Sanquin

PeliKine™
human TNFα ELISA kit

96 tests

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

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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

At arrival the Pelikine human TNFα ELISA kit will be stable for 3 months when kept at 2-8°C. Stability until the expiration date shown on the box label can be achieved by storing the TNFα standard, the biotynilated TNFα antibody conjugate, and the streptavidine-HRP conjugate separately at -18 to -32°C.

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	Cap colour
1 pieces	Precoated microtiterplate	12 x 8 strips + plate-frame	-	
2 vial	TNFα standard (lyophilized)	3400 pg/ml	500 µl**	black
1 vial	biotinylated antibody*	100-fold concentrated	200 µl	yellow
1 vial	streptavidin-poly-HRP conjugate*	10,000-fold concentrated	20 µl	brown
1 bottle	Wash buffer*	20-fold concentrated	50 ml	
1 bottle	HPE dilution buffer*	5- fold concentrated	55 ml	
1 bottle	TMB substrate solution	Ready-for-use	12.5 ml	brown
1 botle	Stop solution (0.18 M H_2SO_4)	Ready-for-use	13.5 ml	white
5 pcs	plate seals	-	-	

* These reagent contains Merthiolate (0.001% w/v) as a preservative.
** Volume after reconstitution of lyophilised material.

V. 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) Centrifuge all vials before use (1 minute 3000 x g).
6) With the exception of the substrate blank wells, do not allow wells to stand uncovered or dry for extended periods between incubation steps.

VI. ADDITIONAL INFORMATION

Additional materials required

– Pipetting devices for accurate delivery of 1-10 µl, 50 µl, 100 µl and 1 ml volumes.
– Beakers, flasks, cylinders necessary for preparation of reagents.
– Device for delivery of wash buffer (wash bottle / automated plate washer).
– Microtiter plate reader.

Sensitivity

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

References

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VII. ASSAY PROCEDURE

- *BRING ALL REAGENT TO ROOM REMPERATURE (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. Centrifugate all vials before use (1 minute 3000 x g).
- It is advised to test all samples and standard dilutions in duplicate.
- Mix all reagents thoroughly before use (without foaming).
- **For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this leaflet.**

1. **MICROTITER PLATE** **Precoated strips**

The kit contains one plate-frame with 12 strips of 8 wells, vacuum-sealed and packed in a resealable pouch. The PeliKine™ human TNFα ELISA kit provides the flexibility to run two partial plates on separate occasions.

Before opening the plastic pouch, determine the number of strips required to test the desired number of samples plus 18 wells needed for running standards and blanks in duplicate. Remove non-used strips from the plate-frame and restore them in the plastic pouch containing the desiccant for up to 1 month at 2-8°C

2. **BUFFER PREPARATIONS**

Wash buffer

The kit contains one bottle with 20 fold concentrated wash buffer.

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

Note: The concentrated buffer may contain salt crystals. Before preparing the working-strength buffer, warm the concentrated buffer BRIEFLY to 37°C to dissolve the crystals.

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 HPE-dilution buffer required (approximately 2 ml concentrated HPE buffer per microwell strip). Prepare a working-strength solution by diluting the opalescent concentrated buffer 5 fold in distilled water. The diluted buffer can be stored for up to one week at 2-8°C.

3. TNFα **standard** **Standard curve preparation**

A natural human TNFα standard has been 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). In former Sanquin TNFα reagents sets [Batch TNF-RS0001, TNF-RS0002, 1923-00-03 to 1923-00-04] 1 pg TNFα standard is comparable with 0.6 pg of the WHO standard).
The kit contains two lyophilized vials with 3400 pg/ml natural human TNFα.

Reconstitute one vial by adding 500 µl of distilled water. Allow 10 minutes at room temperature to dissolve and mix gently. The reconstituted standard can be stored for two hours at 2-8°C and for 1 week between -18 to -32°C.
The second vial of lyophilized standard can be used in later assays.

Label 8 tubes, one tube for each dilution: 1000, 333, 111, 37, 12.4, 4.1, 1.4 and 0 pg/ml.
Pipette 168 µl of working-strength dilution buffer into the tube labeled 1000 pg/ml and 150 µl of working-strength dilution buffer into the other tubes.
Transfer 70 µl of the TNFα standard (3400 pg/ml) into the first tube labeled 1000 pg/ml, mix well and transfer 75 µl of this dilution into the second tube labeled 333 pg/ml.
Repeat the serial dilution's six more times by adding 75 µl of the previous tube of diluted standard to the 150 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.

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. Thaw the reconstituted standard in tap water (18-25°C), do not use 37°C or 56°C water baths for this purpose.

4. SAMPLES

Serum, EDTA-anti-coagulated plasmas, and culture fluids are suitable for use in the assay. Do not use grossly haemolyzed or lipemic specimens. If rheumatoid factors are expected in serum or plasma samples, it is recommended to add normal mouse serum (Sanquin product M1250, final concentration in the diluted sample should be 5%) 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 tap water (18-25°C), do not use a 37°C or 56°C water bath for this purpose.

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.

5. **FIRST WASH STEP**

Prepare wash buffer as described on page 4 of this leaflet.

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

6. **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)**.

Just before washing, prepare next incubation reagent as described in point 8

7. **SECOND WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate as described in point 5 above.

8. **SECOND INCUBATION STEP** **biotinylated antibody**

The kit contains one yellow-capped vial with biotinylated antibody.

Calculate the required amount of conjugate (10 µl per strip) and dilute 1:100 in HPE-dilution buffer.

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)**.

Just before washing prepare next incubation reagents as described in point 10.

9. **THIRD WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate as described in point 5 above.

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

The kit contains one brown 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.

Per microtiter plate, 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)**.

11. **FOURTH WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate as described in point 5 above.

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

The kit contains one brown-capped bottle with a ready-for-use TMB substrate solution containing a mixture 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.
This solution must be on room temperature before use!

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

Cover microtiter plate(s) with lid, 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 aluminum foil.

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

13. STOP ENZYMATIC REACTION

The kit contains one white-capped bottle with a ready-for-use stop solution of 0.18 M H2SO4.

Add 100 µl of stop solution to all wells.

After stopping the colour is stable for maximally 30 minutes.

14. PLATE READ-OUT

Read at 450 nm in an ELISA reader.

VIII. RESULTS

Substrate blank

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

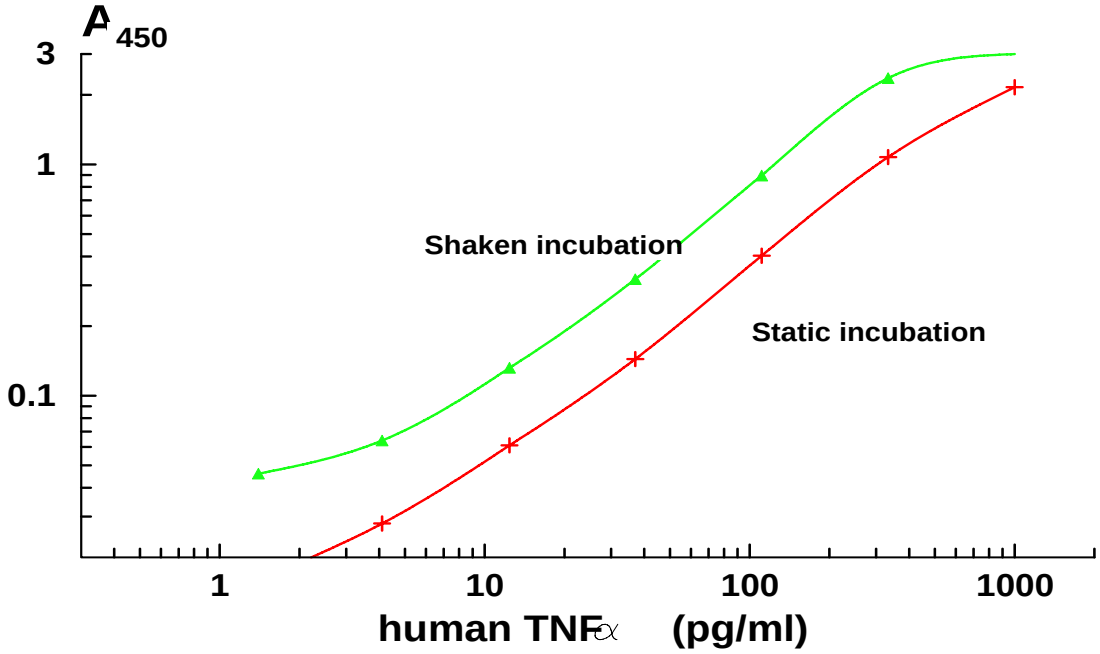
Standard curve

- 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.

A computer program to calculate ELISA results (developed by Mr E.J. Nieuwenhuys, CLB Amsterdam) is available free of charge to our kit users.
This program can be downloaded from internet:
http://www.xs4all.nl/~ednieuw/Calibration/Logit/logit.htm



Typical standard curve for the PeliKine compact™ human TNFα ELISA kit

	STATIC INCUBATION	SHAKEN INCUBATION
	Calculated mean absorbance at 450 nm	
substrate blank	0	0
0 pg/ml	0.012	0.029
1.4 pg/ml	0.016	0.046
4.1 pg/ml	0.028	0.064
12.4 pg/ml	0.061	0.132
37 pg/ml	0.144	0.391
111 pg/ml	0.403	0.895
333 pg/ml	1.076	2.361
1000 pg/ml	2.157	> 3.000

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

Plate plan proposed for the PeliKine™ human TNFα ELISA kit:

	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

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

Protocol summery and checklist PeliKine™ human TNFα ELISA kit

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