BioVendor Group

ENG

Product Data Sheet:

MOUSE TNF-ALPHA ELISA

Catalogue number:

RAF129R

For research use only!

BioVendor R&D

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CONTENTS

1. INTENDED USE

The mouse TNF-α ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of mouse TNF-α. Cell culture supernatant, serum, and plasma (EDTA, citrate) have been tested with this assay.

2. SUMMARY

TNF-α 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.

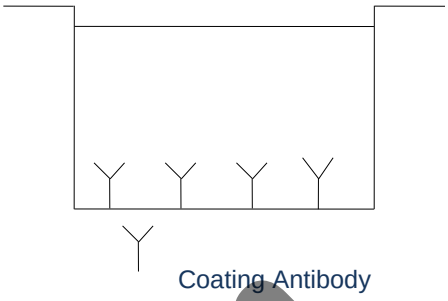
TNF-α, also known as cachectin, was first detected as a cytotoxic factor inducing lysis of certain tumor cells. The TNF-α gene is member 2 of the TNF-superfamily (consisting of at least 20 distinct members).

3. PRINCIPLES OF THE TEST

An anti-mouse TNF-α coating antibody is adsorbed onto microwells.

Coated Microwell

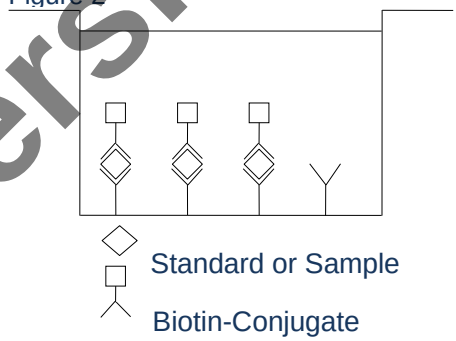
Figure 1



Mouse TNF-α present in the sample or standard binds to antibodies adsorbed to the microwells and a biotin-conjugated anti-mouse TNF-α antibody is added and binds to mouse TNF-α captured by the first antibody.

First Incubation

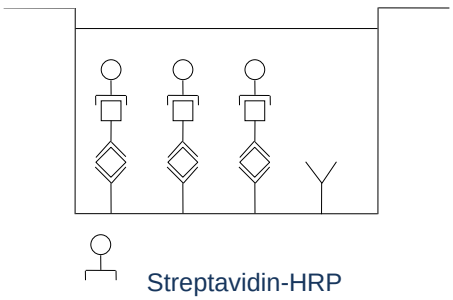
Figure 2



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

Second Incubation

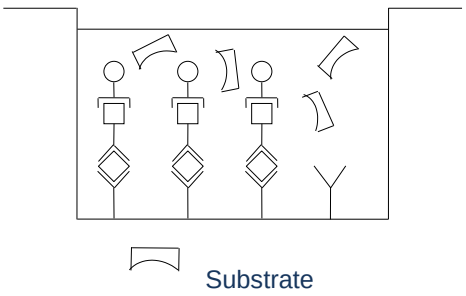
Figure 3



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

Third Incubation

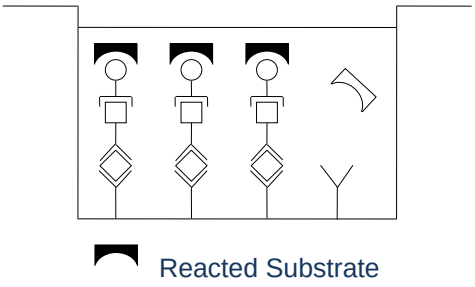
Figure 4



A coloured product is formed in proportion to the amount of mouse TNF-α 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 mouse TNF-α standard dilutions and mouse TNF-α sample concentration determined.

Stop Reaction

Figure 5



4. REAGENTS PROVIDED

1 aluminium pouch with **a Antibody Coated Microtiter Strips** with monoclonal antibody to mouse TNF-α

1 vial (70 µl) **Biotin-Conjugate** anti-mouse TNF-α polyclonal antibody
1 vial (150 µl) **Streptavidin-HRP**
2 vials mouse TNF-α **Standard** lyophilized, 2 ng/ml upon reconstitution
1 vial (5 ml) **Calibrator Diluent**
1 vial (12 ml) **Sample Diluent**
1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 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**

5. STORAGE INSTRUCTIONS – ELISA KIT

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C 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, serum and plasma (EDTA, citrate) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells 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.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive mouse TNF-α. If samples are to be run within 24 hours, they may be stored at 2°C to 8°C.

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 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl 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 samples.
- 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.
- To avoid microbial contamination or cross-contamination of reagents or samples 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°C 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°C 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

Note: The Biotin-Conjugate should be used within 30 minutes after dilution.

For serum and plasma samples, 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

For cell culture supernatant samples, make a 1:200 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	0.015	2.985
1 - 12	0.03	5.97

9.4 Streptavidin-HRP

Note: 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 Mouse TNF-α Standard

Reconstitute mouse TNF-α standard by addition of Calibrator Diluent (for subsequent measurement of serum or plasma samples) or Sample Diluent (for subsequent measurement of cell culture supernatant samples).

Reconstitution volume is stated in the Quality Control Sheet. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 2 ng/ml). Allow standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

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

9.5.1 External Standard Dilution

Label 6 tubes, one for each standard point.

S2, S3, S4, S5, S6, S7.

The reconstituted Standard serves as S1.

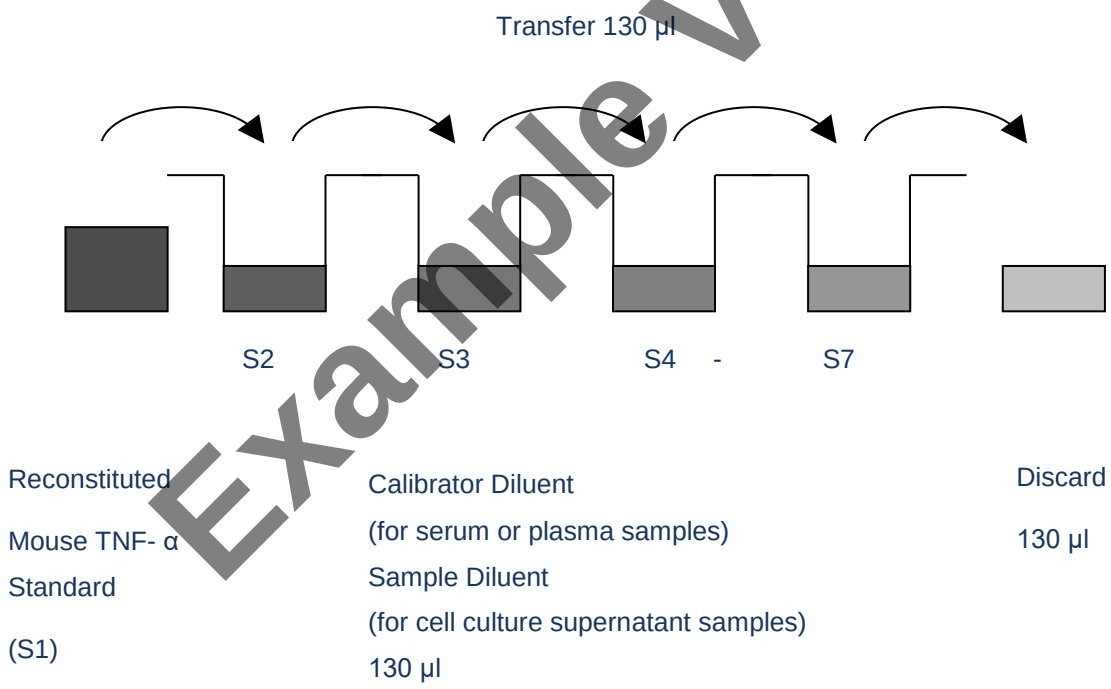
Then prepare 1:2 serial dilutions for the standard curve as follows:

Pipette 130 µl of Calibrator Diluent (for subsequent measurement of serum or plasma samples) or Sample Diluent (for subsequent measurement of cell culture supernatant samples) into each tube.

Pipette 130 µl of reconstituted standard (S1= 2 ng/ml) into the tube, labelled S2, and mix (concentration of standard S2 = 1 ng/ml). Pipette 130 µl if this dilution into the second tube, labelled S3, and mix thoroughly before the next transfer. Repeat serial dilutions 4 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. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).
c. 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**.
d. Add 50 µl **Sample Diluent** in duplicate to **all wells**.
e. Add 50 µl of external diluted **Standards** (refer to 9.5.1) in duplicate to the corresponding **standard wells** (see Table 1).
f. Add 50 µl of each sample in duplicate to the sample wells (see Table 1).
g. Add 50 µl of **Calibrator Diluent** (for serum or plasma samples) or **Sample Diluent** (for cell culture supernatant samples) in duplicate to the **blank wells.**
h. Add 50 µl of diluted **Biotin-Conjugate** to all wells, including the blank wells.

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 (2000.0 pg/ml)	Standard 1 (2000.0 pg/ml)	Sample 1	Sample 1
B	Standard 2 (1000.0 pg/ml)	Standard 2 (1000.0 pg/ml)	Sample 2	Sample 2
C	Standard 3 (500.0 pg/ml)	Standard 3 (500.0 pg/ml)	Sample 3	Sample 3
D	Standard 4 (250.0 pg/ml)	Standard 4 (250.0 pg/ml)	Sample 4	Sample 4
E	Standard 5 (125.0 pg/ml)	Standard 5 (125.0 pg/ml)	Sample 5	Sample 5
F	Standard 6 (62.5 pg/ml)	Standard 6 (62.5 pg/ml)	Sample 6	Sample 6
G	Standard 7 (31.3 pg/ml)	Standard 7 (31.3 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

i. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, on a microplate shaker
j. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP).
k. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol.
l. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blanks.
m. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, on a microplate shaker.

n. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol.
o. Pipette 100 µl of **TMB Substrate Solution** to all wells.
p. Incubate the microwell strips at room temperature (18°C to 25°C) for about 30 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 µl 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°C - 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: Shaking is absolutely necessary for an optimal test performance.

Note: If instructions in this protocol have been followed samples have not been diluted, the concentration read from the standard curve must not be multiplied by a dilution factor.

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 mouse TNF-α 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 mouse TNF-α 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 mouse TNF-α concentration.
– **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low mouse TNF-α levels (Hook Effect). Such samples require further external predilution according to expected mouse TNF-α values with Sample Diluent in order to precisely quantitate the actual mouse TNF-α level.**
– It is suggested that each testing facility establishes a control sample of known mouse TNF-α 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 7. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 7

Representative standard curve for mouse TNF-α ELISA. Mouse TNF-α was diluted in serial 2-fold steps in Sample Diluent.

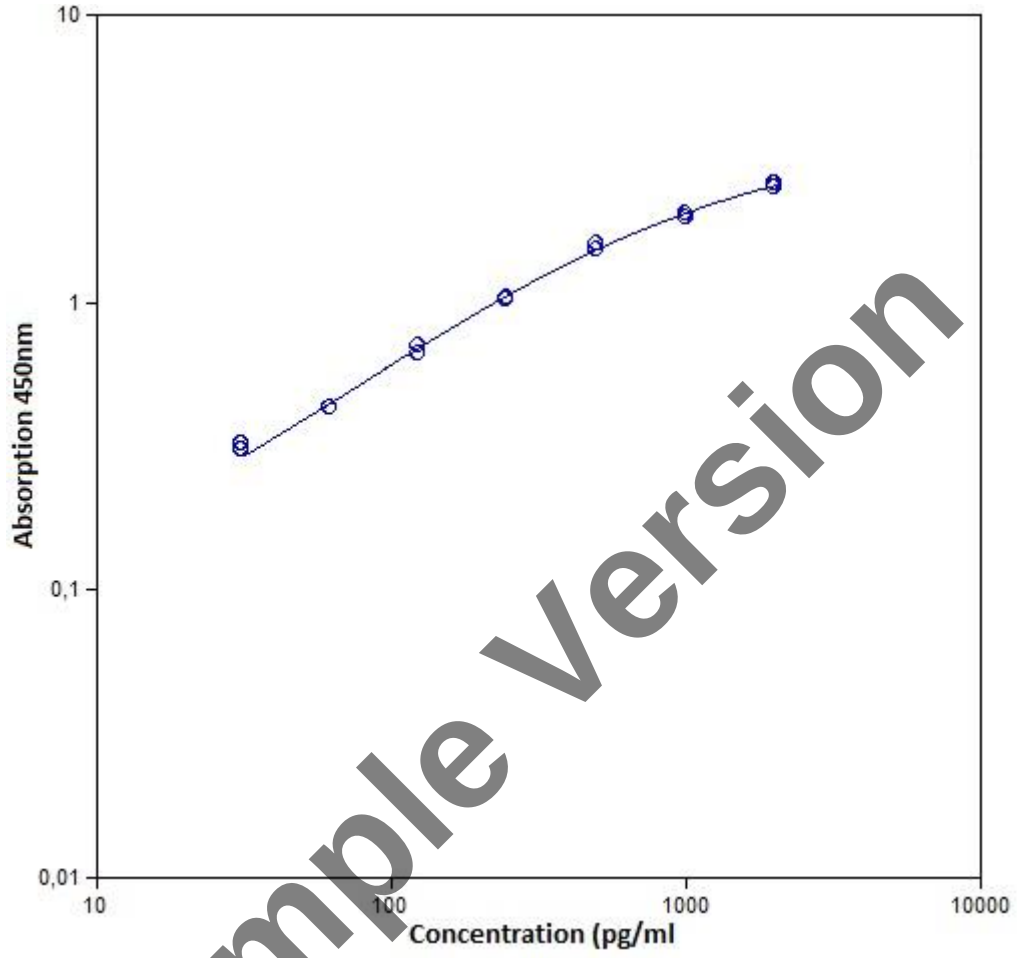


Table 2

Typical data using the mouse TNF-α ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Mouse TNF-α Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	2000.0	2.492	2.526	1.4%
		2.560		
2	1000.0	1.998	1.979	1.0%
		1.960		
3	500.0	1.521	1.551	1.9%
		1.580		
4	250.0	1.018	1.024	0.6%
		1.031		
5	125.0	0.657	0.676	2.8%
		0.695		
6	62.2	0.424	0.426	0.3%
		0.427		
7	31.3	0.320	0.313	2.2%
		0.306		
Blank	0	0.071	0.073	2.4%
		0.074		

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 mouse TNF-α 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 3.7 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 7 serum samples containing different concentrations of mouse TNF-α. 2 standard curves were run on each plate. Data below show the mean mouse TNF-α concentration and the coefficient of variation for each sample. The calculated overall intra-assay coefficient of variation was 6.5%.

Table 3

The mean mouse TNF-α concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Mouse TNF-α Concentration (pg/ml)	Coefficient of Variation (%)
1	1	2240	4.9%
	2	1950	3.4%
	3	2001	4.6%
2	1	635	5.6%
	2	586	8.0%
	3	558	5.4%
3	1	256	7.0%
	2	252	9.3%
	3	227	7.0%
4	1	1503	5.2%
	2	1439	6.9%
	3	1465	5.5%
5	1	768	4.9%
	2	774	9.7%
	3	775	4.4%
6	1	581	5.2%
	2	512	8.0%
	3	535	6.0%
7	1	363	6.8%
	2	310	7.6%
	3	300	8.7%

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 7 serum samples containing different concentrations of mouse TNF-α. 2 standard curves were run on each plate. Data below show the mean mouse TNF-α concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall inter-assay coefficient of variation was 5.7%.

Table 4

The mean mouse TNF-α concentration and the coefficient of variation of each sample

Sample	Mean Mouse TNF-α Concentration (pg/ml)	Coefficient of Variation (%)
1	2064	7.5%
2	593	6.6%
3	242	6.3%
4	1469	2.2%
5	773	0.5%
6	538	6.5%
7	326	10.3%

13.3 Spiking Recovery

The spike recovery was evaluated by spiking 3 levels of mouse TNF-α into serum. Recoveries were determined in 3 independent experiments with 4 replicates each. Recoveries were determined with 4 replicates each.

The amount of endogenous mouse TNF-α in unspiked samples was subtracted from the spike values.

Table 5

Sample matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	113	106	85
Plasma (citrate)	120	109	100
Plasma (EDTA)	90	104	72
Cell culture supernatant	105	93	100

13.4 Dilution Linearity

Serum, plasma and cell culture supernatant samples with different levels of mouse TNF-α were analysed at serial 2 fold dilutions with 4 replicates each.

Table 6

Sample matrix		Recovery of Exp. Val.	
	Dilution	Mean (%)	Range (%)
Serum	1:2	91	86 – 100
	1:4	89	82 – 102
	1:8	99	77 – 119
Plasma (EDTA)	1:2	82	72 – 92
	1:4	103	98 – 109
	1:8	94	87 – 101
Plasma (citrate)	1:2	99	-
	1:4	90	-
	1:8	113	-
Cell culture supernatant	1:2	96	94 – 97
	1:4	82	78 – 86
	1:8	93	71 - 116

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

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

13.6 Storage Stability

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

13.7 Specificity

The assay detects both natural and recombinant mouse TNF-α.

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into mouse IL-10 positive serum.

There was no crossreactivity detected.

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

For serum / plasma samples: 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

For cell culture supernatant samples: Make a 1:200 dilution of **Biotin-Conjugate** in Assay Buffer (1x):

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.015	2.985
1 - 12	0.03	5.97

14.4 Streptavidin-HRP

Make a 1:100 dilution of **Streptavidin-HRP** in 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

14.5 Mouse TNF-α Standard

Reconstitute lyophilized **mouse TNF-α standard** with Calibrator Diluent (serum or plasma samples) or Sample Diluent (cell culture supernatant samples). (Reconstitution volume is stated in the Quality Control Sheet.)

15. TEST PROTOCOL SUMMARY

1. Determine the number of microwell strips required.
2. Prepare Biotin-Conjugate.
3. Wash microwell strips twice with Wash Buffer.
4. Add 50 µl Sample Diluent in duplicate to all wells.
5. Add 50 µl of externally diluted Standards in duplicate to standard wells.
6. Add 50 µl sample in duplicate, to designated sample wells.
7. Add 50 µl of Calibrator Diluent (for serum or plasma samples) or Sample Diluent (for cell culture supernatant samples) in duplicate to the blank wells.
8. Add 50 µl diluted Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18°C to 25°C) on a microplate shaker.
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 6 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°C to 25°C) on microplate shaker.
14. Empty and wash microwell strips 6 times with Wash Buffer.
15. Add 100 µl of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 30 minutes at room temperature (18°C to 25°C).
17. Add 100 µl Stop Solution to all wells.
18. Blank microwell reader and measure colour intensity at 450 nm.

Note: Shaking is absolutely necessary for an optimal test performance.
Note: If instructions in this protocol have been followed samples have not been diluted, the concentration read from the standard curve must not be multiplied by a dilution factor.

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