PeliKine Compactä
human TNFa ELISA kit

288 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 compact™ human TNFα ELISA 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 compact™ 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 compact™ human TNFα ELISA kit should be stored at -18°C to -32°C. The performance of the kit is guaranteed until the expiration date shown on the case label.

IV. CONTENTS OF THE KIT

The PeliKine compact™ human TNFα ELISA kit contains material sufficient for 288 tests, including standard curve samples. The reagents provided are:

Quantity	Kit component		Volume	Cap colour
1 vial	coating antibody	100-fold concentrated	375 μl	red
1 vial	blocking reagent	50-fold concentrated	2 ml	transparent
1 vial	TNFα standard (lyophilized)	2900 pg/ml	500 μl	black
1 vial	biotinylated antibody	100-fold concentrated	375 μl	yellow
1 vial	streptavidin-poly-HRP conjugate	10,000-fold concentrated	20 μl	brown
1 bottle	dilution buffer	5- fold concentrated	60 ml	
3 pcs	microtiter plate + lid	-	-	
10 pcs	plate seals	-	-	

V. PRECAUTIONS FOR USE

1) The PeliKine compact™ 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) The TNFα standard contains human serum which has been found to be non-reactive for Hepatitis B surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV). Nevertheless the standard should be handled as potentially hazardous and capable of transmitting diseases.
7) Centrifuge all vials before use (1 minute 3000 x g).
8) 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 BUFFERS & SOLUTIONS REQUIRED

Coating buffer: 0.1 M Carbonate/bicarbonate buffer pH 9.6

Solution A: 1.24 g $Na_2CO_3.H_2O$ in 100 ml distilled water
Solution B: 1.68 g $NaHCO_3$ in 200 ml distilled water

Take 70 ml of solution A, and add solution B until the pH is 9.6
(approximately 175 ml of solution B required)

The prepared buffer can be stored up to one week at 2-8°C.

PBS stock solution [20 x]: 0.2 M Phosphate Buffered Saline (PBS)

Dissolve 32 g $Na_2HPO_4.2H_2O$
6 g $NaH_2PO_4.2H_2O$
164 g NaCl

in 900 ml distilled water
(intensive stirring and some heating will speed dissolution).
Bring the temperature of the solution back to room temperature (18-25°C) and check pH; if necessary adjust pH to 6.8 - 6.9 with concentrated HCl or NaOH, and add distilled water to a volume of 1 liter (when diluted 20 times the obtained buffer will have a pH of 7.2 - 7.4).
Add 20 mg thiomersal as preservative. Do not use sodium azide (NaN_3) since this preservative reduces the quality of the enzymatic label.

The prepared buffer can be stored up to three months at 2-8°C.

Note: in the concentrated buffer salt crystals may appear when stored at 2-8°C. Before preparing the working-strength buffer, first warm the concentrated buffer BRIEFLY to 37°C to dissolve the precipitate.

Washing buffer: PBS with 0.005% TWEEN20

Make 1 liter of working-strength PBS by diluting the PBS stock solution (see above) 20 fold with distilld water.

Add 50 μl TWEEN 20.

The prepared buffer can be stored up to one month at 2-8°C.

Substrate buffer: 0.11 M acetate buffer pH 5.5

Dissolve 15.0 g sodium-acetate ($CH_3COONa.3H_2O$) in 800 ml distilled water.
Adjust pH to 5.5 with glacial acetic acid, add distilled water to a volume of liter.
Do not add any preservative (e.g. merthiolate, sodium azide) since this may affect the quality of the enzymatic colour development.

The prepared buffer can be stored up to two weeks at 2-8°C.

3,5,3',5'-tetramethylbenzidine (TMB) stock solution: 6 mg/ml TMB in DMSO

Dissolve 30 mg 3,5,3',5'-tetramethylbenzidine (TMB) in 5 ml dimethylsulfoxide (DMSO).
The prepared stock solution can be stored up to 1 month at room temperature (18 25°C) and protected against light.

Hydrogen peroxide stock solution: 3% H2O2 solution in distilled water.
The prepared stock solution can be stored up to one month at 2-8°C.

Substrate solution:

For each plate mix the following reagents:

12 ml substrate buffer
200 µl TMB stock solution
12 µl H_2O_2 stock solution

The substrate solution should be prepared just before use and has to be at room temperature (18-25°C) for optimal reproducible results.

Stop solution: 1.8 M H_2SO_4 solution in distilled water.

VII. 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 washing 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)

Note: the sensitivity is dependent of the type and quality of enzymatic substrate.

Expected values

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

Specificity

No crossreactivity was observed with the following recombinant human proteins: IL-1α, IL-1ß, IL-2, IL-3, IL-4, IL-5, IL-6, sIL-6r (GP80), IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, Macrophage Colony Stimulating Factor (M-CSF), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF), Leukemia Inhibitory Factor (LIF), RANTES, Stem Cell Factor/ Mast Cell Factor (SCF/MCF), Transforming Growth Factor ß-1 (TGFß-1), Tumour Necrosis Factor ß (TNFß/Lymphotoxin), and Interferon γ (IFNγ).

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VIII. ASSAY PROCEDURE

1. *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).
   For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this leaflet.

2. **DILUTION BUFFER**
   The kit contains one bottle with 5-fold concentrated dilution buffer.
   For optimal assay results, dilute samples and standards 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

3. **MICROTITER PLATES** **Coating antibody**
   Coating
   The kit contains three micotiter plates for 96 tests each, including the standard curve samples.

Prepare coating buffer as decribed on page 3 of the information leaflet.
Per mivrotiter plate ass 120 µl of coating antibody (red-capped vial) to 12 ml coating buffer.
Add 100 µl to all wells, cover micrititer plate(s) with lid and **incubate overnight at room temperature (18-25°C).**

Washing procedure

Prepare working-strength PBS (1:20 dilution of stock PBS as described on page 3 of the information leaflet).
Aspirate supernatants from wells and completely fill the wells (> 300 µl) with working-strength PBS and aspirate. Repeat this four times, after the final aspiration the wells should be dry.

Blocking procedure
The kit contains one transparent-capped vial with 2 ml blocking reagent.

Prepare blocking buffer by adding 500 µl blocking reagent to 25 ml working-strength PBS (1:20 dilution of stock PBS as described on page 3 of the information leaflet).
Add 200 µl blocking buffer to all wells, cover microtiter 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).**

4. TNFa 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 one lyophilized vial with 2900 pg/ml natural human TNFα.
Reconstitute the lyophilized standard by adding 500 µl of distilled water to the vial. Incubate for 10 minutes at room temperature and mix gently. After reconstitution the standard must be kept cold (2-8°C) and stored frozen after use (<-18°C, preferably<-70°C).

Label 7 tubes, one tube for each dilution: 1000, 333, 111, 37, 12.4, 4.1, and 1.4 pg/ml.
Pipette 133 µl of working-strength dilution buffer into the tube labelled 1000 pg/ml and 150 µl of working-strength dilution buffer into the other tubes.
Transfer 70 µl of the TNFα standard (2900 pg/ml) into the first tube labelled 1000 pg/ml, mix well and transfer 75µl of this dilution into the second tube labelled 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, and 1.4 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.

5. 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 (CLB 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 37°C or 56°C waterbaths 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.

6. 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.

7. 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).

8. SECOND WASH STEP

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

9. SECOND INCUBATION STEP **biotinylated antibody**

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

Per microtiter plate, add 120 μl biotinylated antibody to 12 ml 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).

10. WASH STEP

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

11. **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).

12. **FOURTH WASH STEP**

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

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

Approximately 10 minutes before use, prepare the substrate solution as decribed on page 4 of this leaflet.

The substrate solution should be at room temperature (18-25°C) for optimal reproducible results.

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

Cover mictrotiter 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 aluminium foil.

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

14. STOP ENZYMATIC REACTION

Add 100 μl of stop solution to all wells.

After stopping the colour is stable for maximally 30 minutes.

15. 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

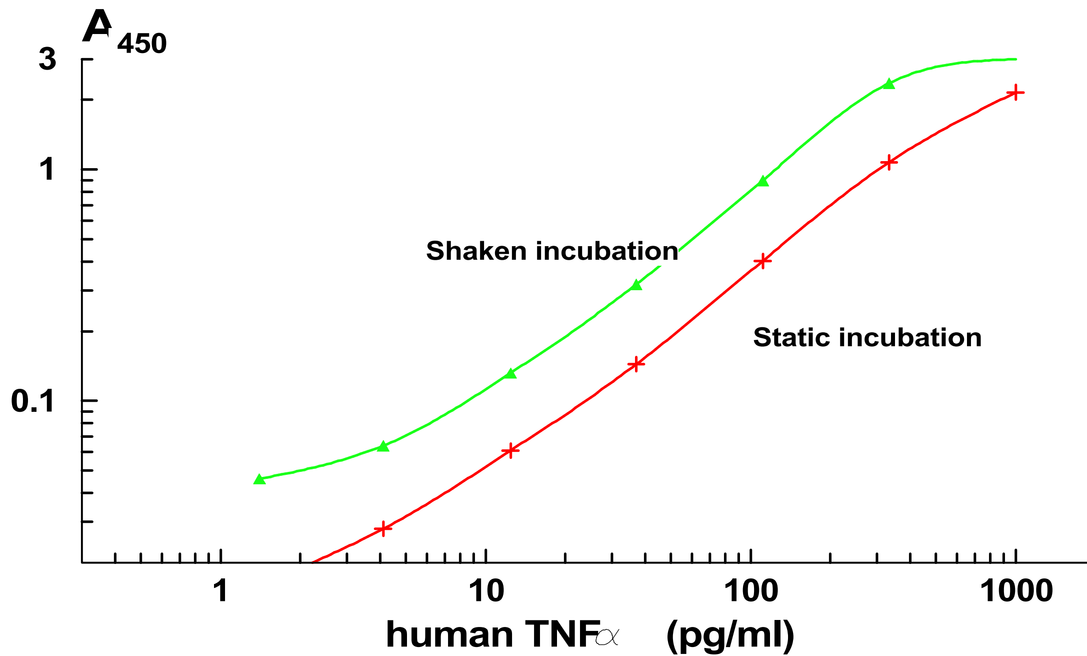
- Record the absorbance at 450 nm for each well containing standard and average the duplicate values.
- Calculate the net average absorbances by subtracting the average of the substrate blank wells.
- Plot the net average absorbances (ordinate) versus the TNFα concentration in pg/ml (abscissa) on log-linear 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.

X. 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 VIII), 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 small effects on the background levels (see figure next page).



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 compact™ 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 0-300 pg/ml Empty: samples

Protocol summery and checklist PeliKine compactÔ human TNFa ELISA kit

Day 0:

- ○ Bring coating antibody to room temperature (18-25°C).
- ○ Prepare coating buffer.
- ○ Dilute coating antibody 1:100 in coating buffer, add 100 μl to all wells, cover the plate(s) and incubate overnight at room temperature.

Day 1:

- ○ Bring all reagents, with the exception of streptavidin-HRP, to room temperature.
- ○ Prepare blocking buffer.
- ○ Wash the plate(s) five times with PBS.
- ○ Add 200 μl blocking buffer to all wells and incubate for one hour at room temperature.
- ○ Prepare standard and sample dilutions.
- ○ Prepare washing buffer.
- ○ Wash the plate(s) five times with washing buffer.
- ○ Leaving the substrate blank wells empty, add 100 μl of the diluted biotinylated antibodiy to all wells, cover the plate(s) and incubate for one hour at room temperature.
- ○ Dilute the streptavidin-HRP conjugate 1:10,000 in dilution buffer.
- ○ Wash the plate(s) five times with washing buffer.
- ○ Leaving the substrate blank wells empty, add 100 μl of the streptavidin-HRP conjugate to all wells, cover the plate(s) and incubate for 30 minutes at room temperature.
- ○ Just before use, prepare substrate solution.
- ○ Wash the plate(s) five times with washing buffer.
- ○ Add 100 μl substrate solution to all wells, including the substrate blank wells, 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.