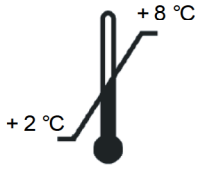
Arbeitsanleitung/Manual

TNF α ELISA Kit

For the in vitro determination of TNF α in serum, plasma, stool and cell culture supernatant

Valid from 06.04.2011

K 9610



Immundiagnostik AG, Stubenwald-Allee 8a, D 64625 Bensheim
Tel.: ++49 6251 70190-0
Fax: ++ 49 6251 849430
e.mail: Info@immundiagnostik.com
www.Immundiagnostik.com

1. INTENDED USE

The *Immundiagnostik* Assay is a sandwich Enzyme Immuno Assay intended for the quantitative determination of **TNF α** in plasma, serum, stool and cell culture supernatant. For *in vitro* diagnostic use only.

2. CLINICAL RELEVANCE

TNF-α (**tumor-necrosis-factor-alpha**) belongs to the group of pro-inflammatory cytokines with cytotoxic activity. Activated monocyctes/ macrophages, lymphocytes and natural killer cells as well as many other malignant cells and mast cells are capable of producing **TNF-α.**

Recent studies using antibodies against **TNF-α** such as the "ATTRACT study - Anti TNF Trial in Rheumatoid Arthritis with Concomitant Therapy" confirm that **TNF-α** in the case of rheumatoid arthritis plays a key pathogenic role. **TNF-α** is produced by macrophages in the synovial tissue once the inflammatory macrophages have infiltrated the mucus membrane of the joints. There, **TNF-α** activates the fibroblasts to proliferate, induces vascular growth and stimulates the macrophages in a positive feedback reaction for further production of pro-inflammatory cytokines such as IL-1 and IL-6 and stimulates thereby the inflammatory processes of the joints. Altogether, these mechanisms play an important role in the destruction of inflamed joints.

3. TEST PRINCIPLE

This Enzyme-Linked-Immunosorbent Assay (ELISA) allows the quantitative determination of Tumor Necrosis Factor-α. In a first incubation step, TNFα in the samples is bound to monoclonal mouse antibodies against TNFα, which are immobilized on the surface of the microtiter plate. After a washing step, to remove all interfering substances, the quantification of the bound TNFα is carried out by adding a second monoclonal anti TNFα antibody and a horseradish peroxidase labeled conjugate. The amount of the converted substrate by the peroxidase is directly proportional to the amount of bound TNF α and can be determined photometrically at 450 nm.

4. MATERIAL SUPPLIED

Cat. No.	Content	Kit Components	Quantity
K 9610MTP	PLATE	One holder with precoated strips	12 x 8 wells
K 9610WP	WASHBUF	ELISA wash buffer concentrate 10x	100 ml
K 9610A2	AB	2nd Antibody (Mouse anti hTNF-α, biotinylated)	1 x 150 µl
K 9610K	CONJ	Conjugate, (Streptavidin Peroxidase-labeled)	1 x 200 µl
K 9610ST	STD	Calibrator concentrate (1000 pg/ml), lyophilized	3 x 1 vial
K 9610KO1	CTRL	Control, lyophilized	3 x 1 vial
K 9610KO2	CTRL	Control, lyophilized	3 x 1 vial
K 9610PV	STDBUF	sample dilution buffer, ready-to-use	1 x 25 ml
K 9610TMB	SUB	TMB substrate (Tetramethylbenzidine), ready to use	1 x 15 ml
K 9610AC	STOP	ELISA stop solution, ready to use	1 x 15 ml

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Bidistilled water (aqua bidest.)
- Laboratory balance
- Precision pipettors and disposable tips to deliver 10-1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 3000 x g
- Vortex-Mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 or 405 nm
  (reference wave length 620 or 690 nm)

6. PREPARATION AND STORAGE OF REAGENTS

- To run assay more than once, ensure that reagents are stored at conditions stated on the label. **Prepare only the appropriate amount necessary for each assay**. The kit can be used up to 3 times within the expiry date stated on the label.

- Reagents with a volume less than **100 µl** should be centrifuged before use to avoid loss of volume.

- The **WASHBUF** (wash buffer concentrate) should be diluted with aqua bidest. **1:10** before use (100 ml concentrate + 900 ml aqua bidest.), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved in a water bath at 37°C before dilution. The **WASHBUF** (wash buffer concentrate) is stable at **2-8°C** until the expiry date stated on the label. Diluted **buffer solution** can be stored in a closed flask at **2-8°C for one month**.

- The **AB** (2nd antibody) is diluted **1:101** in wash buffer (100 µl **2nd antibody** + 10 ml wash buffer). Undiluted antibody is stable at 2-8°C until the expiry date given on the label. **Diluted antibody solution is not stable and cannot be stored.**

- The **CONJ** (conjugate, Streptavidin Peroxidase-labeled) must be diluted **1:101 in** wash buffer (100 µl CONJ + 10 ml wash buffer). The undiluted conjugate is stable at **2-8 °C** until the expiry date stated on the label. **Diluted conjugate is not stable and cannot be stored.**

- The lyophilized **CTRL** (controls) are stable at **2-8°C** until the expiry date stated on the label. The **CTRL** (controls) must be reconstituted with **500 µl** of **STDBUF** (standard dilution buffer). Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to insure complete reconstitution. **Reconstituted controls are not stable and cannot be stored.**

- The lyophilized **STD** (standard) **1000 pg/ml** must be reconstituted with **800 µl STDBUF** (standard dilution buffer). Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to insure complete reconstitution. For the **TNF-α** calibration curve dilute the concentrate (S=1000 pg/ml) **in 1:2 dilution steps in standard dilution buffer** as described in the following example:

S 1 = **500 µl** S + **500 µl** standard dilution buffer

S 2 = **500 µl** S1 + **500 µl** standard dilution buffer

S 3 = **500 µl** S2 + **500 µl** standard dilution buffer

S 4 = **500 µl** S3 + **500 µl** standard dilution buffer

S 5 = **500 µl** S4 + **500 µl** standard dilution buffer

S 6 = **500 µl** S5 + **500 µl** standard dilution buffer

As zero standard use the standard dilution buffer.

Reconstituted standard is not stable and cannot be stored.

- All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at **2-8°C**.

7. PRECAUTIONS

- For *in vitro* diagnostic use only.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, all kit components should be treated as potentially infectious.
- Kit reagents contain sodium azide or thimerosal as bactericides. Sodium azide and thimerosal are toxic. Substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- Stop solution is composed of sulfuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water.
- Reagents should not be used beyond the expiration date shown on the kit label.

8. SPECIMEN COLLECTION AND PREPARATION

Faeces

Add a stool sample of about 100 mg (size of a pea, please note the exact weight for the calculation) to 5 ml of the ELISA wash buffer and homogenize very thoroughly for 15 seconds on a Vortex-mixer. Centrifuge the suspension for 10 min at 3000 rpm. 1 ml of the supernatant is given into an Eppendorf tube and centrifuged once more at 13,000 rpm for 5 min. **The supernatant not stable and cannot be stored**.

100 µl of the supernatant is used in the assay.

Immundiagnostik recommends the use of Roche Diagnostics / Mannheim sample preparation tubes, article No. 10745804332, for sample preparation.

PLEASE NOTE:

For TNF-α measurement in stool, please freeze the stool samples at -20 °C immediately after collection (not later than two hours after collection). If samples have to be transported, please make sure to keep them frozen. The stability of the samples must be checked before using the method in routine.

Serum, plasma

Collection and storage of **serum**: Collect sufficient blood (at least 1 ml) by venipuncture into a tube or a plastic syringe, avoid hemolysis, centrifuge for 15 minutes at 1,000 x g and 4°C and collect the serum.

Collection and storage of **plasma**: Collect sufficient blood (at least 1 ml) by venipuncture into an EDTA venipuncture tube or a plastic syringe, centrifuge for 15 minutes at 1,000 x g and 4°C within 10 minutes after blood collection and separate the plasma from the cells.

Serum/Plasma can be used without further dilution. Samples must be stored at –20 °C.

9. ASSAY PROCEDURE

Procedural notes

- Do not mix different lot numbers of any kit component within the same assay.
- The quality control guidelines should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the test results. Immundiagnostik can therefore not be held responsible for any damage.
- Carry out the assay with the actual manual delivered with the kit.

Test procedure

Bring all reagents to room temperature (18-26°C) and mix well before use. Avoid direct sun light during all incubation steps. Cover the microtiter plate during the different incubation steps. Carry out the tests in duplicate.

Wash the precoated microtiter plate **5 x with 250 µl** ELISA wash buffer before use. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper to remove excess solution.

1. Add **100 µl STD** (Standard), **SAMPLE** (Sample) or **CTRL** (Controls) into respective well.
2. Incubate for **2 hours**, shaking on a horizontal mixer, at room temperature.
3. Decant the contents of the plate and wash the cavities **5 x with 250** µl of washing buffer solution. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper to remove excess solution.
4. Add **100 µl** diluted **AB** (2. antibody, Mouse anti hTNF-α, biotinylated) solution.
5. Incubate for **1 hour** on plate shaker at room temperature.

6. Decant the contents of the plate and wash the cavities **5 x with 250** µl of washing buffer solution. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper to remove excess solution.

7. Add **100 µl** diluted **CONJ** (Streptavidin Peroxidase-labeled).

8. Incubate for **1 hour**, shaking on a horizontal mixer, at room temperature in the dark.

9. Decant the contents of the plate and wash the cavities **5 x with 250** µl of washing buffer solution. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper to remove excess solution.

10. Add **100 µl SUB** (TMB substrate).

11. Incubate for **10-20 minutes** at room temperature.

12. Add **50 µl STOP** (stop solution) and mix shortly.

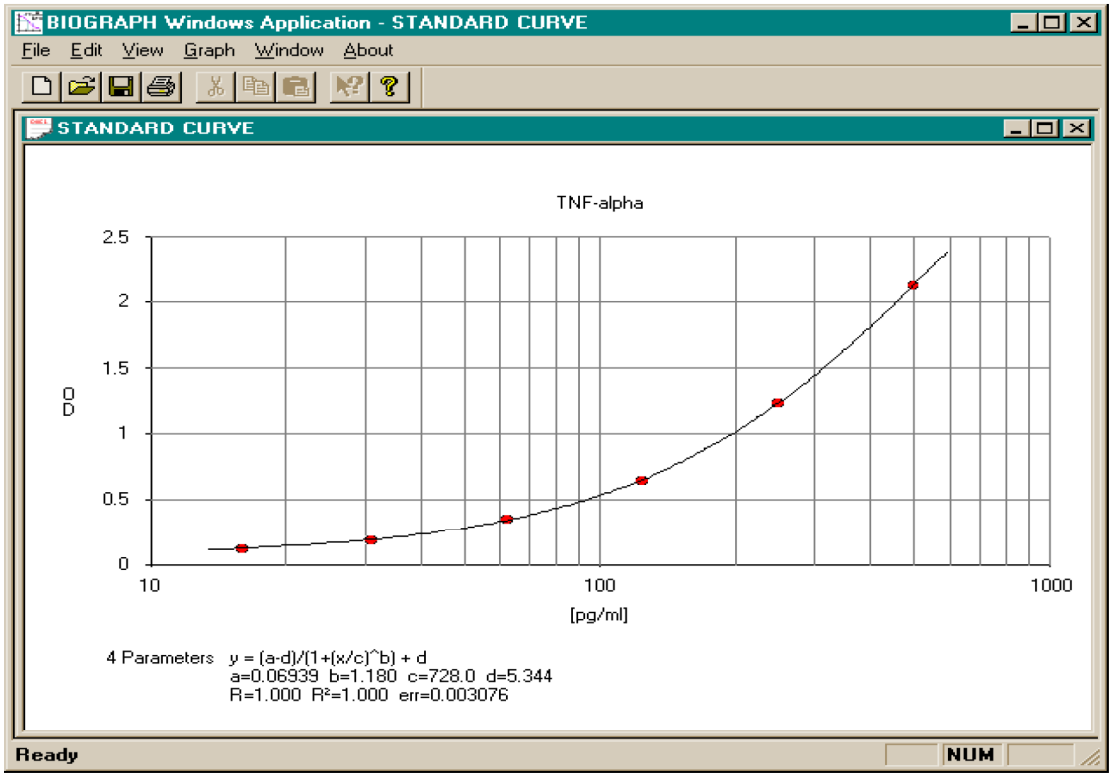
13. Determine **absorption immediately** with an ELISA reader at **450 nm** against 620 nm (or 690 nm) as reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the measurement range of the photometer, absorption must be measured immediately at 405 nm against 620 nm (or 690 nm) as reference.

10. RESULTS

A calibration curve is constructed from the standards. Commercially available software can be used as well as graph paper. Results of the samples are read from this calibration curve.

THE CALIBRATION CURVE IS NOT LINEAR, therefore a spline- or 4PL algorithm is recommended.

Typical calibration curve



Concentration [pg/ml]	16	31	62	125	250	500
OD mean value	0.127	0.191	0.348	0.651	1.235	2.131

These data are for demonstration only and cannot be used instead of data obtained from the actual assay

Faecal samples

To calculate the TNF α concentration of faecal specimen see the following example:

weight:	80 mg (1ml stool = 1g) = 0.08 ml
dilution step:	5 ml / 0,08 ml = 62.5
dilution factor:	62.5

Multiply the results with the calculated dilution factor (in this case 62.5) to get the TNF α concentration of the stool samples. **Please note:** the dilution factor depends on the weight of the used faecal specimen.

11. LIMITATIONS

Samples with TNF α levels greater than the highest calibrator, should be diluted and re-assayed.

12. QUALITY CONTROL

Control samples should be analyzed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid, if within the same assay one or more values of the quality control sample are outside the acceptable limits.

Expected values

Plasma (n=40): < 20pg/ml

13. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

The precision (intra-assay variation) of the Immundiagnostik TNF α ELISA test was calculated from 20 replicate determinations on each of one samples.

Intra-Assay CV n= 20

Sample	TNFα mean [pg/ml]	Intra-Assay CV [%]
1	155	6.3

Inter-Assay-Variation

The total precision (inter-assay variation) of the Immundiagnostik TNFα ELISA test was calculated from data one sample obtained in 20 different assays by three technicians on two different lots of reagents over a period of three months.

Inter-Assay CV n= 20

Sample	TNFα mean [pg/ml]	Inter-Assay CV [%]
1	142	8.2

Sensitivity

The detection limit of TNF α ELISA is calculated as B_0 + 2SD = 10 pg/ml.

Cross reactivity

GM-CSF < 0.1

M-CSF < 0.1

G-CSF < 0.1

Linearity

Linearity of the test was evaluated by dilution of TNFα-containing material. Linearity was obtained in the range of 10 - 500 pg/ml.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- For *in vitro* diagnostic use only.
- Quality control guidelines should be followed.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, all kit components should be treated as potentially infectious.
- Kit reagents contain sodium azide or thimerosal as bactericides. Sodium azide and thimerosal are toxic. Substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- Stop solution is composed of sulfuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water.
- Do not mix different lot numbers of any kit component.
- Reagents should not be used beyond the expiration date shown on the kit label.
- The assay should always be performed according the enclosed manual.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from wrong use.
- Warranty claims and complaints in respect of deficiencies must be logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.

Used symbols:


Temperature limitation

Catalogue Number

In Vitro Diagnostic Medical Device

Contains sufficient for <n> tests


Manufacturer


Use by

Lot number