid foaming. Wash Buffer could be stored at 2-8°C for 4 weeks.

9.2. Preparation of sTNF RII Standards. Reconstitute the sTNF RII Standard with 0.5 mL of Assay Buffer. This reconstitution produces the high standard solution of sTNF RII at 10 ng/mL (S1). Allow the high standard to sit for a minimum of 15 minutes with gentle agitation prior to making further dilutions. Label 4 polypropylene tubes (S2, S3, S4, S5), one for each standard point. Then prepare 1:3 serial dilutions for the Standard Curve as follows: Pipette 0.5mL of the Assay Buffer into each tube. Use 0.25 mL of the high standard solution (S1) to produce a dilution series (see the figure below); Pipette 0.25 mL of high Standard (S1, 10ng/mL) into the first tube, labelled as S2, and then mix (at this step the concentration of S2 is 3.3 ng/mL). Pipette 0.25 mL of S2 into the second tube, labelled as S3, and then mix thoroughly before next transfer. Repeat serial dilutions for creating the points of the Standard Curve. The Assay Buffer itself serves as the

zero standard (0 pg/mL). Store the unused reconstituted high standard aliquoted at -20°C. Avoid repeated freeze and thaw cycles.

Figure:Preparation of sTNF-RII Standards



10. TEST PROCEDURE

1.	Pipette 20 µL of each Standards, Assay Buffer or Samples into the respective wells of microtiter plate. <u>Wells</u> A1: Standard S1(10 ng/mL) B1: Standard S2 (3.3 ng/mL) C1: Standard S3 (1.1 ng/mL) D1: Standard S4 (0.36 ng/mL) E1: Standard S5 (0.12 ng/mL) F1: Assay Buffer (zero standard) G1 and on: Sample
2.	Pipette 100 µL of ready-to use Peroxidase Conjugate into each well.
3.	Cover the plate with adhesive film. Incubate 60 min at room temperature (18-25°C).
4.	Remove adhesive film. Discard incubation solution. Wash plate 3 times each with 300 µL of Wash Buffer (1X) . Remove excess solution by tapping the inverted plate on a paper towel.
5.	Pipette 100 µL of TMB Substrate Solution into each well.
6.	Incubate 20 min (without adhesive film) at room temperature (18-25°C) in the dark.
7.	Stop the substrate reaction by adding 100 µL of Stop Solution into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow.
8.	Measure OD with a photometer at 450 nm (reference at 620 nm is optional) within 30 min after pipetting of the Stop Solution.

11. QUALITY CONTROL

The test results are only valid only if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All standards must be found within the acceptable ranges as stated above and/or label. If the criteria are not met, the run is not valid and should be repeated. In case

of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

12. CALCULATION & INTERPRETATION OF RESULTS

12.1. Construct a Standard Curve by plotting the absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and the concentration on the horizontal (X) axis.

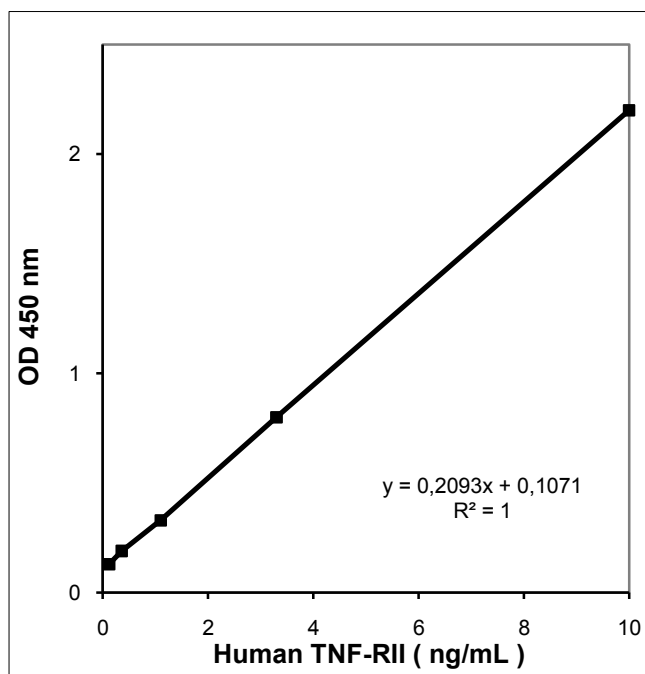
12.2. The concentration of the samples can be read directly from this standard curve. Using the absorbance value for each sample, determine the corresponding concentration sTNF-RII from the standard curve. Find the absorbance value on the Y-axis and extend a horizontal line to the curve. At the point of intersection, extend a vertical line to the X-axis and read the sTNF-RII concentration for the unknown sample.

12.3. Any sample undiluted or diluted still reading greater than the highest standard must not be extrapolated and should be further diluted appropriately with **Assay Buffer** and retested. **If the samples have been diluted, the concentration determined from the standard-curve must be multiplied by the dilution factor.**

Typical Calibration Curve

(Example. Do not use for calculation!)

Standard	Concentration (ng/mL)	Mean OD450 nm
S1	10.00	2,200
S2	3.30	0.800
S3	1.10	0.330
S4	0.36	0.190
S5	0,12	0,150
Zero Stnd	0	0,060



13. ASSAY CHARACTERISTICS

13.1. SPECIFICITY

Matriks Biotek, Human sTNF-RII ELISA recognizes both natural and recombinant human sTNF-RII (p75/80). Because of the Etanercept (Enbrel®) is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1, if present in sample, produces positive reaction in a concentration-dependent manner. Cross reaction was **not** observed with the other anti-TNF therapeutic immunoglobulins (i.e. infliximab (Remicade®) and adalimumab (Humira®)) tested at the concentrations up to 500 µg/mL. This kit exhibits no detectable cross-reactivity with human TNF-RI (p55/60), TNF-α, TNF-β, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TGF-β, M-CSF, GM-CSF, EGF tested at concentrations up to 100 ng/mL.

13.2. SENSITIVITY

The lowest detectable level that can be distinguished from the zero standard is 0.08 ng/mL.

13.3. PRECISION OF KIT

Intra-assay CV: <8% for human TNF-RII at a range of 1-10 ng/mL.

Inter-assay CV: <8% for human TNF-RII at a range of 1-10 ng/mL.

13.4. RECOVERY

Recovery rate was found to be higher than 98% when spiked using RPMI-1640 medium supplemented with 10% Fetal Calf Serum, normal human serum or urine samples with known concentrations.

14. REFERENCES

1. Sybil M. Santee and Laurie B. Owen-Schaub, Human Tumor Necrosis Factor Receptor p75/80 (CD120b), Gene Structure and Promoter Characterization, *The Journal of Biological Chemistry*, 1996; 271(35): 21151–21159.
2. Takada Y, Aggarwal BB., Evidence that genetic deletion of the TNF receptor p60 or p80 in macrophages modulates RANKL-induced signaling, *Blood*. 2004;104(13): 4113-4121.
3. Holtmann MH, Schuchmann M, Zeller G, Galle PR, Neurath MF, The emerging distinct role of TNF-receptor 2 (p80) signaling in chronic inflammatory disorders. *Arch Immunol Ther Exp (Warsz)*. 2002; 50(4): 279-88.
4. Holtmann MH, Schütz M, Galle PR, Neurath MF., Functional relevance of soluble TNF-alpha, transmembrane TNF-alpha and TNF-signal transduction in gastrointestinal diseases with special reference to inflammatory bowel diseases. *Z Gastroenterol*. 2002; 40(8): 587-600.
5. Odamaki M, Kato A, Takita T, Furuhashi M, Maruyama Y, Yonemura K, Hishida A. Role of soluble receptors for tumor necrosis factor alpha in the development of hypoalbuminemia in hemodialysis patients. *Am J Nephrol*. 2002; 22(1): 73-80.
6. Kato A, Odamaki M, Takita T, Furuhashi M, Maruyama Y, Hishida A., High blood soluble receptor p80 for tumour necrosis factor-alpha is associated with erythropoietin resistance in haemodialysis patients. *Nephrol Dial Transplant*. 2001; 16(9): 1838-44.
7. Mukhopadhyay A, Suttles J, Stout RD, Aggarwal BB. Genetic deletion of the tumor necrosis factor receptor p60 or p80 abrogates ligand-mediated activation of nuclear factor-kappa B and of mitogen-activated protein kinases in macrophages., *J Biol Chem*. 2001; 276(34): 31906-12.
8. Chan FK, Lenardo MJ., A crucial role for p80 TNF-R2 in amplifying p60 TNF-R1 apoptosis signals in T lymphocytes. *Eur J Immunol*. 2000; 30(2): 652-60.
9. Ulich TR, Yi ES, Yin S, Smith C, Remick D., Intratracheal administration of endotoxin and cytokines. VII. The soluble interleukin-1 receptor and the soluble tumor necrosis factor receptor II (p80) inhibit acute inflammation. *Clin Immunol Immunopathol*. 1994; 72(1): 137-40.
10. Odamaki M, Kato A, Takita T, Furuhashi M, Maruyama Y, Yonemura K, Hishida A., Role of soluble receptors for tumor necrosis factor alpha in the development of hypoalbuminemia in hemodialysis patients. *Am J Nephrol*. 2002; 22(1): 73-80.
11. Kato A, Odamaki M, Maruyama Y, Hishida A., Association between circulating leptin and soluble receptors for tumor necrosis factor-alpha in hemodialysis patients. *Clin Nephrol*. 2001; 56(5): 370-7.
12. Mohler KM, Torrance DS, Smith CA, Goodwin RG, Stremler KE, Fung VP, Madani H, Widmer MB., Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. *J Immunol*. 1993; 151(3): 1548-61.