PeliKine
human TNFa ELISA kit

96 tests

An enzyme immunoassay for the quantitative
determination of human Tumor Necrosis Factor alpha.

PRODUCT INFORMATION

Sanquin Reagents

PO Box 9190
1006 AD Amsterdam
The Netherlands

reagents@sanquin.nl
www.sanquin.nl

Cat.No. M1920

For The Netherlands
Tel. 020 - 512 3599
Fax. 020 - 512 3570

For other countries:
Contact your local distributor

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Protocol summary and checklist PeliKine TNFα ELISA kit

- ○ Bring all reagents, with the exception of streptavidin-HRP, to room temperature.
- ○ Prepare dilution buffer.
- ○ Prepare standard and sample dilutions.
- ○ Prepare washing buffer.
- ○ Wash the plate five times with washing buffer.
- ○ Leaving the substrate blank wells empty, add 100 µl of standard and sample dilutions to the appropriate wells, cover the plate and incubate for one hour at room temperature.
- ○ Dilute biotinylated antibody 1:100 in dilution buffer.
- ○ Wash the plate five times with washing buffer.
- ○ Leaving the substrate blank wells empty, add 100 µl of the diluted biotinylated antibody to all wells, cover the plate and incubate for one hour at room temperature.
- ○ Dilute the streptavidin-HRP conjugate 1:10,000 in dilution buffer.
- ○ Wash the plates five times with washing buffer.
- ○ Leaving the substrate blank wells empty, add 100 µl of the streptavidin-HRP conjugate to all wells, cover plate and incubate for 30 minutes at room temperature.
- ○ Wash the plate five times with washing buffer.
- ○ Add 100 µl substrate solution to all wells, including the substrate blank wells, and incubate for 30 minutes at room temperature in the dark.
- ○ Add 100 µl stop solution to all wells and read the plate at 450 nm.
- ○ Calculate the amount of TNFα in the samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1										
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	S8	S8									B	B

Plate plan proposed for the Pelikine human TNFα ELISA kit:

Key: B: substrate blank S1-S8: TNFα standards 0 - 1000 pg/ml Empty: samples

I. INTRODUCTION

Tumour necrosis factor α (TNFα) is an extremely potent peptide cytokine which serves as an endogenous mediator of inflammatory, immune and host defence functions. Several substances originally described for their biological activities have been identified as TNFα; cachectin, macrophage cytotoxin (MCT), necrosin, cytotoxin (CTX), haemorrhagic factor, macrophage cytotoxic factor (MCF) and differentiation-inducing factor (DIF).
TNFα is capable of acting independently and in conjunction with a variety of other factors to affect the phenotype and metabolism of cells in every tissue of the body. It is generally thought that TNFα is not produced constitutively by normal cells, but rather to be induced potently by invasive stimuli in the setting of both neoplastic and infectious disease. In this role, macrophages and monocytes are thought to be the cells which contribute most to the local and systemic TNFα response to bacterial, viral and parasitic organisms and products.
Bioassays for the quantification of TNFα, including the cytotoxic assay on murine fibroblasts have been used for several years. However, TNFα shares many of the biological effects of IL-1 and for this reason the two commonly interfere in bioassays. Although the cytotoxic assay mentioned above, is unaffected by IL-1, it remains time consuming and might be susceptible to interference by other substances.
The Pelikine human TNFαELISA kit has been developed for faster, more reproducible and specific quantification of human TNFα in serum, plasma and other body fluids, as well as in cell-culture supernatant.

II. PRINCIPLE OF THE TEST

The Pelikine human TNFα ELISA kit is a "sandwich-type" of enzyme immunoassay in which a monoclonal anti human TNFα antibody is bound onto polystyrene microtiter wells.
Human TNFα, present in a measured volume of sample or standard is captured by the antibody on the microtiter plate, and non-bound material is removed by washing.
Subsequently, a biotinylated second monoclonal antibody to human TNFα is added. This antibody binds to the TNFα-antibody complex present in the microtiter well. Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the TNFα sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution is added to the wells. A coloured product is formed in proportion to the amount of TNFα present in the sample or standard. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microtiter plate reader. From the absorbance of samples and those of a standard curve, the concentration of TNFα can be determined by interpolation with the standard curve.

III. STORAGE AND STABILITY

The Pelikine human TNFα ELISA kit should be stored at 2-8°C. At arrival the components of the Pelikine human TNFα ELISA kit will be stable for at least 9 months when kept at 2-8°C. Prolonged stability up to the expiration date shown on the case label can be achieved by storing the TNFα standard, the biotynilated TNFα antibody conjugate, and the streptavidine-HRP conjugate separately below -18°C and store the remainder of the Pelikine human TNFα ELISA kit at 2-8°C.

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IV. CONTENTS OF THE KIT

The Pelikine human TNFα ELISA kit contains material sufficient for 96 tests, including standard curve samples. The reagents provided are:

Quantity	Kit component		Volume
1 pc	precoated microtiterplate	12 x 8 strips	- -
2 vial	TNFα standard (lyophilised)	2.9 ng/ml	500 µl*
1 vial	biotinylated antibody	100-fold concentrated	200 µl
1 vial	streptavidin-HRP conjugate	10,000-fold concentrated	20 µl
1 bottle	wash buffer	20-fold concentrated	50 ml
1 bottle	HPE dilution buffer	5-fold concentrated	60 ml
1 bottle	TMB substrate solution	ready for use	12.5 ml
1 bottle	stop solution (0.18 M H_2SO_4	ready for use	13.5 ml
5 pcs	plate seals	-	-

* After reconstitution

V. ADDITIONAL MATERIALS REQUIRED

- Pipetting devices for accurate delivery of 1-10 µl, 50 µl, 100 µl and 1 ml volumes.
- Distilled or de-ionized water.
- Polypropylene or polyethylene tubes for making sample dilutions, **do not use polystyrene, polycarbonate or glass tubes**.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Device for delivery of wash buffer (wash bottle / automated plate washer)
- Microtiter plate reader, capable of measuring absorbance at 450 nm.

VI. PRECAUTIONS FOR USE

1) The Pelikine human TNFα ELISA kit is intended *for research purposes only*.
2) Only use the reagents and microtiter plates supplied with the kit, do not mix reagents from different kit lots.
3) Handle all plasma and serum samples with care to prevent transmission of blood-borne infections.
4) Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.
5) All reagents contain thiomersal (0.001 % w/v) and may be toxic upon ingestion, inhalation or skin contact. Avoid contact of skin, eyes or clothing with dilution, washing or substrate buffer. In case of contact, wash skin or eyes with water and consult a physician.
6) Centrifuge all vials before use (1 minute 3000 x g).
7) With the exception of the substrate blank wells, do not allow wells to stand uncovered or dry for extended periods between incubation steps.

MATERIAL SAFETY DATA SHEET

Hazardous ingredients

3,3',5,5'-Tetramethylbenzidine may be harmful by inhalation, ingestion, or skin absorption. May cause irritation. To our best knowledge the chemical, physical and toxicological properties have not been thoroughly investigated. CAS No. 54827-17-7.

Thiomersal may be toxic upon ingestion, inhalation or skin contact. Avoid contact of skin, eyes or clothing with dilution, washing or substrate buffer. In case of contact, wash skin or eyes with water and consult a physician. CAS No. 54-64-8

Sulphuric Acid: CAS No. 7664-93-9

Physical data
No information is available on physical data for the chemical mixture as a whole.

Health hazard
Please refer to "Precautions for use", page 2 and 3 of this information leaflet.

Protection information
Please refer to "Precautions for use", page 2 and 3 of this information leaflet.

Disclaimer
The above information is believed to be accurate and represents the best information available to us. However, CLB neither warrants the accuracy of this information nor assumes any legal responsibility in connection with its dissemination. All materials and mixtures may present unknown hazards and should be used with caution. Users should make their own investigations to determine the suitability of this information for their particular purpose.

VII. ASSAY PROCEDURE

1. *BRING ALL REAGENTS TO ROOM TEMPERATURE (18-25°C)*, with the exception of the streptavidin-HRP conjugate which has to be kept at -18°C to -32°C to ensure stability.
   For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this leaflet.
2. Mix all reagents thoroughly without foaming before use.

3. **MICROTITER PLATE**

The kit contains one frame with twelve pre-coated strips of eight microwells, vacuum sealed in a plastic bag. The CLB TNFα ELISA provides the flexibility to run two partial plates on separate occasions. Before opening the plastic bag determine the number of strips required to test the desired number of samples plus 16 wells needed for running standards in duplicate. Remove extra strips from holder and repack these in the plastic bag with the desiccant

4. **BUFFERS** **buffer preparation**

Wash buffer concentrate
Prepare a working-strength solution by adding 50 ml of the wash buffer concentrate (total content of the bottle) to 950 ml distilled water. The working-strength solution wash buffer can be stored for up to 2 months at 2-8°C.

HPE dilution buffer
The kit contains one bottle with 5-fold concentrated dilution buffer.
For optimal assay results, dilute samples and standard in working-strength dilution buffer.

Calculate the quantity of dilution buffer required (approximately 15 ml undiluted buffer per microtiter plate) and prepare a working-strength solution by diluting the opalescent concentrated buffer 5 times in distilled water before use. The working-strength dilution buffer can be stored for up to one week at 2-8°C.

5. **TNFa STANDARD** **standard curve preparation**

The kit contains two vials with 2.9 ng/ml of a lyophilized natural human TNFα standard, calibrated against the WHO International Standard (TNFα 87/650; National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, U.K. 1 WHO Unit = 25 pg TNFα).

Reconstitute one vial by adding 500 µl of distillled water. Allow 10 minutes at room temperature to dissolve and mix gently. The reconstituted standard can be stored for 2 hours at 2-8°C and for 1 week at <-18°C. Avoid repeated freeze-thawing of the standard, although experimental data have shown that up to 3 freeze-thaw cycles have no effect on the TNFα levels of the standard.
The second vial of lyophilized standard can be used in later assays.

Label 8 tubes, one tube for each dilutions: 1000, 333, 111, 37, 12.4, 4.1, 1.4 and 0 pg/ml. Pipette 190 µl of working-strength dilution buffer into the first tube (1000 pg/ml) and 300 µl of workingstrength dilution buffer into the other tubes.
Transfer 100 µl of the reconstituted TNFα standard (2.9 ng/ml) into the first tube labelled 1000 pg/ml, mix well and transfer 150 µl of this dilution into the second tube labelled 333 pg/ml. Repeat the serial dilutions five more times by adding 150 µl of the previous tube of diluted standard to the 300 µl of dilution buffer.
The standard curve will contain 1000, 333, 111, 37, 12.4, 4.1, 1.4 and 0 pg/ml (dilution buffer).

It is recommended to prepare two separate series for each assay.

6 SAMPLES

Serum, heparin or EDTA-anti-coagulated plasmas, and culture fluids are suitable for use in the assay. (**caution**: separate plasma/serum and blood cells within 4 hours after collection, non-separated samples must be kept on temperatures from 2 to 8°C). Do not use grossly haemolyzed or lipemic specimens. If samples are to be run within 24 hours, they may be stored at 2-8°C; otherwise samples should be stored frozen (<-18°C).
Up to 3 freeze-thaw cycles have no effect on the TNFα levels of serum or plasma samples. Nonetheless, excessive freeze-thaw cycles should be avoided. Prior to the assay, frozen samples should be thawed **as quickly as possible** in a 37°C waterbath and then brought to room temperature (18-25°C).

It is recommended to dilute the test samples at least 1:2 in working-strength dilution buffer. If high levels of TNFα (>750 pg/ml) are expected in the test samples, additional dilutions of sample i.e. 1:10 and 1:50 should also be prepared.

7. FIRST WASH STEP

Prepare washing buffer as described on page 3 of this leaflet.

Wash the required microtiter plates five times with washing buffer in a plate washer.
In case of manual washing, completely fill the wells (> 300 µl) with washing buffer and aspirate, repeat this four times. After the final aspiration the wells should be dry.

8. FIRST INCUBATION STEP — Standards and samples

Leaving the substrate blank wells empty, transfer 100 µl of the prepared standards and samples in duplicate into the appropriate wells (see recommended plate plan).
Cover plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well **and incubate for 1 hour at room temperature (18-25°C).**

IX. ADDITIONAL INFORMATION

Increased sensitivity

The assay sensitivity can be increased by a small adaptation of the incubation methodology. Just follow all the instructions as stated in the assay procedure (chapter VII), but incubate at room temperature (18-25°C) on a horizontal plate shaker at 700 ± 100 rpm. All incubations, including the enzymatic colour development, have to be completed on the shaker, in the same time as stated in the static assay procedure. This will result in an increase in assay sensitivity, with little effects on the background levels (see figure opposite page).

Sensitivity

MEAN calculated zero signal + 3 SD : 1 - 3 pg/ml (shake - static incubation)
2 x (MEAN calculated zero signal) : 4 - 6 pg/ml (shake - static incubation)

Expected values

TNFα values in fresh serum and plasma samples of healthy individuals are below 10 pg/ml.

Specificity

No crossreactivity was observed with the following recombinant human proteins: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, Macrophage Colony Stimulating Factor (M-CSF), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF), Leukaemia Inhibitory Factor (LIF), RANTES, Stem Cell Factor/ Mast Cell Factor (SCF/MCF), Transforming Growth Factor β-1 (TGFβ-1), Tumour Necrosis Factor β (TNFβ/Lymphotoxin) and Interferon gamma (IFNγ).

X. REFERENCES

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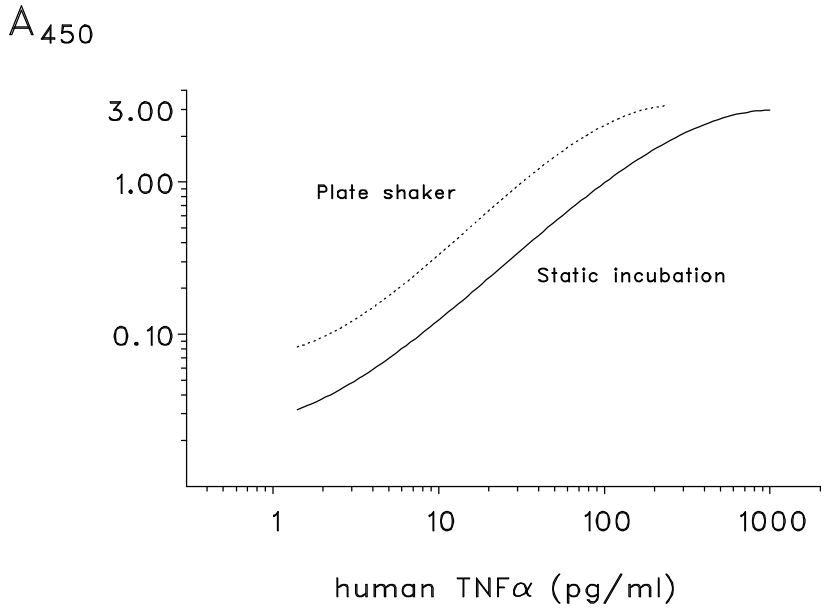

Typical standard curve for the PeliKine human TNFa ELISA kit
The assay is completed static or shaken at room temperature

	STATIC INCUBATION	SHAKEN INCUBATION
	Calculated mean absorbance at 450 nm	
substrate blank	0	0
0 pg/ml	0.014	0.028
1.4 pg/ml	0.031	0.081
4.1 pg/ml	0.065	0.159
11.4 pg/ml	0.139	0.402
37 pg/ml	0.415	1.069
111 pg/ml	1.102	2.726
333 pg/ml	2.196	> 3.000
1000 pg/ml	2.962	> 3.000

DO NOT USE THESE DATA TO CONSTRUCT A STANDARD CURVE FOR SAMPLE VALUE CALCULATIONS

9. **SECOND WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate(s) as described in point 6 above.

10. **SECOND INCUBATION STEP** **biotinylated antibody**

The kit contains one yellow-capped vial with concentrated antibody-biotin conjugate. Calculate the quantity of the antibody-biotin conjugate required (10 µl conjugate antibody per microwell strip) and prepare a working-strength solution by diluting the conjugate 1:100 in working-strength dilution buffer just before use.

Leaving the substrate blank wells empty, add 100 µl of diluted biotinylated antibody to all wells.

Cover plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 1 hour at room temperature (18-25°C)**.

11. THIRD WASH STEP

Aspirate supernatant from wells and wash the microtiter plate(s) as decribed in point 6 above.

12. **THIRD INCUBATION STEP** **Streptavidin-HRP conjugate**

The kit contains one brown capped vial of concentrated streptavidin-HRP conjugate, which must be stored at –18°C to –32°C to maintain maximal stability. The contents of the vial will not be frozen at this temperature.

Add 3 µl streptavidin-HRP conjugate to 30 ml of working-strength dilution buffer just before use. **Do not prepare in advance of assay.**

Leaving the substrate blank wells empty, add 100 µl of streptavidin-HRP conjugate to all wells.

Cover the microtiter plate(s) with adhesive seal, agitate the microtiter plate by tapping the edge of the frame for a few seconds to mix contents of each well and **incubate for 30 minutes at room temperature (18-25°C).**

13 **FOURTH WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate(s) as decribed in point 6 above.

14. **FOURTH INCUBATION STEP** **Enzymatic colour development**

The kit contains one brown capped bottle with a ready for use solution of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide. Take care not to contaminate the TMB substrate reagent; if the solution is blue prior to use the reagent cannot be used any more. Protect from prolonged exposure to light.

Add 100 µl of substrate solution to all wells, **including the substrate blank wells**.

Cover microtiter plate, gently agitate the microtiter plate by tapping the edge of the frame for a few seconds to mix contents of each well and **incubate for 30 minutes at room temperature (18-25°C) in the dark**.
Do not cover the plate with aluminium foil.

Note: The speed of enzymatic coulour development is influenced by many factors including temperature and quality of the used TMB.

15. **STOP ENZYMATIC REACTION**

The kit contains one white capped bottle with a ready for use stop solution of 0.18 M H_2SO_4.

Add 100 µl of stop solution to all wells.

After stopping the colour is stable for maximally 30 minutes.

16. **PLATE READ-OUT**

Read at 450 nm in an ELISA reader.

IX. RESULTS

Substrate blank

- Record the absorbance at 450 nm for the substrate blank wells and average the duplicate values.

Standard curve

- Record the absorbance at 450 nm for each well containing standard and average the duplicate values.
- Calculate the net average absorbances by subtracting the average of the substrate blank wells.
- Plot the net average absorbances (ordinate) versus the TNFα concentration in pg/ml (abscissa) on log-linear paper and draw the best fitting curve. An example of a standard curve is given on the next page.

Samples

- Record the absorbance at 450 nm for each standard well, and average the duplicate values.
- Calculate the net average absorbances by subtracting the average of the substrate blank wells.
- Locate the net average absorbance value found for each sample on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the TNFα concentration (pg/ml) from the horizontal axis. Multiply the obtained TNFα concentration with the dilution factor of the sample and record this figure.