Human TNFα ELISA Kit

Instructions for use

Catalogue numbers: 1x48 tests: EA102163
1x96 tests: EA102164
2x96 tests: EA102165

For research use only

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Table of Contents

1. Intended use....................3
2. Introduction....................3
2.1. Summary....................3
2.2. Principle of the method....................4
3. Reagents provided and reconstitution....................4
4. Materials required but not provided....................5
5. Storage Instructions....................5
6. Specimen collection, processing & storage....................5
7. Safety & precautions for use....................6
8. Assay Preparation....................7
8.1. Assay Design....................7
8.2. Preparation of Wash Buffer....................7
8.3. Preparation of Standard Diluent Buffer....................7
8.4. Preparation of Standard....................8
8.5. Preparation of Controls....................8
8.6. Preparation of Biotinylated anti- TNFα....................9
8.7. Preparation of Streptavidin-HRP....................9
9. Method....................10
10. Data Analysis....................11
11. Assay limitations....................11
12. Performance Characteristics....................12
12.1. Sensitivity....................12
12.2. Specificity....................12
12.3. Precision....................12
12.4. Dilution Parallelism....................13
12.5. Spike Recovery....................14
12.6. Stability....................14
12.7. Expected serum values....................14
12.8. Standard Calibration....................14
13. Bibliography....................15
14. OriGeneHuman TNFα ELISA references....................16
15. Assay Summary....................17
16. International Summaries....................18
16.1. French....................18
16.2. Spanish....................20

Human TNFα ELISA KIT

1. Intended use

The OriGene TNFα ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of TNFα in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human TNFα.

This kit has been configured for research use only. Not suitable for use in therapeutic procedures.

2. Introduction

2.1. Summary

Tumor Necrosis Factor (TNFα), also known as cachectin, is a polypeptide cytokine produced by and macrophages. It functions as a multipotent modulator of immune response and further acts as a pyrogen (4, 17). TNFα circulates throughout the body responding to stimuli (infectious agents or tissue injury), activating neutrophils, altering the properties of vascular endothelial cells, regulating metabolic activities of other tissues, as well as exhibiting tumoricidal activity by inducing localized blood clotting. TNFα also inhibits lipoprotein lipase activity resulting in cachexia, a physical wasting condition (4, 17). Activation of B-cells by the Epstein Barr virus can be inhibited by TNFα (15). Due to its varied actions throughout the immune system, TNFα may play a role in the pathogenesis of many disease states.
TNFα production is mediated by the action of lymphokines and endotoxins on the macrophage. Purified monocytes produce TNFα within four hours of stimulation by recombinant IL-2 (9) and there is some in vitro evidence to suggest that TNFα is expressed at high levels and with prolonged kinetics in T cells stimulated by both CD2 and CD28 (5). Secretion of TNFα is enhanced by gamma interferon. TNFα then induces or enhances the specific production of Class I MHC antigen, GM-CSF, and IL-1. Recent evidence has suggested an intracellular role for this peptide (23).

TNFα may play a significant role in the pathogenesis of inflammatory disease of the joints and other tissues. Chin et al. (6) found that TNFα, along with gamma interferon and IL-1 increased cell surface expression of ICAM -1 on synovial fibroblasts. Alvaro-Garcia et al. (3) reported that TNFα stimulates synovial proliferation.

Waage et al. (25) found that increased levels of TNFα in patients with septicemia and meningococcal disease correlated with fatal outcome. Scuderi et al. (22) suggest that increased levels of this cytokine may play a role in the host defense mechanism against parasitic infections. Girardin et al. (12) reported that increased serum TNFα levels correlated with the number of risk factors involved in children with gram-negative sepsis and purpura fulminians. Elevated levels of TNFα were also found in individuals suffering from myocarditis (11).

Recently, a growing body of information has pointed to a role for TNFα in the pathogenesis of AIDS. Alveolar macrophages (AM) from HIV positive individuals with opportunistic lung infections have been shown to spontaneously produce higher levels of TNFα in vitro than those HIV positive individuals without infection and HIV negative controls (14, 16). Krishnan et al. (16) report that higher TNFα production by AM was associated with lower counts of pneumocystis carinii in broncheoalveolar lavage fluid, indicating that TNFα may play a role in the control of this infection in AIDS. Israel-Biet et al. (14) also reported in in-vitro studies, that AM that express HIV (p24+) released significantly higher levels of TNFα than p24- alveolar macrophages and controls. Reddy et al. (20) found persistently elevated levels of circulating TNFα in HIV seropositive individuals and suggest a possible involvement of this cytokine in the development of AIDS.

Measurement of TNFα levels has also been shown to be useful in transplant research, where Maury et al. (18) and McLaughlin et al. (19). Both reported TNFα to be markedly elevated in renal allograft rejection episodes. Recent evidence has been presented on increased TNFα levels in Bone Marrow Transplant (BMT) (13, 21). BMT patients with major transplant related complications such as interstitial pneumonitis and severe acute graft-versus - host disease had TNFα levels significantly increase over controls (13).

2.2. Principle of the method

A capture Antibody highly specific for TNFα has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of TNF-α samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti-TNFα secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of TNFα present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of TNFα in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8°C)	**Quantity 1x48 well kit** Cat no. EA102163	**Quantity 1x96 well kit** Cat no. EA102164	**Quantity 2x96 well kit** Cat no. EA102165	**Reconstitution**
96 well microtitre strip plate	1/2	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	2	4	n/a
Standard: 800pg/ml	1	2	4	Reconstitute as directed on the vial (see reagent preparation, section 8)
Standard Diluent (Buffer)	1 (25ml)	1 (25ml)	1 (25ml)	10x Concentrate, dilute in distilled water (see reagent preparation, section 8)
Standard Diluent (Serum)	1 (7ml)	1 (7ml)	2 (7ml)	Ready to use
Control	1	2	4	Reconstitute as directed on the vial (see reagent preparation, section 8)
Biotinylated anti-TNFα	1 (0.4ml)	1 (0.4ml)	2 (0.4ml)	Dilute in biotinylated antibody diluent (see reagent preparation, section 8)
Biotinylated Antibody diluent	1 (7ml)	1 (7ml)	1 (13ml)	Ready to use
Streptavidin-HRP	1 (5µl)	2 (5µl)	4 (5µl)	Add 0.5ml of HRP diluent prior to use (see reagent preparation, section 8)
HRP Diluent	1 (23ml)	1 (23ml)	1 (23ml)	Ready to use
Wash Buffer	1 (10ml)	1 (10ml)	2 (10ml)	200x Concentrate dilute in distilled water (see reagent preparation, section 8)
TMB Substrate	1 (11ml)	1 (11ml)	1 (24ml)	Ready to use
H_2SO_4 stop reagent	1 (11ml)	1 (11ml)	2 (11ml)	Ready to use

4. Materials required but not provided

- Microtitre plate reader fitted with appropriate filters (450nm required with optional 620nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The 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, the reagent is not contaminated by the first handling.

6. Specimen collection, processing & storage

Cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Serum: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.
Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Laboratory gloves should be worn at all times.
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels.
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and substrate solution, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly.
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance's within 1 hour after completion of the assay.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Follow incubation times described in the assay procedure.
- Dispense the TMB solution within 15 min of the washing of the microtitre plate.

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard, zero and control should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.

Example plate layout (example shown for a 6 point standard curve)

	Standards / Controls		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	800	800										
B	400	400										
C	200	200										
D	100	100										
E	50	50										
F	25	25										
G	zero	zero										
H	Ctrl	Ctrl										

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of Wash Buffer

Dilute the (200x) wash buffer concentrate 200 fold with distilled water to give a 1x working solution. Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2˚-25˚C.

8.3. Preparation of Standard Diluent Buffer

Add the contents of the vial (10x concentrate) to 225 ml of distilled water before use.

This Solution can be stored at 2-8°C for up to 1 week.

8.4. Preparation of Standard

Depending on the type of samples you are assaying, the kit may include two standard diluents. Because biological fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine you want to measure, you should reconstitute standard vials with the most appropriate Standard Diluent.

For **serum and plasma** samples: use Standard Diluent - Serum.
For **cell culture supernatants**: use Standard Diluent Buffer.

Standard vials must be reconstituted with the volume of standard diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 800pg/ml of TNFα. Mix the reconstituted standard gently by repeated aspiration /ejection. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 800 to 25 pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to well's A1 and A2, which provides the highest concentration standard at 800pg/ml.

- Add 100µl of appropriate standard diluent to the remaining standard wells B1 and B2 to F1 and F2.

- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells.

- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 800pg/ml to 25pg/ml.

- Discard 100µl from the final wells of the standard curve (F1 and F2).

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

8.5. Preparation of Controls

Freeze-dried control vials should also be reconstituted with the most appropriate Standard Diluent to your samples.

For serum and plasma samples: use Standard Diluent - Serum.
For cells culture supernatants: use Standard Diluent Buffer.

The supplied Controls must be reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution at the concentration stated on the vial. Do not store after use.

8.6. Preparation of Biotinylated anti- TNFα

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-TNFα with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of wells required	Biotinylated Antibody (μl)	Biotinylated Antibody Diluent (μl)
16	40	1060
24	60	1590
32	80	2120
48	120	3180
96	240	6360

8.7. Preparation of Streptavidin-HRP

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom.

Dilute the 5μl vial with 0.5ml of HRP diluent **immediately before use.** Do not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

Number of wells required	Streptavidin-HRP (μl)	Streptavidin-HRP Diluent (ml)
16	30	2
24	45	3
32	60	4
48	75	5
96	150	10

9. Method

We strongly recommend that every vial is mixed without foaming prior to use.

Prepare all reagents as shown in section 8.
Note: final preparation of Biotinylated Secondary Antibody (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

Assay Step		Details
1.	Addition	**Prepare Standard curve** as shown in section 8.4 above
2.	Addition	Add 100µl of each**, Sample, Standard, Control and zero (appropriate standard diluent)** in duplicate to appropriate number of wells
3.	Addition	Add 50µl of diluted **biotinylated anti-TNFα** to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **3 hours**
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min**
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
10.	Incubation	Incubate in the dark for **12-15 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
11.	Addition	Add 100µl of **H_2SO_4:Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).		

*****_Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range_

10. Data Analysis

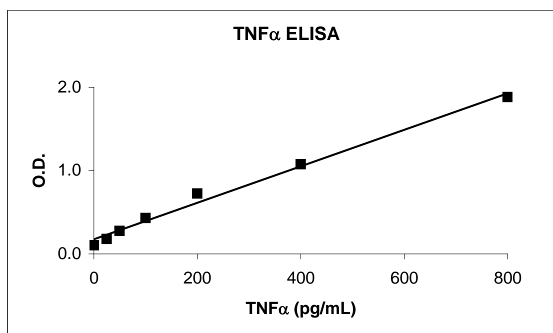
Calculate the average absorbance values for each set of duplicate standards, controls and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding TNFα standard concentration on the horizontal axis.

The amount of TNF-α in each sample is determined by extrapolating OD values against TNF-α standard concentrations using the standard curve**.**

Example TNF-α Standard curve

Standard	**TNFα Conc**	**OD (450nm) mean**	**CV (%)**
1	800	1.883	3.7
2	400	1.076	10.8
3	200	0.724	4.3
4	100	0.43	7.4
5	50	0.277	0.3
6	25	0.18	10.2
zero	0	0.102	4.2



Note: curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The sensitivity, minimum detectable dose of TNFα using this OriGeneTNFα ELISA kit was found to be less than **8pg/ml**. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 40 times.

12.2. Specificity

This assay recognises both natural and recombinant human TNFα. To asses the specificity of this OriGeneTNFα ELISA, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1β, IL-6, IL-12, IL-4, IL-2, IFNγ, IL-10, IL-8, and IL-13).

12.3. Precision

Intra-assay

Reproducibility within the assay was evaluated in three independent experiments by two different analysts. Each assay was carried out with 6 replicates (3 duplicates) of 3 spiked human pooled serum samples containing different concentrations of TNFα. Data below shows the mean TNFα concentration and the coefficient of variation for each sample. **The overall intra-assay coefficient of variation has been calculated to be 3.3%.**

Sessions	Samples	Assay 1 [TNFα] pg/ml	Assay 2 [TNFα] pg/ml	Assay 3 [TNFα] pg/ml	Mean	SD	CV(%)
1	1	837.0	870.0	781.0	829.3	45.0	5.43
	2	531.0	518.0	539.0	529.3	10.6	2.0
	3	199.0	203.0	201.0	201.0	2.0	1.0
2	1	817.0	814.0	790.0	807.0	14.8	1.8
	2	447.0	470.0	449.0	455.3	12.5	2.8
	3	168.0	165.0	182.0	171.7	9.1	5.3
3	1	827.0	845.0	827.0	833.0	10.4	1.2
	2	501.0	492.0	476.0	489.7	12.7	2.6
	3	170.0	175.0	173.0	172.7	2.5	1.5

Note: Example data shown above is the data generated by analyst A only.

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in four independent experiments by two analysts. Each assay was carried out with 6 replicates of 3 spiked serum human pooled serum samples containing different concentrations of TNFα. **The calculated overall coefficient of variation was 9.0%.**

Technician	Session	Sample 1 TNFα pg/ml	Sample 2 TNFα pg/ml	Sample 3 TNFα pg/ml
A	1	837	531	199
		870	518	203
		781	539	201
	2	817	446	168
		814	469	164
		790	449	182
	3	827	501	170
		845	492	175
		827	476	173
	4	843	429	149
		810	412	148
		869	392	164
B	1	848	443	157
		860	455	155
		778	492	158
	2	817	431	161
		820	466	158
		815	438	144
	3	772	393	156
		771	381	140
		711	388	134
	4	787	444	138
		781	420	142
		758	442	162
Mean		810	452	163
SD		38	44	19
CV		5	10	12

12.4. Dilution Parallelism

Four human pooled serum samples with different levels of TNFα were analysed at different serial two fold dilutions (1:2 To 1:8) with four replicates each. Recoveries ranged from 101 to 115% with an overall **mean recovery of 107%.**

12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 concentrations of TNFα in human serum in 2 separate experiments. Recoveries ranged from 74 to 90% with an overall **mean recovery of 81%.**

12.6. Stability

Storage Stability

Aliquots of spiked serum samples were stored at –20°C, 2-8°C, room temperature (RT) and at 37°C and the TNFα level determined after 24h. There was no significant loss of TNFα reactivity during storage at RT, and 2-8°C, however there is a significant loss of reactivity when stored at 37°C.

Freeze-thaw Stability

Aliquots of spiked serum were stored frozen at –20°C and thawed up to 5 times and the TNFα level was determined. There was no significant loss of TNFα after 5 cycles of freezing and thawing.

12.7. Expected serum values

A panel of 50 human sera was tested for TNFα. All were below the detection level of 8pg/ml.

12.8. Standard Calibration

This immunoassay is calibrated against the International Reference Standard NIBSC 87/650. NIBSC 87/650 is quantitated in International Units (IU). 1IU corresponding to 75pg OriGeneTNFα.

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15. Assay Summary

Total procedure length: 3h45mn

Add 100 µl of sample and diluted standard/controls and 50µl Biotinylated anti-TNFα

↓

Incubate 3 hours at room temperature

↓

Wash three times

↓

Add 100µl of Streptavidin-HRP

↓

Incubate 30min at room temperature

↓

Wash three times

↓

Add 100 µl of ready-to-use TMB
Protect from light. Let the color develop for 12-15 mn.

↓

Add 100µl H_2SO_4

↓

Read Absorbance at 450 nm

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16. International Summaries

16.1. French

PREPARATION DES REACTIFS : RESUME

1. Tampon de Lavage *(Washing Buffer)*	Ajouter 10 ml de **Tampon de Lavage concentré** (*Washing Buffer Concentrate*) 200 fois (200X) à 1990 ml d'eau distillée
2 Tampon de Dilution du Standard *(Standard Diluent Buffer)*	Ajouter 25 ml de **Tampon de Dilution du Standard concentré** (*Standard Diluent Buffer Concentrate)* 10 fois (10X) à 225 ml d'eau distillée
3. Standard TNFα *(TNFα Standard)*	Reconstituer le **Standard TNFα** (*TNFα Standard*) en ajoutant la quantité indiquée sur le tube de Standard de **Tampon de Dilution du Standard** (*Standard Diluent Buffer*) approprié.
4. Controls *(Controls)*	Reconstituer les **controls** (*controls*) en ajoutant la quantité indiquée sur le tube de contrôle de **Tampon de Dilution du Standard** (*Standard Diluent Buffer*) approprié.

5. Anti-TNFα Biotinylé (*Biotinylated TNFα)*

Nombre de barrettes	Anti-TNFα Biotinylé **Concentré** (µl) (*Biotinylated anti TNFα)*	**Diluent de l'Anticorps Biotinylé** µl) (*Biotinylated Antibody Diluent*)
2	40	1060
3	60	1590
4	80	2120
6	120	3180
12	240	6360

6. Streptavidin-HRP (*Streptavidin-HRP*)

Number of barrettes	**Streptavidin-HRP pré-diluée** (µl) (*Pre-diluted Streptavidin-HRP*)	**Diluent HRP** (ml) (*HRP-Diluent*)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

RESUME DU PROTOCOLE OPERATOIRE: durée totale : 3h45mn

1. Ajouter 100 µl de **Tampon de Dilution du Standard** (*Standard Diluent Buffer*) approprié, en duplicate, dans les puits Standards (B1 à F2).
2. Ajouter à la pipette 200 µl de **Standard TNFα** (*TNFα Standard*) reconstitué dans les puits A1 et A2 puis réaliser des dilutions du Standard allant de 800 à 25 pg/ml en transférant 100 µl d'un puit à l'autre. Jeter les 100 µl des derniers puits (F1 et F2).
3. Ajouter 100 µl de **Tampon de Dilution du Standard** approprié (*Standard Diluent Buffer ou Standard Diluent : serum*) en duplicate dans les puits "blancs".
4. Ajouter 100 µl d'**échantillon** (*Sample*), en duplicate, dans les puits désignés et 100 µl de **contrôle** *(control)*, en duplicate dans les puits contrôles.
5. Préparer **l'anticorps TNFα Biotinylé** (*Biotinylated anti TNFα).*
6. Ajouter 50 µl **d'anticorps anti TNFα Biotinylé dilué** (*diluted biotinylated anti TNFα)* dans tous les puits.
7. Couvrir les barrettes de puits et incuber pendant 3 heures à température ambiante (18-25°C).
8. Vider et laver les puits 3 fois avec le **Tampon de Lavage** (*Washing Buffer*).
9. Préparer la Streptavidin-HRP.
10. Ajouter 100 µl de **Streptavidin-HRP diluée** (*diluted HRP-Streptavidin*) dans tous les puits.
11. Couvrir les puits et incuber pendant 30 minutes à température ambiante (18°-25°C).
12. Vider et laver les puits 3 fois avec le **Tampon de Lavage** (*Washing Buffer*).
13. Ajouter 100 µl de **solution de TMB** (*TMB solution*) prête à l'emploi dans tous les puits y compris les "blancs".
14. Incuber les barrettes de puits pendant environ 12-15 minutes à température ambiante (18°-25°C) à l'obscurité.
15. Ajouter 100 µl d'**H_2SO_4: Solution Stop** (*H_2SO_4 : Stop Solution)* dans tous les puits y compris les "blancs".
16. Mesurer l'absorbance à la longueur d'onde 450 nm et optionnellement à 620 nm (entre 610 et 650 nm) comme longueur d'onde de référence.

Remarque: Les échantillons présentant une valeur de D.O. excédant la gamme de la courbe Standard peuvent résulter à des taux de TNFα incorrects. C'est pourquoi, il est recommandé de diluer de tels échantillons avec le Tampon de Dilution du Standard *(Standard Diluent Buffer)* **approprié afin de quantifier précisément le véritable taux de TNFα**

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16.2. Spanish

PREPARACIÓN DE LOS PRODUCTOS

1. Tampón de Lavado	Añadir **Tampón de Lavado Concentrado** 200 X (10 ml) a 1990 ml de agua destilada.
2 Tampón diluyente del estándar	Añadir **Tampón Diluyente del Estándar Concentrado** 10 X (25 ml) a 225 ml de agua destilada.
3. Estándar TNFα	Reconstituir el **Estándar TNFα** añadiendo el Diluyente del Estándar apropiado, como indica la etiqueta del vial.
4. Controles	Reconstituir los **controles** añadiendo el Diluyente del Estándar apropiado, como indica la etiqueta del vial.

5. Anti-TNFα biotinilado

Número de tiras	Anticuerpo biotinilado concentrado (µl)	Diluyente del anticuerpo biotinilado (µl)
2	40	1060
3	60	1590
4	80	2120
6	120	3180
12	240	6360

6. Estreptavidina-HRP

Número de tiras	Estreptavidina-HRP prediluida (µl)	Diluyente de HRP (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

RESUMEN DEL PROTOCOLO. El procedimiento total tiene una duración de 3h45min.

1. Añadir 100 µl del **Tampón Diluyente del Estándar** apropiado, por duplicado, a los pocillos designados para el estándar (B1 to F2).
2. Pipetear 200 µl del **Estándar TNFα** reconstituido en los pocillos A1 y A2 y hacer diluciones seriadas del estándar con el rango de concentraciones de 800 a 25 pg/ml, transfiriendo 100 µl de un pocillo al siguiente. Descartar 100 µl de los últimos pocillos.
3. Añadir 100 µl del **Tampón Diluyente del Estándar** apropiado, por duplicado, a los pocillos que van a ser el "blanco".
4. Añadir 100 µl de las muestras, por duplicado, a los pocillos designados para ello, y 100 µl del Control reconstituido, por duplicado, a los pocillos designados como "control".
5. Preparar el anticuerpo **Anti-TNFα Biotinilado**.
6. Añadir 50 µl del **anti-TNFα Biotinilado** y diluido, a todos los pocillos.
7. Cubrir la placa e incubar durante 3 horas a temperatura ambiente (18-25°C).
8. Vaciar y lavar la placa 3 veces con **Tampón de Lavado**.
9. Preparar la **Estreptavidina-HRP**.
10. Añadir 100 µl de **Estreptavidina-HRP** diluida a todos los pocillos.
11. Cubrir la placa e incubar 30 minutos a temperatura ambiente (18° to 25°C).
12. Vaciar y lavar la placa 3 veces con **Tampón de Lavado**.
13. Añadir 100 µl de solución **TMB preparado para utilizar**, a todos los pocillos, incluidos los pocillos con "blancos".
14. Incubar la placa durante 12-15 minutos a temperatura ambiente (18° to 25°C) y en oscuridad.
15. Añadir 100 µl de H_2SO_4: **Solución de Parada**, a todos los pocillos, incluidos los pocillos con los "blancos".
16. Medir la intensidad de color a 450 nm y a 620 nm como longitud de onda de referencia (de 610 nm a 650 nm sería aceptable).

Nota: **El cálculo de concentraciones de muestras con densidad óptica que supere el rango de la curva estándar, resultaría incorrecto, dando niveles de TNFα más bajos de lo real. Estas muestras, requerirían ser diluidas con el Tampón de Dilución de Estándar adecuado, para poder precisar la cantidad real de TNFα .**