



TNF-R2, Soluble (human) ELISA Kit

Manufactured by Bender MedSystems.

ALX-850-048-KI01

96 wells (~80 tests)

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For laboratory use only. Not for human or diagnostic use.

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1. INTENDED USE

The sTNF-R (80 kDa) ELISA is an enzyme-linked immunosorbent assay for quantitative detection of soluble human 80 kDa Tumor Necrosis Factor-Receptor levels in cell culture supernatants, human serum, plasma, urine, or other body fluids. **The sTNF-R (80 kDa) ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

2. SUMMARY

Tumor Necrosis Factor (TNF) was originally discovered in sera of animals and was found to cause hemorrhagic necrosis of some transplantable mouse and human tumors and to exhibit primarily cytotoxic activities against tumor but not normal cells in vitro (12,28). The TNF family consists of two proteins designated TNF- α , also called cachectin (10), and TNF- β , also called lymphotoxin (29), which are pleiotropic cytokines that can mediate a wide variety of biological effects (4).

Both TNF- α and TNF- β have been shown to interact with a cell through specific high affinity receptors with a few hundred up to more than 20,000 copies per cell (7,12,13,25,33,35,36,40). TNF-receptors have been demonstrated on a wide variety of human somatic cells including fibroblasts (23), endothelial cells, adipocytes, liver membranes (9), granulocytes and several tumor cell lines (3,11,13,39). Normal and malignant human myeloid cells as well as mitogen-stimulated lymphocytes express similar numbers of TNF receptors (400 - 1,900 per cell), whereas resting lymphoid cells have fewer, red blood cells and platelets have no detectable TNF receptors (26).

In most cases no correlation is observed between receptor number and sensitivity to TNF. Based on gel filtration experiments the receptor appears to be a complex of different proteins with a molecular weight of 350 kDa. In a variety of cell lines two different types of TNF receptors with 75 - 80 and 55 - 60 kDa respectively have been identified (11,20, 38).

The cDNAs encoding the two different TNF receptors have been cloned (19,25,34,37). The predicted amino acid sequences of the extracellular regions of the two TNF-R reveal significant similarities. The 60 kDa receptor consists of 426 amino acids with a single membrane span, an extracellular domain of 182 amino acids and an intracellular domain of 223 amino acids. The 80 kDa receptor is also a single membrane-spanning receptor of 439 amino acids with an extracellular domain of 235 amino acids and an intracellular domain of 178 amino acids. They share 28 % identity on their extracellular domain, but their intracellular parts are totally different from each other.

The present assay provides a simple, rapid and highly sensitive method for the determination of soluble TNF-R (80 kDa) levels in body fluids or cell culture supernatants. This assay will help to clarify the possible diagnostic and prognostic value of circulating sTNF-R (80 kDa) in various neoplastic and inflammatory diseases.

- **autoimmune diseases:** in patients with systemic lupus erythematosus (SLE) and progressive systemic sclerosis (PSS), plasma concentrations of both types of TNF receptors and in mixed connective tissues disease (MCTD) patients, type TNF-R (80 kDa) receptors are significantly elevated compared to controls. In rheumatoid arthritis patients, concentrations of TNF-R (60 kDa) and TNF-R (80 kDa) are significantly higher than in controls. (1,8,18)
- **hematology:** upregulation of TNF-R is found in reactive hyperplasia. Expression of TNF-R is mainly seen in high-grade malignant Non-Hodgkin's lymphoma. Concentrations of the 60 kDa type sTNF-R are significantly higher in Hodgkin's Disease patients than in healthy controls. (17)
- **HIV:** sTNF-R is upregulated following seroconversion, remains persistently high during the asymptomatic phase and becomes even more elevated in some ARC and AIDS patients. (14,21)
- **kidney:** sTNF-R is elevated in serum and urine of patients on chronic hemodialysis. This may have beneficial effects on inflammatory conditions. (30)

- **liver:** differential diagnosis of ascites: sTNF-R levels in ascites are significantly elevated in patients with malignancy-related and infected ascites compared with patients with uncomplicated hepatic ascites. (5)
- **Malaria:** the excessive release of TNF induced by malaria parasites is controlled by sTNF-R that binds and deactivates TNF. (22)
- **ovary:** elevated levels of sTNF-R inhibit the cytolytic activity of recombinant TNF given in the course of immunotherapy. (15,16,27)
- **pancreas:** human pancreatic carcinoma cells express receptors for TNF. Elevated sTNF-R levels may therefore be of prognostic value for detection of pancreatic carcinoma. (31)
- **pregnancy:** sTNF-R levels are a physiologic constituent of amniotic fluid (AF). Elevations of sTNF-R levels in AF are directly related to intrauterine infection and preterm parturition. (6,32)
- **skin:** sTNF-R is significantly elevated in patients with severe burns. (2)
- **therapy:** recombinant TNF as a cancer therapeutic can provoke release of sTNF-R. (24)

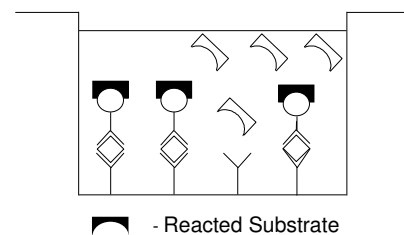
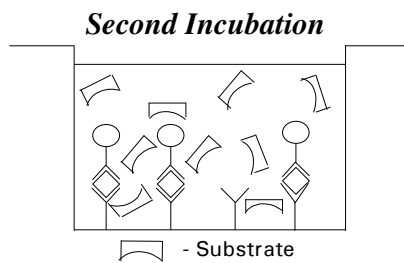
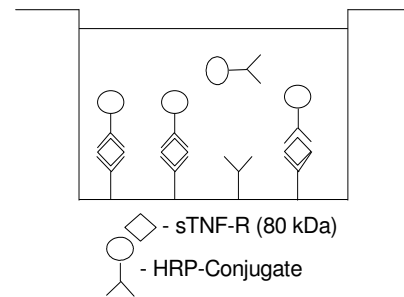
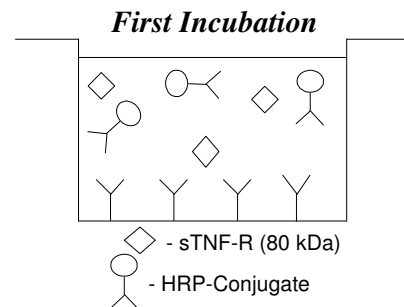
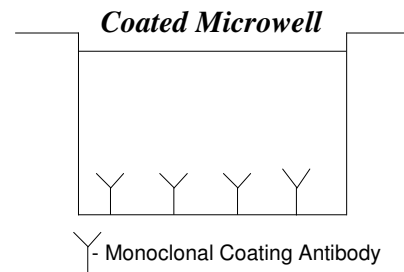
3. PRINCIPLES OF THE TEST

An anti-sTNF-R (80 kDa) monoclonal coating antibody is adsorbed onto microwells.

sTNF-R (80 kDa) present in the sample or standard binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-sTNF-R (80 kDa) antibody is added and binds to sTNF-R (80 kDa) captured by the first antibody.

Following incubation unbound enzyme conjugated anti-sTNF-R (80 kDa) is removed during a wash step and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of soluble TNF-R (80 kDa) 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 sTNF-R (80 kDa) standard dilutions and sTNF-R (80 kDa) sample concentration determined.



4. REAGENTS PROVIDED

- 1 aluminium pouch with a **Microwell Plate coated with Monoclonal Antibody** (murine) to human sTNF-R (80 kDa)
- 1 vial (0.1 ml) **HRP-Conjugate** anti-sTNF-R (80 kDa) monoclonal (murine) antibody
- 2 vials (0.5 ml) 10 ng/ml **sTNF-R (80 kDa) Standard**
- 1 vial lyophilized **Control**
- 1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)
- 1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)
- 1 vial (15 ml) **Substrate Solution**
- 1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)
- 1 vial (0.4 ml) **Blue-Dye**
- 1 vial (0.4 ml) **Green-Dye**
- 2 adhesive **Plate Covers**

Reagent Labels

5. STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C except control. Store lyophilized control at -20°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

6. SPECIMEN COLLECTION

Cell culture supernatants, human serum, EDTA, heparinized or citrate plasma, urine, or other body fluids are suitable for use in the assay. Remove serum or plasma from the clot or red cells, respectively, as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive sTNF-R (80 kDa). If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

For stability and suitability of samples refer to 13. E and F.

7. MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 μ l to 1,000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ l adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis.

8. PRECAUTIONS FOR USE

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.

- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9. PREPARATION OF REAGENTS

Except for the HRP-Conjugate (reagent C.) the reagents should be prepared before starting with the test procedure.

A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

B. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Preparation of HRP-Conjugate

The HRP-Conjugate must be diluted 1:100 with Assay Buffer (reagent B.) just prior to use in a clean plastic test tube.

Please note that the HRP-Conjugate should be used within 30 minutes after dilution. HRP-Conjugate may be prepared as needed according to the following table:

Number of Strips	HRP-Conjugate (ml)	Assay Buffer (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

D. Preparation of Control

Solubilize by adding 100µl distilled water to lyophilized **Control**. Swirl or mix gently to ensure complete and homogeneous solubilization. Further treat the control like your samples in the assay. For control range please refer to certificate of analysis or vial label. Store reconstituted control aliquoted at -20 °C. Avoid repeated freeze and thaw cycles.

E. Addition of colour-giving reagents: Blue-Dye, Green-Dye

In order to help our customers to avoid any mistakes in pipetting the Bender MedSystems ELISAs, Bender MedSystems now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet. Alternatively, the dye solutions from the stocks provided (**Blue-Dye**, **Green-Dye**) can be added to the reagents according to the following guidelines:

1. Diluent: Before sample dilution add the **Blue-Dye** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) according to the test protocol. After addition of **Blue-Dye**, proceed according to the instruction booklet.

5 ml Assay Buffer	20 µl Blue-Dye
12 ml Assay Buffer	48 µl Blue-Dye
50 ml Assay Buffer	200 µl Blue-Dye
60 ml Assay Buffer	240 µl Blue-Dye

2. HRP-Conjugate: Before dilution of the concentrated conjugate, add the **Green-Dye** at a dilution of 1:100 (see table below) to the Assay Buffer used for the final conjugate dilution. Proceed after addition of **Green-Dye** according to the instruction booklet, preparation of HRP-conjugate.

3 ml Assay Buffer	30 μ l Green-Dye
6 ml Assay Buffer	60 μ l Green-Dye
12 ml Assay Buffer	120 μ l Green-Dye

10. TEST PROTOCOL

- a. Mix all reagents thoroughly without foaming before use.
- b. 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. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with Monoclonal Antibody** (murine) to human sTNF-R (80 kDa) from holder and store in foil bag with the desiccant provided at 2°-8 °C sealed tightly.
- c. Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

- d. Add 100 µl of **Assay Buffer** in duplicate to all standard wells leaving the first wells (10 ng/ml) empty. Prepare standard dilutions by pipetting 200 µl **sTNF-R (80 kDa) Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Transfer 100 µl to well B1 and B2, respectively. Mix the contents of wells B1 and B2 by repeated aspiration and ejection and transfer 100 µl to well C1 and C2. Take care not to scratch the inner surface of the microwells. Continue this procedure four times, creating two rows of sTNF-R (80 kDa) standard dilutions ranging from 10 to 0.16 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of sTNF-R (80 kDa) standard dilutions:

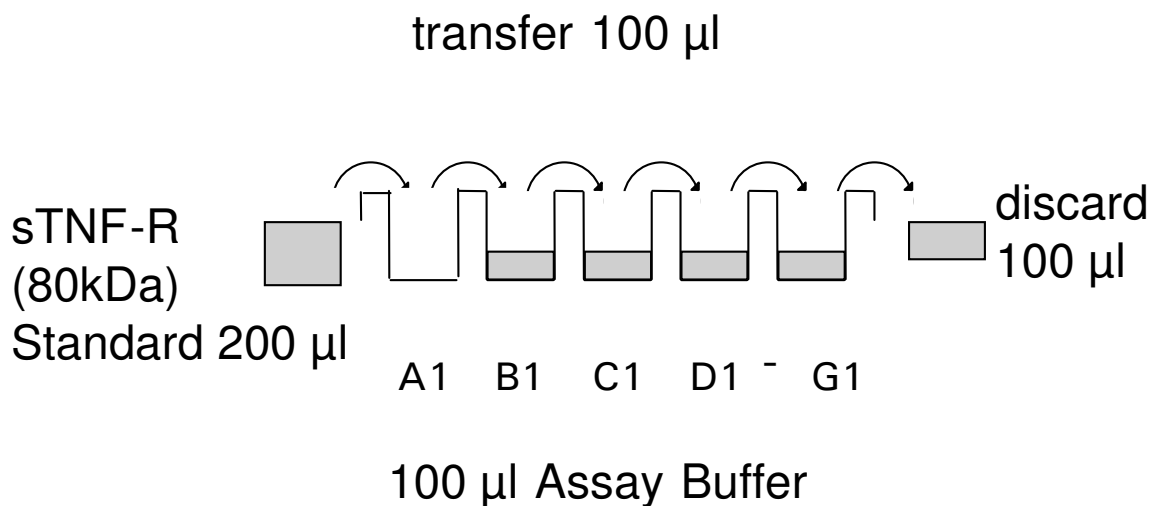


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (10 ng/ml)	Standard 1 (10 ng/ml)	Sample 1	Sample 1
B	Standard 2 (5 ng/ml)	Standard 2 (5 ng/ml)	Sample 2	Sample 2
C	Standard 3 (2.5 ng/ml)	Standard 3 (2.5 ng/ml)	Sample 3	Sample 3
D	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Sample 4	Sample 4
E	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.31 ng/ml)	Standard 6 (0.31 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.16 ng/ml)	Standard 7 (0.16 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- e. Add 100 µl of **Assay Buffer** in duplicate to the blank wells.
- f. Add 90 µl of **Assay Buffer** to all wells designated for samples.
- g. Add 10 µl of each **Sample**, in duplicate, to the designated wells and mix the contents.
- h. Prepare **HRP-Conjugate**. (Refer to preparation of reagents 9.C.)
- i. Add 50 µl of diluted (1:100) **HRP-Conjugate** to all wells, including the blank wells.
- j. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours, if available on a rotator set at 100 rpm.
- k. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- l. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.
- m. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes. Avoid direct exposure to intense light.
The colour development on the plate should be monitored and the substrate reaction stopped (see point n. of this protocol) before positive wells are no longer properly recordable.
It is recommended to add the stop solution when the highest standard has developed a dark blue colour.
Alternatively the colour development can be monitored by the ELISA reader at 620nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

- n. Stop the enzyme reaction by quickly pipetting 100 μ l of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- o. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the TNF-R (80kDa) standards.

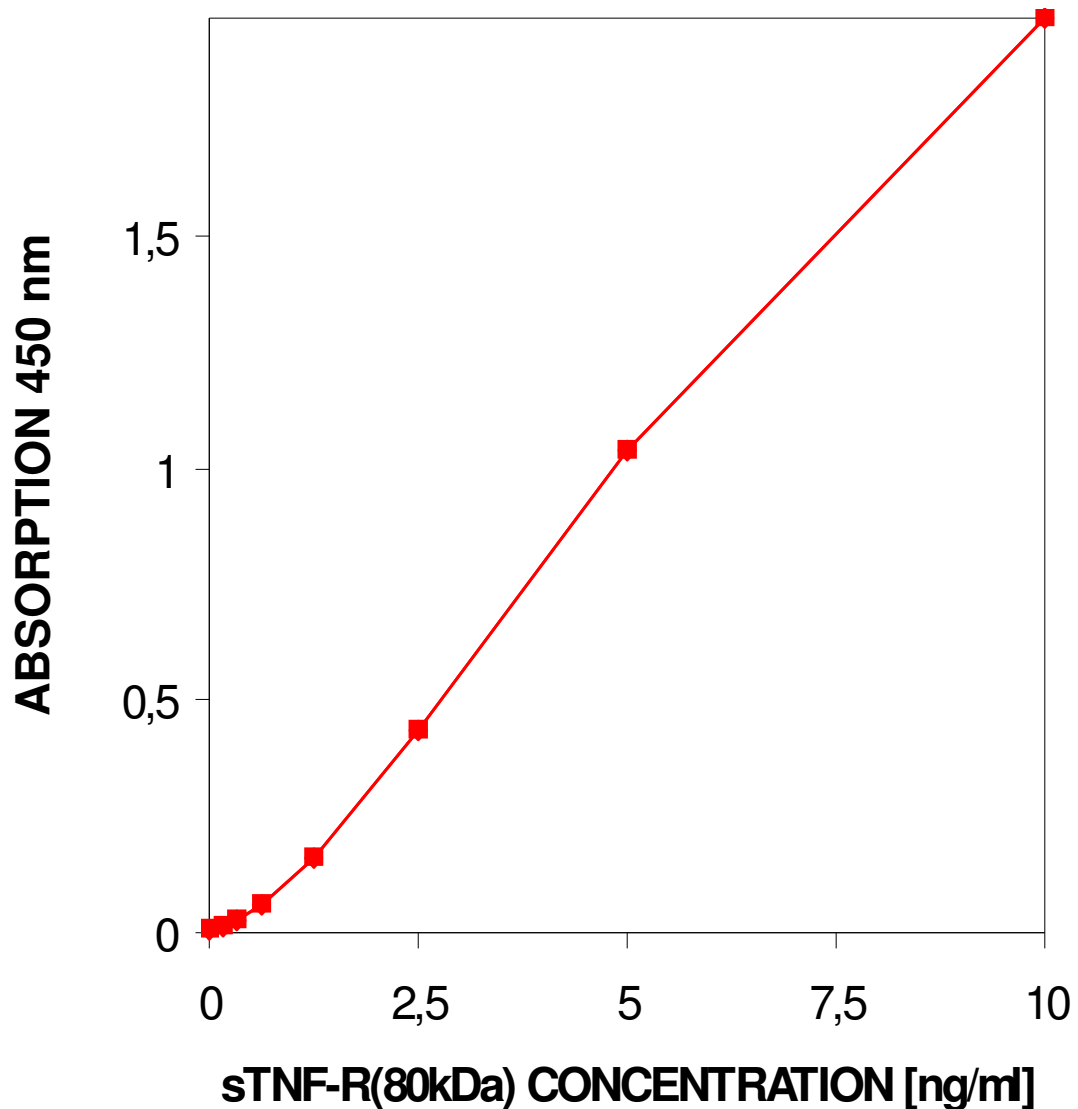
Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

11. CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the sTNF-R (80 kDa) concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating sTNF-R (80 kDa) for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding sTNF-R (80 kDa) concentration.
- **For samples which have been diluted according to the instructions given in this manual 1:10 within the microwells, the concentration read from the standard curve must be multiplied by the dilution factor (x 10).**
- **Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low sTNF-R (80 kDa) levels. Such samples require further dilution of 1:20 - 1:40 with Assay Buffer in order to precisely quantitate the actual sTNF-R (80 kDa) level.**
- It is suggested that each testing facility establishes a control sample of known sTNF-R (80 kDa) concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.
- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for sTNF-R (80 kDa) ELISA. sTNF-R (80 kDa) was diluted in serial two-fold steps in Assay Buffer, symbols represent the mean of three parallel titrations.

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



Typical data using the sTNF-R (80 kDa) ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	sTNF-R (80 kDa) Concentration (ng/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	10	1.978	1.971	0.5
	10	1.963		
2	5	1.045	1.042	0.4
	5	1.039		
3	2.5	0.443	0.435	2.6
	2.5	0.427		
4	1.25	0.162	0.160	2.2
	1.25	0.157		
5	0.63	0.055	0.058	7.3
	0.63	0.061		
6	0.32	0.025	0.026	5.4
	0.32	0.027		
7	0.16	0.015	0.014	10.1
	0.16	0.013		
Blank	0	0.008	0.008	
	0	0.007		

12. LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of immunotherapy has significantly increased the number of patients with human anti-mouse IgG antibody (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Assay Buffer.

13. PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of sTNF-R (80 kDa) defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be less than 0.099 ng/ml (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sTNF-R (80 kDa). Two standard curves were run on each plate. Data below show the mean sTNF-R (80 kDa) concentration and the coefficient of variation for each sample. The overall Intra-assay coefficient of variation has been calculated to be 1.4 %.

Positive Sample	Experiment	sTNF-R (80 kDa) Concentration (ng/ml)	Coefficient of Variation (%)
1	1	16.25	0.4
	2	15.94	0.9
	3	15.81	1.3
2	1	35.71	2.0
	2	35.79	0.5
	3	35.52	0.4
3	1	16.36	0.3
	2	16.13	0.4
	3	16.14	0.3
4	1	72.26	0.1
	2	77.74	1.7
	3	75.40	0.8
5	1	22.35	1.5
	2	21.06	2.5
	3	21.74	1.7
6	1	75.79	1.8
	2	82.35	2.1
	3	81.14	1.3
7	1	21.56	0.5
	2	21.52	0.9
	3	21.31	1.6
8	1	36.65	4.1
	2	35.79	3.6
	3	36.89	3.0

Negative Sample	O.D. Experiment	Coefficient of 450 nm	Variation (%)
1	1	0.008	17.7
	2	0.008	17.7
	3	0.006	23.6

b. Inter-Assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by three technicians. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sTNF-R (80 kDa). Two standard curves were run on each plate. Data below show the mean sTNF-R (80 kDa) concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 2.0 %.

Sample	sTNF-R (80 kDa) Concentration (ng/ml)	Coefficient of Variation (%)
1	16.0	1.4
2	35.7	0.4
3	16.2	0.8
4	75.1	3.7
5	21.7	3.0
6	79.8	4.4
7	21.5	0.7
8	36.4	1.6

C. Spike Recovery

The spike recovery was evaluated by spiking four levels of sTNF-R (80 kDa) into pooled normal human serum and 1:10 dilution. As shown below recoveries were determined in three independent experiments with 8 replicates each. The amount of endogenous sTNF-R (80 kDa) in unspiked 1:10 diluted serum was subtracted from the two spike values. Recoveries ranged from 87 to 109 % with an overall mean recovery of 94 %.

sTNF-R (80 kDa) Spike (ng/ml)	Experiment	Recovery % sTNF-R (80 kDa)
50	1	102
	2	106
	3	109
40	1	88
	2	90
	3	94
30	1	87
	2	88
	3	92
20	1	89
	2	89
	3	93

D. Dilution Parallelism

Four serum samples with different levels of sTNF-R (80 kDa) were assayed at four serial two-fold dilutions (1:10 - 1:80) with 4 replicates each. In the table below the percent recovery of expected values is listed. Recoveries ranged from 86.6 % to 108.9 % with an overall mean recovery of 93.5 %.

Sample	Dilution	sTNF-R (80 kDa) Concentration (ng/ml)		
		Expected Value	Observed Value	% Recovery of Exp. Value
1	1:10	--	15.95	--
	1:20	8.0	7.48	93.8
	1:40	4.0	3.48	87.3
	1:80	2.0	1.78	89.4
2	1:10	--	34.14	--
	1:20	17.1	15.85	92.9
	1:40	8.5	7.64	89.5
	1:80	4.3	3.93	92.2
3	1:10	--	16.24	--
	1:20	8.1	7.71	94.8
	1:40	4.1	4.12	101.1
	1:80	2.0	2.20	108.9
4	1:10	--	78.58	--
	1:20	39.3	36.29	92.4
	1:40	19.6	17.01	86.6
	1:80	9.8	9.16	93.2

E. Sample Stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -70°C and thawed up to 5 times, and sTNF-R (80 kDa) levels determined. There was no significant loss of sTNF-R (80 kDa) by repeated freezing and thawing.

b. Storage Stability

Aliquots of a serum sample (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the sTNF-R (80 kDa) level determined after 24 h. There was no significant loss of sTNF-R (80 kDa) immunoreactivity during storage under above conditions.

F. Comparison of Serum and Plasma

From two individuals, serum as well as EDTA plasma, citrate plasma, and heparin plasma obtained at the same time point were evaluated. As shown below, sTNF-R (80 kDa) concentrations were somewhat different, however, all these body fluids are suitable for the assay.

Sample	sTNF-R (80 kDa) Concentration (ng/ml)	
	Donor 1	Donor 2
Serum	3.4	5.8
EDTA Plasma	2.8	5.1
Citrate Plasma	2.5	4.1
Heparin Plasma	3.9	7.4

G. Interference with TNF

The interference of tumor necrosis factor alpha (TNF- α) and beta (TNF- β) with the assay was evaluated by spiking with different concentrations of above cytokines in the sTNF-R (80 kDa) ELISA. No interference has been observed.

H. Expected Values

A panel of 20 sera from apparently healthy blood donors (males and females) was tested for sTNF-R (80 kDa). The detected sTNF-R (80 kDa) levels ranged between 3.4 and 10.8 ng/ml with a mean level of 5.2 ng/ml and a standard deviation of $\pm 2,1$ ng/ml.

I. Specificity

The assay recognizes both natural and recombinant human sTNF-R (80 kDa). To define the specificity of this ELISA several proteins were tested for cross reactivity. Notably there was no cross reactivity observed for sTNF-R (60 kDa).

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16. REAGENT PREPARATION SUMMARY

A. Wash Buffer Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

B. Assay Buffer	Number of Strips	Assay Buffer Concentr. (ml)	Distilled Water (ml)
	1 - 6	2.5	47.5
	1 - 12	5.0	95.0

C. HRP-Conjugate	Number of Strips	HRP-Conjugate (ml)	Assay Buffer (ml)
	1 - 6	0.03	2.97
	1 - 12	0.06	5.94

17. TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Assay Buffer**, in duplicate, to standard wells except the first
- Pipette 200 µl (10 ng/ml) **sTNF-R (80 kDa) Standard** into the first wells and create standard dilutions ranging from 10 to 0.16 ng/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells
- Add 100 µl **Assay Buffer**, in duplicate, to the blank wells
- Add 90 µl **Assay Buffer** to the sample wells
- Add 10 µl **Sample**, in duplicate, to designated wells
- Prepare **HRP-Conjugate**
- Add 50 µl of diluted **HRP-Conjugate** to all wells
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C)
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 15 minutes at room temperature (18° to 25°C).
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

Note: For samples which have been diluted according to the instructions given in this manual 1:10, the concentration read from the standard curve must be multiplied by the dilution factor (x 10). Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low sTNF-R (80 kDa) levels. Such samples require further dilution of 1:20 - 1:40 with Assay Buffer in order to precisely quantitate the actual sTNF-R (80 kDa) level.