



ELISA PRODUCT INFORMATION & MANUAL

Rat TNF-alpha ELISA Kit (Colorimetric)

NBP1-92681

Enzyme-linked Immunosorbent Assay for quantitative
detection. For research use only.

Not for diagnostic or therapeutic procedures.

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Novus kits are guaranteed for 6 months from date of receipt

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1 Intended Use

The rat TNF alpha ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of rat TNF alpha. **The rat TNF alpha ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2 Summary

TNF alpha is a multifunctional cytokine involved in many different pathways, in homeostasis and pathophysiology of mammals. It can show opposing biological effects suggesting complex regulatory mechanisms.

TNF alpha, also known as cachectin, was first detected as a cytotoxic factor inducing lysis of certain tumor cells. The TNF alpha gene is member 2 of the TNF-superfamily (consisting of at least 20 distinct members).

TNF alpha release is mainly triggered by viral infections and endotoxins, lipopolysaccharides or other bacterial components, by tissue injury, DNA-damage and by IL-1, PDGF and TNF alpha itself. It is primarily expressed in macrophages, but also in monocytes, neutrophils, NK-cells, mast-cells, endothelial cells and activated lymphocytes. TNF alpha expression in endothelial cells and fibroblasts can be induced by IL-17. The expression of other cytokines, chemokines, reactive oxygen intermediates, nitric oxide and prostaglandins is stimulated by TNF alpha.

The initially membrane bound TNF alpha is enzymatically cleaved by TACE (= ADAM17). The soluble monomers aggregate to homotrimers and are secreted into blood and other biological fluids. The membrane bound and the soluble form are biologically active and bind to the TNF-receptors TNFR1 (= TNFRSF1A, p55-60) and TNFR2 (= TNFRSF1B, TNFBR2, p75-80).

Upon ligand binding, receptors form trimers leading to conformational changes, protein dissociation (SODD = silencer of death domains, BAG4, Bcl2-associated athanogene 4) and association (TRADD = TNF-R1 associated death domain protein) and yielding the following biological activities:

- transcription of anti-apoptotic factors and proteins involved in cell proliferation and inflammation via binding of TRAF2 (TNF-R associated

factor 2) and RIPK1 (TNF-R interacting serine-threonine kinase 1) and activation of the transcription factor NF kappa B.

- cell proliferation, differentiation but also apoptosis via TRAF2 binding, kinases activation, activation of c-Jun and ATF2 (JNK-MAPK-pathway).

- apoptosis via the binding of FADD (Fas associating protein with death domain) to TRADD and activation of caspases (including caspase 8 = FLICE).

- necrosis, a caspase independent cell death, mediated by NADPH oxidases, which form a complex with TRADD and RIPK1, leading to the generation of oxygen species.

TNF-R2 contains no DD (death domain), but exhibits its function via direct TRAF binding.

Thus the multiple biological functions of TNF alpha comprise cellular proliferation and differentiation, tumorigenesis, apoptotic or necrotic cell death (including certain tumor cell lines), immunoregulatory activities, lipid metabolism, coagulation and endothelial function. It promotes local or systemic inflammation (TNF alpha is a potent pyrogen) and stimulates the acute phase response. Very high expressions of TNF alpha after infection can lead to septic shock (TNF alpha is highly cytotoxic.), whereas sustained low levels induce cachexia and inflammation. Dysregulation of TNF alpha is involved in many diseases.

3 Principles of the Test

An anti-rat TNF alpha coating antibody is adsorbed onto microwells.

Rat TNF alpha present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-rat TNF alpha antibody is added and binds to rat TNF alpha captured by the first antibody.

Following incubation unbound biotin-conjugated anti-rat TNF alpha antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-rat TNF alpha antibody.

Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 1

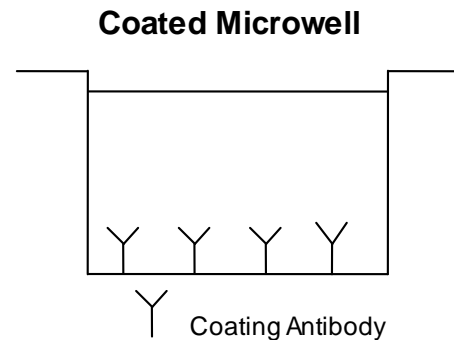


Figure 2

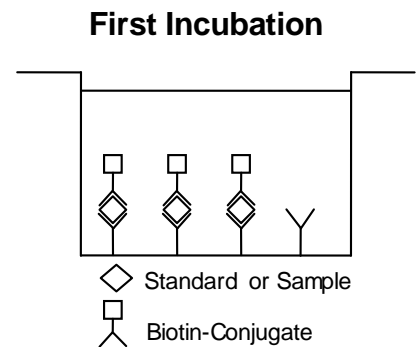


Figure 3

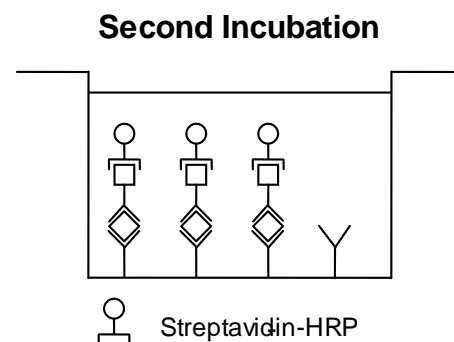
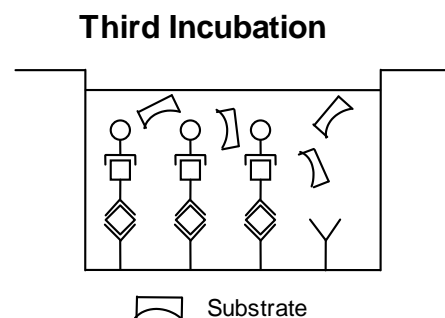
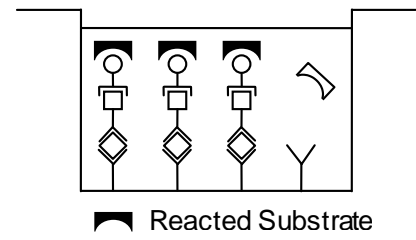


Figure 4



A coloured product is formed in proportion to the amount of rat TNF alpha present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 rat TNF alpha standard dilutions and rat TNF alpha sample concentration determined.

Figure 5



4 Reagents Provided

4.1 Reagents for rat TNF alpha ELISA NBP1-92681 (96 tests)

- 1 aluminium pouch with a **Microwell Plate coated** with polyclonal antibody to rat TNF alpha
- 1 vial (70 µl) **Biotin-Conjugate** anti-rat TNF alpha polyclonal antibody
- 1 vial (150 µl) **Streptavidin-HRP**
- 2 vials rat TNF alpha **Standard** lyophilized, 5 ng/ml upon reconstitution
- 1 vial (12 ml) **Sample Diluent**

Please note: *In some, very rare cases, an insoluble precipitate has been seen in the Sample Diluent vial. This precipitate does not interfere in any way with the performance of the test and can thus be ignored.*

- 1 vial (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20, 10% BSA)
- 1 bottle (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
- 4 **Adhesive Films**

4.2 Reagents for rat TNF alpha ELISA NBP1-92681 (2x96 tests)

- 2 aluminium pouches with a **Microwell Plate coated** with polyclonal antibody to rat TNF alpha
- 2 vials (70 µl) **Biotin-Conjugate** anti-rat TNF alpha polyclonal antibody
- 2 vials (150 µl) **Streptavidin-HRP**
- 4 vials rat TNF alpha **Standard** lyophilized, 5 ng/ml upon reconstitution
- 2 vials (12 ml) **Sample Diluent**

Please note: *In some, very rare cases, an insoluble precipitate has been seen in the Sample Diluent vial. This precipitate does not interfere in any way with the performance of the test and can thus be ignored.*

- 1 vial (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20, 10% BSA)
- 1 bottle (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
- 2 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 2 vials (15 ml) **Stop Solution** (1M Phosphoric acid)
- 8 **Adhesive Films**

4.3 Reagents for rat TNF alpha ELISA NBP1-92681 (10x96 tests)

- 10 aluminium pouches with a **Microwell Plate coated** with polyclonal antibody to rat TNF alpha
 - 10 vials (70 µl) **Biotin-Conjugate** anti-rat TNF alpha polyclonal antibody
 - 10 vials (150 µl) **Streptavidin-HRP**
 - 10 vials rat TNF alpha **Standard** lyophilized, 5 ng/ml upon reconstitution
 - 7 vials (12 ml) **Sample Diluent**
- Please note:** *In some, very rare cases, an insoluble precipitate has been seen in the Sample Diluent vial. This precipitate does not interfere in any way with the performance of the test and can thus be ignored.*
- 2 vials (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20, 10% BSA)
 - 5 bottles (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
 - 10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)
 - 1 vial (100 ml) **Stop Solution** (1M Phosphoric acid)
 - 20 **Adhesive Films**

5 Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°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.

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, this reagent is not contaminated by the first handling.

6 Specimen Collection and Storage Instructions

Cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Pay attention to a possible “**Hook Effect**” due to high sample concentrations (see chapter 11).

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

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive rat TNF alpha. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 μ l to 1000 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)
- Microwell strip 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 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 statement(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 reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water 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 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

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.

If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate (20x)** into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water.

Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate (20x)** into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

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

9.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.5 Rat TNF alpha Standard

Reconstitute **rat TNF alpha standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 5 ng/ml). Allow the reconstituted standard to sit for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded.

Standard dilutions can be prepared directly on the microwell plate (see 10.c) or alternatively in tubes (see 9.5.1).

9.5.1 External Standard Dilution

Label 7 tubes, one for each standard point.

S1, S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 225 µl of Sample Diluent into each tube.

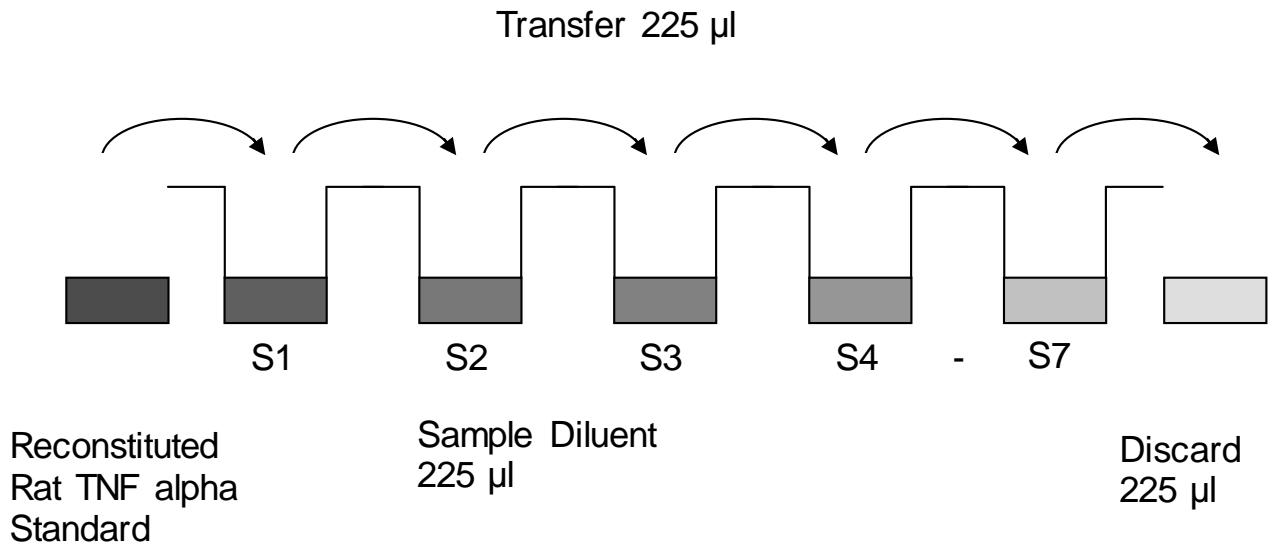
Pipette 225 µl of reconstituted standard (concentration = 5 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 2.5 ng/ml).

Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.

Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 6).

Sample Diluent serves as blank.

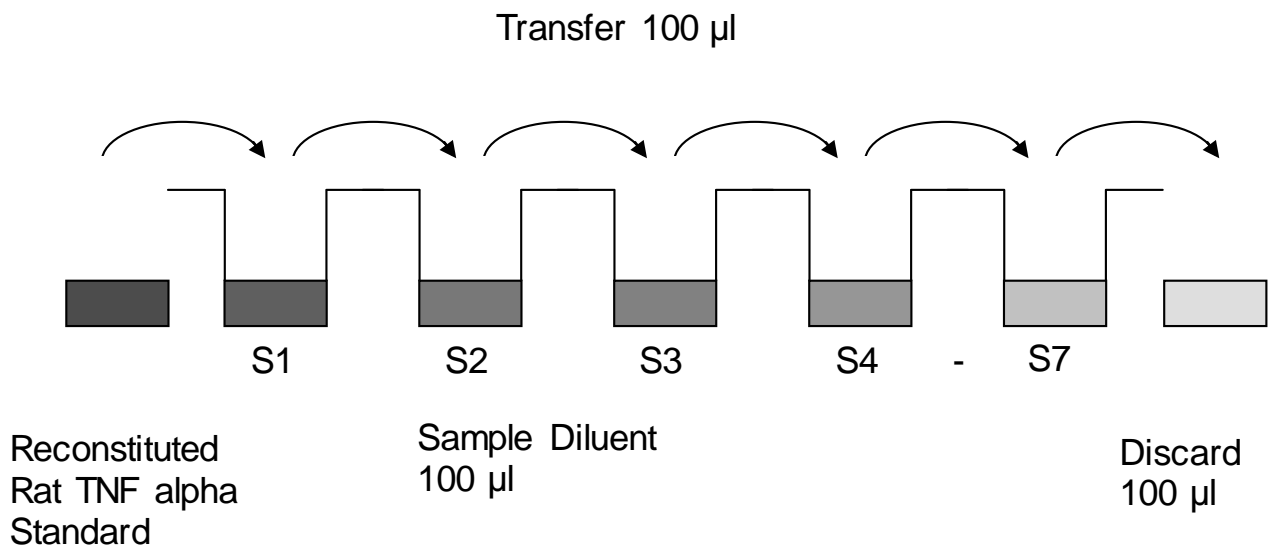
Figure 6



10 Test Protocol

- a. 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 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- b. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**
- c. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.5.1):
Add 100 µl of Sample Diluent in duplicate to all **standard wells**.
Pipette 100 µl of prepared **standard** (see Preparation of Standard 9.5, concentration = 5000.0 pg/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 2500.0 pg/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of rat TNF alpha standard dilutions ranging from 2500.0 to 39.1 . pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 7



In case of an **external standard dilution** (see 9.5.1), pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (2500.0 pg/ml)	Standard 1 (2500.0 pg/ml)	Sample 1	Sample 1
B	Standard 2 (1250.0 pg/ml)	Standard 2 (1250.0 pg/ml)	Sample 2	Sample 2
C	Standard 3 (625.0 pg/ml)	Standard 3 (625.0 pg/ml)	Sample 3	Sample 3
D	Standard 4 (312.5 pg/ml)	Standard 4 (312.5 pg/ml)	Sample 4	Sample 4
E	Standard 5 (156.3 pg/ml)	Standard 5 (156.3 pg/ml)	Sample 5	Sample 5
F	Standard 6 (78.1 pg/ml)	Standard 6 (78.1 pg/ml)	Sample 6	Sample 6
G	Standard 7 (39.1 pg/ml)	Standard 7 (39.1 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- d. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
- e. Add 50 µl of **Sample Diluent** to the **sample wells**.
- f. Add 50 µl of each **sample** in duplicate to the **sample wells**.
- g. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).
- h. Add 50 µl of **Biotin-Conjugate** to all wells.
- i. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 400 rpm.
- j. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.4).
- k. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point b. of the test protocol. Proceed immediately to the next step.
- l. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- m. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 400 rpm.
- n. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point b. of the test protocol. Proceed immediately to the next step.
- o. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- p. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

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 620 nm. The

substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

- q. Stop the enzyme reaction by quickly pipetting 100 μ l of **Stop Solution** into each well. 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.
- r. 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 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 value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the rat TNF alpha concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating rat TNF alpha 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 rat TNF alpha concentration.
- **If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**
- **Calculation of samples with a concentration exceeding standard 1 will result in incorrect, low rat TNF alpha levels (Hook Effect). Such samples require further external predilution according to expected rat TNF alpha values with Sample Diluent in order to precisely quantitate the actual rat TNF alpha level.**
- It is suggested that each testing facility establishes a control sample of known rat TNF alpha concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 8

Representative standard curve for rat TNF alpha ELISA. Rat TNF alpha was diluted in serial 2-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.

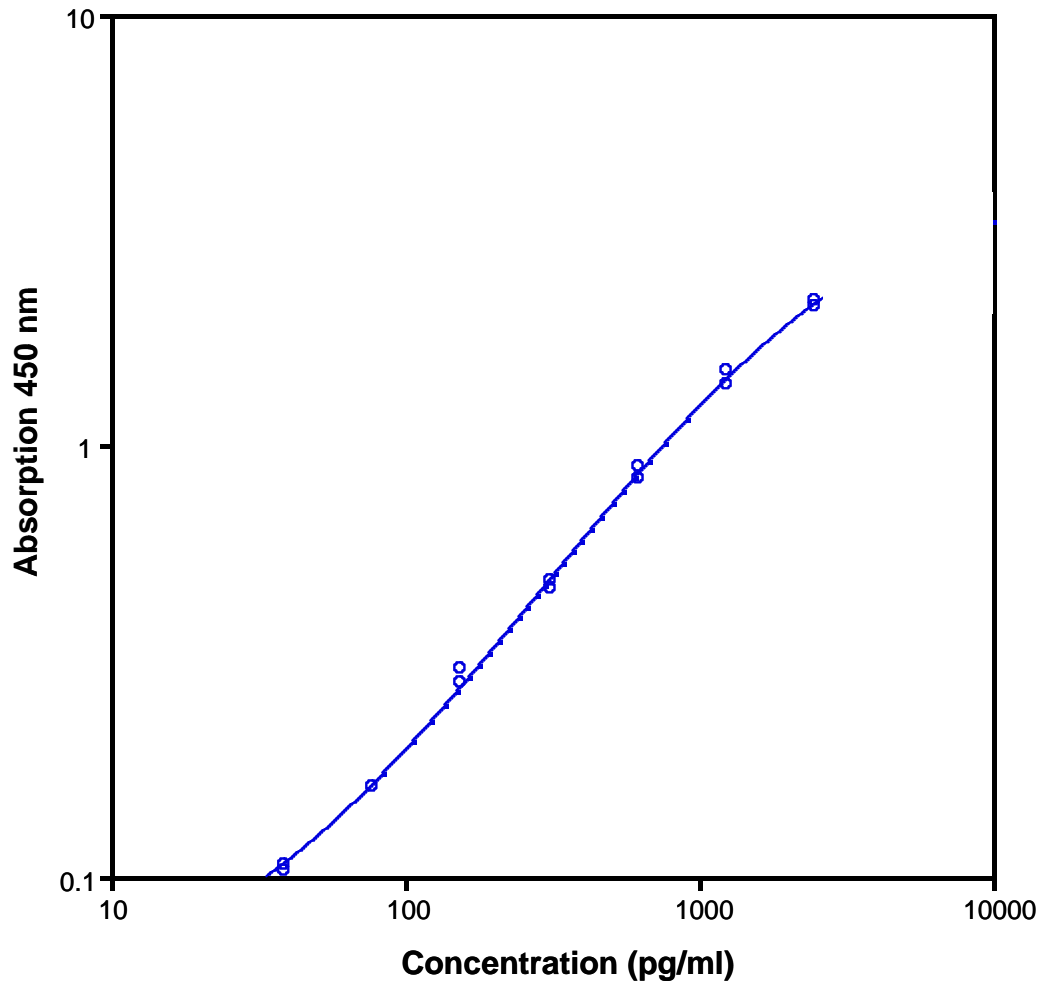


Table 2

Typical data using the rat TNF alpha ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Rat TNF alpha Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	2500.0	2.179 2.104	2.142	1.8
2	1250.0	1.486 1.382	1.434	3.6
3	625.0	0.841 0.892	0.867	2.9
4	312.5	0.468 0.488	0.478	2.1
5	156.3	0.282 0.304	0.293	3.8
6	78.1	0.161 0.161	0.161	0.0
7	39.1	0.107 0.103	0.105	1.9
Blank	0	0.031 0.029	0.030	

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

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. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

13 Performance Characteristics

13.1 Sensitivity

The limit of detection of rat TNF alpha defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 11 pg/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of rat TNF alpha. 2 standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was < 5%.

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of rat TNF alpha. 2 standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was < 10%.

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of rat TNF alpha into a pooled normal rat serum sample. Recoveries were determined in 2 independent experiments with 4 replicates each.

The unspiked serum was used as blank in these experiments.

The overall mean recovery was 109%.

13.4 Dilution Parallelism

Serum samples with different levels of rat TNF alpha were analysed at serial 2 fold dilutions with 4 replicates each. The overall mean recovery was 114%.

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of spiked serum samples were stored at -20°C and thawed 5 times, and the rat TNF alpha levels determined. There was no significant loss of rat TNF alpha immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of spiked serum samples were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the rat TNF alpha level determined after 24 h. There was no significant loss of rat TNF alpha immunoreactivity detected during storage at -20°C, 2-8°C and RT. A significant loss of rat TNF alpha immunoreactivity (50%) was detected during storage at 37°C after 24 h.

13.6 Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a rat TNF alpha positive serum. There was no crossreactivity detected.

13.7 Expected Values

There were no detectable rat TNF alpha levels found in serum samples from healthy rats. Elevated rat TNF alpha levels depend on the type of immunological disorder.

14 Reagent Preparation Summary

14.1 Wash Buffer (1x)

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

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

14.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate 20x** (5 ml) to 95 ml distilled water.

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

14.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Assay Buffer (1x):

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

14.4 Streptavidin-HRP

Make a 1:100 dilution of **Streptavidin-HRP** in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

14.5 Rat TNF alpha Standard

Reconstitute lyophilized **rat TNF alpha standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

15 Test Protocol Summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
Alternatively external standard dilution in tubes (see 9.5.1): Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
5. Add 50 µl Sample Diluent to sample wells.
6. Add 50 µl sample in duplicate, to designated sample wells.
7. Prepare Biotin-Conjugate.
8. Add 50 µl Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C).
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 4 times with Wash Buffer.
12. Add 100 µl diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
14. Empty and wash microwell strips 4 times with Wash Buffer.
15. Add 100 µl of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
17. Add 100 µl Stop Solution to all wells.
18. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 2).