

# PRODUCT INFORMATION & MANUAL

## Human TNF- $\alpha$ High Sensitivity ELISA

***BMS223HS***

Enzyme-linked Immunosorbent Assay for  
quantitative detection of human TNF- $\alpha$ .

For research use only.

Not for diagnostic or therapeutic procedures.



*Human TNF- $\alpha$   
High Sensitivity  
ELISA*

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## 1 Intended Use

The human TNF $\alpha$  ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human TNF $\alpha$ . **The human TNF $\alpha$  ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

## 2 Summary

TNF- $\alpha$  is a multifunctional cytokine involved in many different pathways, in homeostasis and pathophysiology of mammals. It can show opposing biological effects suggesting complex regulatory mechanisms.

TNF- $\alpha$ , also known as cachectin, was first detected as a cytotoxic factor inducing lysis of certain tumor cells. The TNF- $\alpha$  gene is member 2 of the TNF-superfamily (consisting of at least 20 distinct members).

TNF- $\alpha$  release is mainly triggered by viral infections and endotoxins, lipopolysaccharides or other bacterial components, by tissue injury, DNA-damage and by IL-1, PDGF and TNF- $\alpha$  itself. It is primarily expressed in macrophages, but also in monocytes, neutrophils, NK-cells, mast-cells, endothelial cells and activated lymphocytes. TNF- $\alpha$  expression in endothelial cells and fibroblasts can be induced by IL-17.

The expression of other cytokines, chemokines, reactive oxygen intermediates, nitric oxide and prostaglandins is stimulated by TNF- $\alpha$ .

The initially membrane bound TNF- $\alpha$  is enzymatically cleaved by TACE (= ADAM17). The soluble monomers aggregate to homotrimers and are secreted into blood and other biological fluids. The membrane bound and the soluble form are biologically active and bind to the TNF-receptors TNFR1 (= TNFRSF1A, p55-60) and TNFR2 (= TNFRSF1B, TNFR2, p75-80).

Upon ligand binding, receptors form trimers leading to conformational changes, protein dissociation (SODD = silencer of death domains, BAG4, Bcl2-associated athanogene 4) and association (TRADD = TNF-R1 associated death domain protein) and yielding the following biological activities:

- transcription of anti-apoptotic factors and proteins involved in cell proliferation and inflammation via binding of TRAF2 (TNF-R associated factor 2) and RIPK1 (TNF-R interacting serine-threonine kinase 1) and activation of the transcription factor NF- $\kappa$ B.
- cell proliferation, differentiation but also apoptosis via TRAF2 binding, kinases activation, activation of c-Jun and ATF2 (JNK-MAPK-pathway).

- apoptosis via the binding of FADD (Fas associating protein with death domain) to TRADD and activation of caspases (including caspase 8 = FLICE).

- necrosis, a caspase independent cell death, mediated by NADPH oxidases, which form a complex with TRADD and RIPK1, leading to the generation of oxygen species.

TNF-R2 contains no DD (death domain), but exhibits its function via direct TRAF binding.

Thus the multiple biological functions of TNF- $\alpha$  comprise cellular proliferation and differentiation, tumorigenesis, apoptotic or necrotic cell death (including certain tumor cell lines), immunoregulatory activities, lipid metabolism, coagulation and endothelial function. It promotes local or systemic inflammation (TNF- $\alpha$  is a potent pyrogen) and stimulates the acute phase response. Very high expressions of TNF- $\alpha$  after infection can lead to septic shock (TNF- $\alpha$  is highly cytotoxic.), whereas sustained low levels induce cachexia and inflammation.

Dysregulation of TNF- $\alpha$  is involved in many diseases:

Cancer: Diverse roles of TNF- $\alpha$  in cancer have been described, depending on the tumor type, tumor microenvironment and on overall TNF- $\alpha$  expression levels and expression kinetics. A model was proposed, in which single very high TNF- $\alpha$  levels lead to tumor regression and that chronic low-dose levels are associated with tumor progression.

Systemic lupus erythematosus (SLE): Murine models of SLE show contradictory effects of TNF- $\alpha$ : anti-autoimmune effect at low TNF- $\alpha$  levels (TNF- $\alpha$  administration leads to an attenuation of symptoms and TNF- $\alpha$  blockade to generation of SLE-symptoms), pro-inflammatory effect at high TNF- $\alpha$  levels (TNF- $\alpha$  blockade damped the disease).

Chronic intestinal inflammation (Crohn's disease (CD), Inflammatory bowel disease (IBD), Ulcerative Colitis (UC)): An essential effect of TNF- $\alpha$ /TNF-R1 activity on induction of chronic intestinal inflammation was shown. In IBD overexpression of TNF- $\alpha$  in monocytes/macrophages of gut and intestinal tissue has been described.

Psoriasis: In psoriatic patients TNF- $\alpha$  was shown to be increased systemically and in cutaneous tissue. TNF- $\alpha$  expression in peripheral blood mononuclear cells (PBMCs) was highly elevated in patients in active phase of the disease and elevated in chronic psoriasis. In an animal model it was shown that TNF- $\alpha$  dependent activation of resident T cells was essential for Psoriasis development.

Pulmonary disorders (Cystic Fibrosis (CF), Asthma): In cystic fibrosis high levels of TNF- $\alpha$  were observed. TNF- $\alpha$ , is over-expressed in persistent severe asthma. In allergic asthma, under low antigen exposure, TNF- $\alpha$  contributes to elevated histamine release. In a mouse model for asthma, airway inflammation was caused by TNF- $\alpha$  mediated activation of phospholipase A2.

Rheumatoid arthritis (RA), Ankylosing spondylitis (AS): TNF- $\alpha$  has stimulating effects on matrix degrading proteases (matrix metallo-proteinases, MMPs), tissue remodeling and osteoclasts, causing bone resorption leading to joint erosions. In a mouse model for RA, elevated levels of TNF- $\alpha$  were observed in bone marrow. Cytokines, synthesized by TNF- $\alpha$  activated synovial cells, were shown to induce RA. In AS patients, TNF- $\alpha$  concentration was shown to be elevated in the sacroiliac joint.

Transplantation (Graft-versus-host disease, Allograft rejection):

Measurement of TNF- $\alpha$  levels was shown to be useful in transplantation research. TNF- $\alpha$  was reported to be markedly elevated in renal allograft rejection. Evidence has been presented on increased TNF- $\alpha$  levels in Bone Marrow Transplants (BMT). BMT patients with major transplant related complications such as interstitial pneumonitis and severe acute graft-versus-host disease showed significantly increased TNF- $\alpha$  levels.

Atherosclerosis, arterial calcification: Increased risk of recurrent myocardial infarction, atherosclerotic thickening of carotid intima-media, disturbances in triglyceride and glucose homeostasis and age-related atherosclerosis have been connected to circulating TNF- $\alpha$  levels. TNF- $\alpha$  induced mechanisms lead to increased aortic calcium accumulation in animals with type 2 diabetes mellitus.

Insulin Resistance (IR) and Obesity: In adipose tissue of rodent models for obesity and in adipose individuals increased TNF- $\alpha$  expression was observed. In adipose tissue and in IR chronic low-grade inflammation was shown. Increased TNF- $\alpha$  levels affect insulin pathway regulation.

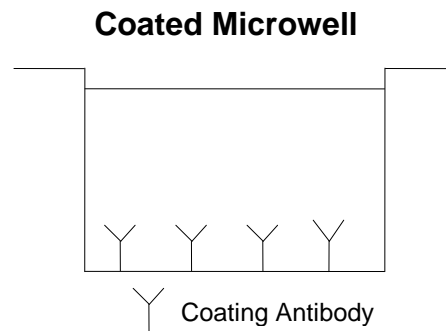
Neurodegenerative diseases (Multiple sclerosis (MS), Alzheimer's Disease, Prion Disease, Parkinson): TNF- $\alpha$  is produced from activated microglial cells and leads to neuronal degeneration, apoptosis of neuronal tissue and increased inflammation. TNF- $\alpha$  administration caused oligodendrocytes cell death - a symptom of MS – in coculture with astrocytes and microglia.

For literature update refer to [www.eBioscience.com](http://www.eBioscience.com)

### 3 Principles of the Test

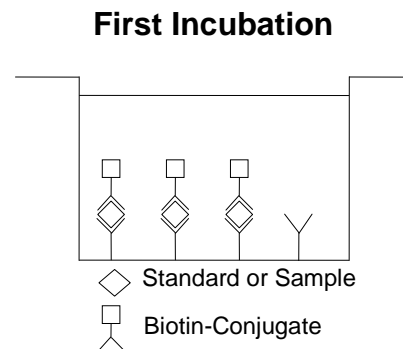
An anti-human TNF- $\alpha$  coating antibody is adsorbed onto microwells.

Figure 1



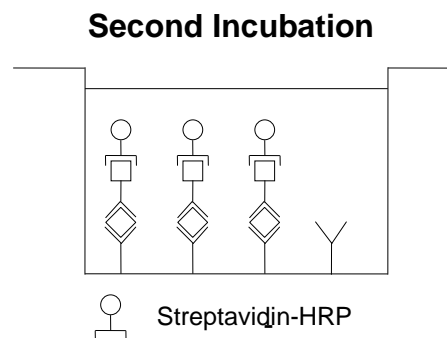
Human TNF- $\alpha$  present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human TNF- $\alpha$  antibody is added and binds to human TNF- $\alpha$  captured by the first antibody.

Figure 2



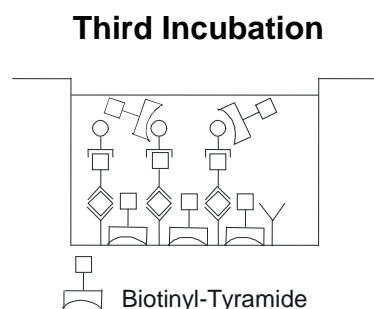
Following incubation unbound biotin-conjugated anti-human TNF- $\alpha$  antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human TNF- $\alpha$  antibody.

Figure 3



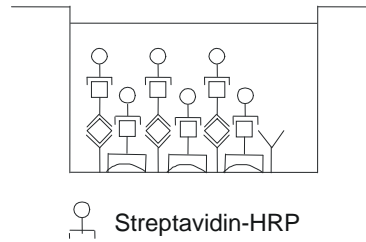
Following incubation unbound Streptavidin-HRP is removed during a wash step, and amplification reagent I (Biotinyl-Tyramide) is added to the wells.

Figure 4



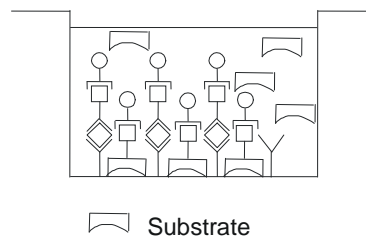
Following incubation unbound amplification reagent I is removed during a wash step and amplification reagent II (Streptavidin-HRP) is added.

Figure 5

**Fourth Incubation**

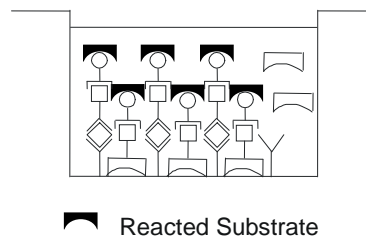
Following incubation unbound amplification reagent II is removed during a wash step and substrate solution reactive with HRP is added.

Figure 6

**Fifth Incubation**

A coloured product is formed in proportion to the amount of human TNF- $\alpha$  present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human TNF- $\alpha$  standard dilutions and human TNF- $\alpha$  sample concentration determined.

Figure 7



## 4 Principle of Amplification Reaction

The amplification reaction is based upon PerkinElmer Life Sciences' TSA (Tyramide Signal Amplification) technology (see 15, References 1 and 2).

Amplification reagent I contains biotinyl-tyramide. HRP converts multiple biotinyl-tyramide molecules into highly reactive derivatives (free radicals). These free radicals bind covalently to any protein in the well.

Thus, the amount of reacted biotinyl-tyramide is proportional to the amount of HRP in the well.

Following incubation unbound biotinyl-tyramide is removed during a wash step. Amplification reagent II contains Streptavidin-HRP, which binds to the biotin sites created during the biotinyl-tyramide reaction, thus multiplying the HRP molecules available at the surface for the substrate reaction.



## 5 Reagents Provided

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human TNF- $\alpha$
- 1 vial (100  $\mu$ l) **Biotin-Conjugate** anti-human TNF- $\alpha$  polyclonal antibody
- 1 vial (150  $\mu$ l) **Streptavidin-HRP**
- 2 vials human TNF- $\alpha$  **Standard** lyophilized, 1 ng/ml upon reconstitution
- 1 vial **Control high**, lyophilized
- 1 vial **Control low**, lyophilized
- 1 bottle (12 ml) **Sample Diluent**
- 1 vial (5 ml) **Assay Buffer Concentrate 20x**  
(PBS with 1% Tween 20 and 10% BSA)
- 1 vial (7 ml) **Amplification Diluent Concentrate (2x)**
- 1 vial (75  $\mu$ l) **Amplification Reagent I\***
- 1 vial (90  $\mu$ l) **Amplification Reagent II**
- 2 bottles (50 ml) **Wash Buffer Concentrate 20x**  
(PBS with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)

## 8 Adhesive Films

\* reagent contains ethyl alcohol

## **6 Storage Instructions – ELISA Kit**

Store kit reagents between 2° and 8°C except controls. Store lyophilized controls at -20°C.

Immediately after use remaining reagents should be returned to cold storage (2° to 8°C), controls to -20°C, respectively. Expiry of the kit and reagents is stated on labels.

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, this reagent is not contaminated by the first handling.

## **7 Specimen Collection and Storage Instructions**

Cell culture supernatant, serum and plasma (EDTA, heparin, citrate) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human TNF- $\alpha$ . If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 14.5).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

## **8 Materials Required But Not Provided**

- 5 ml and 10 ml graduated pipettes
- 5  $\mu$ l to 1000  $\mu$ l adjustable single channel micropipettes with disposable tips
- 50  $\mu$ l to 300  $\mu$ l adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate shaker
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

## 9 Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

## 10 Preparation of Reagents

**Buffer concentrates** should be brought to room temperature and should be diluted before starting the test procedure.

If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

### 10.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

### 10.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

### 10.3 Biotin-Conjugate

**Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.**

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with **Assay Buffer (1x)** in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

### 10.4 Streptavidin-HRP

**Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.**

Make a 1:400 dilution of the concentrated **Streptavidin-HRP** solution with **Assay Buffer (1x)** in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.015	5.985
1 - 12	0.030	11.970

## 10.5 Human TNF- $\alpha$ Standard

Reconstitute **human TNF- $\alpha$  standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 1000 pg/ml). Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded.

The concentrated **human TNF- $\alpha$  standard** must be diluted 1:25 with **Sample Diluent** just prior to use in a clean plastic test tube according to the following dilution scheme:

40  $\mu$ l concentrated **human TNF- $\alpha$  standard** + 960  $\mu$ l **Sample Diluent**. Shake gently to mix (concentration of standard = 40 pg/ml).

**Standard dilutions** can be prepared directly on the microwell plate (see 11.0) or alternatively in tubes (see 10.5.1).

### 10.5.1 External Standard Dilution

Label 7 tubes, one for each standard point.

S1, S2, S3, S4, S5 S6, S7

Then prepare 1:2 serial dilutions for the standard curve as follows:

Pipette 225  $\mu$ l of Sample Diluent into each tube.

Pipette 225  $\mu$ l of diluted standard (concentration of standard = 40 pg/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 20 pg/ml).

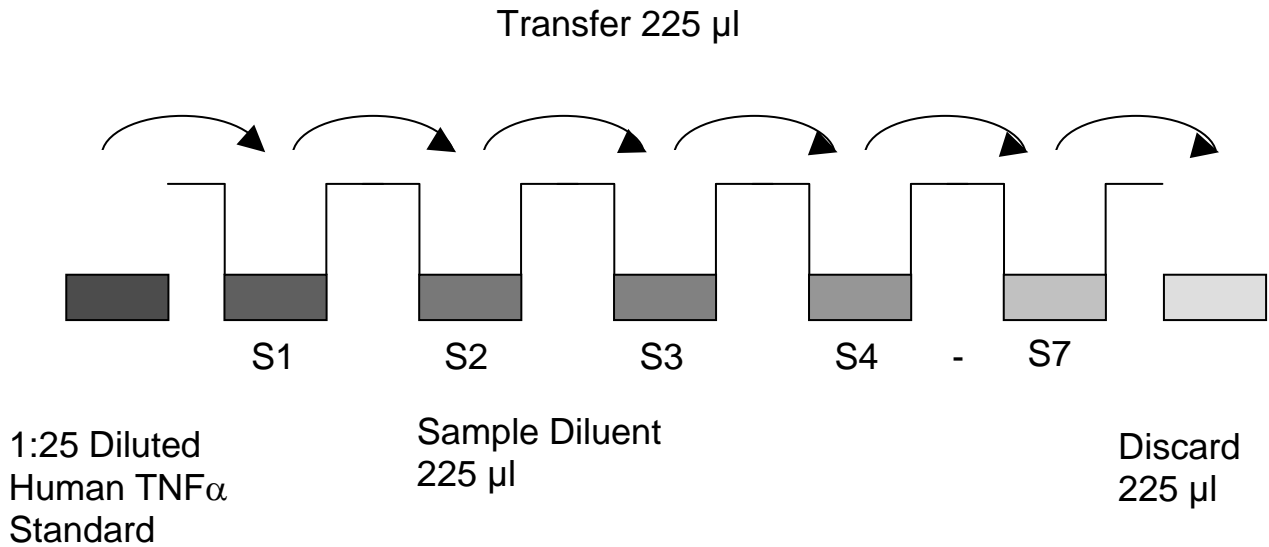
Pipette 225  $\mu$ l of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.

Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 8).

Sample Diluent serves as blank.



Figure 8



## 10.6 Controls

Reconstitute by adding 800  $\mu$ l distilled water to lyophilized **controls** (10-30 minutes). Swirl or mix gently to ensure complete and homogeneous solubilization. Predilute solubilized control 1:20 in **Sample Diluent**: 50  $\mu$ l control + 950  $\mu$ l **Sample Diluent**.

Further treat the controls like your samples in the assay. For control range please refer to certificate of analysis or vial label. Store reconstituted control aliquoted at  $-20^{\circ}\text{C}$ . Avoid repeated freeze and thaw cycles.

## 10.7 Amplification Diluent (1x)

Preparation of Amplification Diluent (1x) has to be done **immediately prior to use**. Make a 1:2 dilution of the concentrated **Amplification Diluent (2x)** as needed according to the following table:

Number of Strips	Amplification Diluent (2x) (ml)	Distilled Water (ml)
1 - 6	3	3
1 - 12	6	6

## 10.8 Amplification Solution I

Preparation of Amplification Solution I has to be done **immediately prior to application** on the plate.

Centrifuge vial for a few seconds in a microcentrifuge before opening to collect liquid trapped in the lid.

Make a 1:300 dilution of **Amplification Reagent I** in **Amplification Diluent (1x)** as needed according to the following table:

Number of Strips	Amplification Reagent I (ml)	Amplification Diluent (1x) (ml)
1 - 6	0.02	5.98
1 - 12	0.04	11.96

Discard immediately any prediluted Amplification Solution I after usage.

## 10.9 Amplification Solution II

Preparation of Amplification Solution II has to be done **immediately prior to application** on the plate.

Centrifuge vial for a few seconds in a microcentrifuge before opening to collect liquid trapped in the lid.

Make a 1:200 dilution of **Amplification Reagent II** in **Assay Buffer (1x)** as needed according to the following scheme:

Number of Strips	Amplification Reagent II (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

Discard immediately any prediluted Amplification Solution II after usage.

## 11 Test Protocol

**As this ELISA is a high sensitive system it is extremely important to stick exactly to the manual (washing procedure; chronology of / and preparation of solutions; incubation time) to obtain optimal test performance! Soaking is highly recommended between the washes to obtain a good test performance!**

**Please note: Amplification Solutions have to be prepared immediately prior to application on the plate! It is extremely important to wash the wells properly to obtain a good test performance!**

- a. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- b. Wash the microwell strips twice with exactly 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Soaking is highly recommended between the washes to obtain a good test performance! Take care not to scratch the surface of the microwells.  
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. **Do not allow wells to dry.**

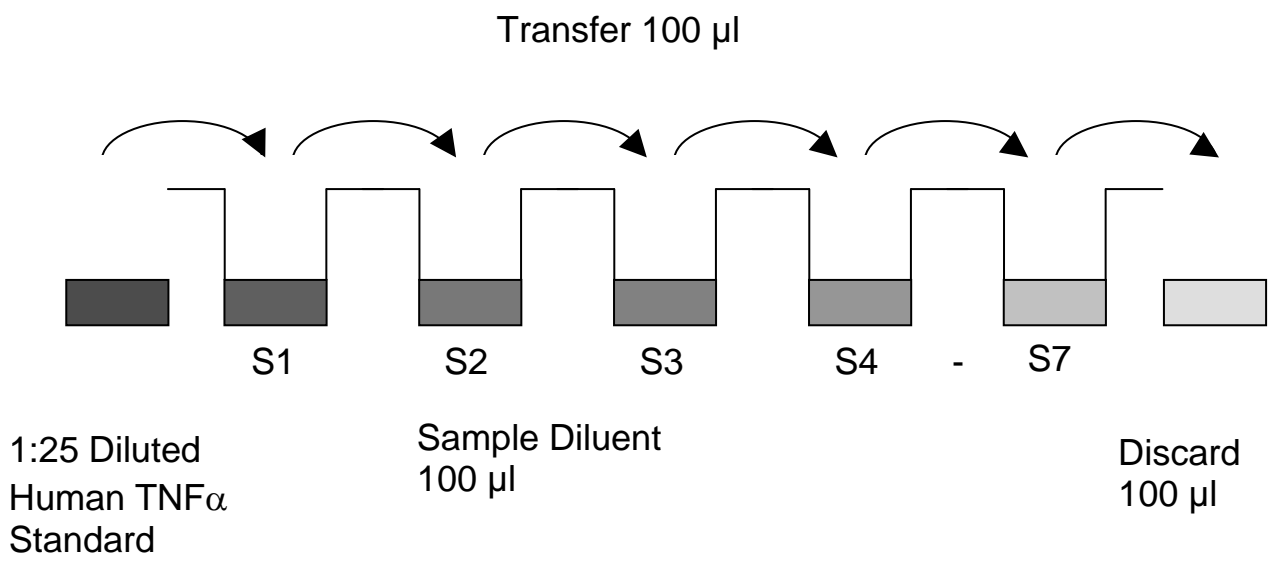
**Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 10.5.1.):

Add 100 µl of Sample Diluent in duplicate to all **standard wells**.

Pipette 100 µl of prepared **standard** (see Preparation of Standard 10.5, concentration = 40 pg/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 20 pg/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 9). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human TNF- $\alpha$  standard dilutions ranging from 20.00 to 0.31 pg/ml. Discard 100 µl of the

contents from the last microwells (G1, G2) used.

Figure 9



In case of an **external standard dilution** (see 10.5.1.), pipette 100  $\mu$ l of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Standard 1 (20.00 pg/ml)	Standard 1 (20.00 pg/ml)	Sample 1	Sample 1
<b>B</b>	Standard 2 (10.00 pg/ml)	Standard 2 (10.00 pg/ml)	Sample 2	Sample 2
<b>C</b>	Standard 3 (5.00 pg/ml)	Standard 3 (5.00 pg/ml)	Sample 3	Sample 3
<b>D</b>	Standard 4 (2.50 pg/ml)	Standard 4 (2.50 pg/ml)	Sample 4	Sample 4
<b>E</b>	Standard 5 (1.25 pg/ml)	Standard 5 (1.25 pg/ml)	Sample 5	Sample 5
<b>F</b>	Standard 6 (0.63 pg/ml)	Standard 6 (0.63 pg/ml)	Sample 6	Sample 6
<b>G</b>	Standard 7 (0.31 pg/ml)	Standard 7 (0.31 pg/ml)	Sample 7	Sample 7
<b>H</b>	Blank	Blank	Sample 8	Sample 8

- c. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
- d. Add 50 µl of **Sample Diluent** to the **sample wells**.
- e. Add 50 µl of each **sample** in duplicate to the **sample wells**.
- f. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 10.3).
- g. Add 50 µl of **Biotin-Conjugate** to all wells.
- h. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 2 hours on a microplate shaker set at 400 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**
- i. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 10.4).
- j. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.
- k. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- l. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker set at 400 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**
- m. Prepare **Amplification Solution I** diluted in **Amplification Diluent (1x)** (see Preparation of Amplification Solution I 10.8) **immediately prior to use**.
- n. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.
- o. Add 100 µl of **Amplification Solution I** to all wells, including the blank wells.

- p. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for **exactly** 15 minutes on a microplate shaker set at 400 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**
- q. Prepare **Amplification Solution II** diluted in **Assay Buffer** (see Preparation of Amplification Solution II 10.9) **immediately prior to use.**
- r. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.
- s. Add 100 µl of **Amplification Solution II** to all wells, including the blank wells.
- t. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for **exactly** 30 minutes on a microplate shaker set at 400 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**
- u. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.
- v. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- w. Incubate the microwell strips at room temperature (18° to 25°C) for about 10-20 min. Avoid direct exposure to intense light.

**The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.**

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

- x. Stop the enzyme reaction by quickly pipetting 100  $\mu$ l of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- y. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

**Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.**



## 12 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human TNF- $\alpha$  concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human TNF- $\alpha$  for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human TNF- $\alpha$  concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:2 (50  $\mu$ l sample + 50  $\mu$ l Sample Diluent) and controls 1:40 (50  $\mu$ l of 1:20 prediluted control + 50  $\mu$ l Sample Diluent). Thus concentrations read from the standard curve must be multiplied by the dilution factor (x 2 for samples, x 40 for controls).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human TNF- $\alpha$  levels. Such samples require further external predilution according to expected human TNF- $\alpha$  values with Sample Diluent in order to precisely quantitate the actual human TNF- $\alpha$  level.**
- It is suggested that each testing facility establishes a control sample of known human TNF- $\alpha$  concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 10. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 10

Representative standard curve for human TNF- $\alpha$  ELISA. Human TNF- $\alpha$  was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

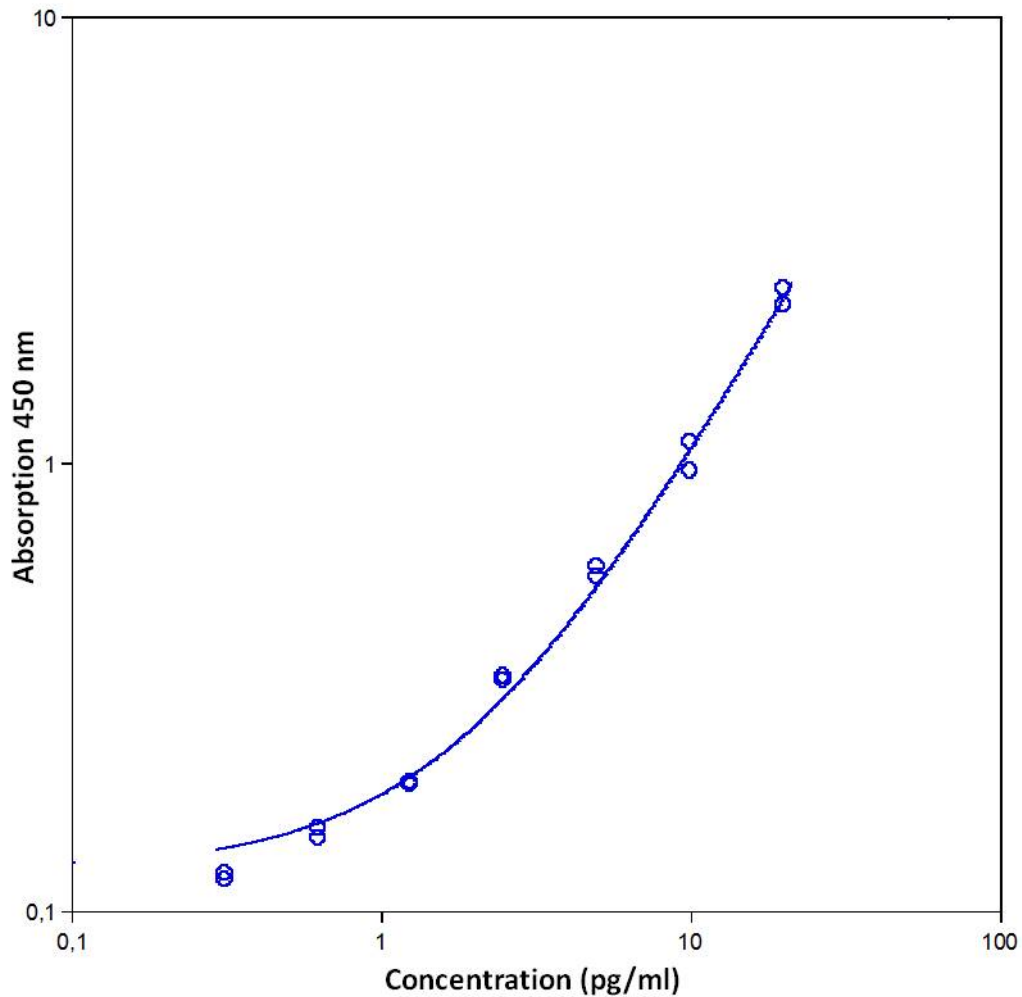


Table 2

Typical data using the human TNF- $\alpha$  ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human TNF- $\alpha$ Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	20.00	2.267 2.466	2.367	4.2
2	10.00	0.963 1.121	1.042	7.6
3	5.00	0.558 0.587	0.572	2.5
4	2.50	0.329 0.335	0.332	0.9
5	1.25	0.192 0.194	0.193	0.6
6	0.63	0.145 0.153	0.149	2.9
7	0.31	0.118 0.121	0.120	1.2
Blank	0.00	0.083 0.079	0.081	2.8

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

### 13 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

## **14 Performance Characteristics**

### **14.1 Sensitivity**

The limit of detection of human TNF- $\alpha$  defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.13 pg/ml (mean of 6 independent assays).

### **14.2 Reproducibility**

#### **14.2.1 Intra-assay**

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of human TNF- $\alpha$ . 2 standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was 8.5%.

#### **14.2.2 Inter-assay**

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of human TNF- $\alpha$ . 2 standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was 9.8%.

### **14.3 Spike Recovery**

The spike recovery was evaluated by spiking recombinant human TNF- $\alpha$  into human serum samples. Recoveries were determined in 3 independent experiments with 8 serum samples. Recoveries ranged from 84% to 97% with an overall mean recovery of 91%.

### **14.4 Dilution Parallelism**

4 serum samples with different levels of human TNF- $\alpha$  were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 81% to 101% with an overall recovery of 95%.

## **14.5 Sample Stability**

### **14.5.1 Freeze-Thaw Stability**

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human TNF- $\alpha$  levels determined. There was a slight loss of human TNF- $\alpha$  immunoreactivity detected by freezing and thawing, which therefore should be avoided.

### **14.5.2 Storage Stability**

Aliquots of serum and cell culture samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human TNF- $\alpha$  level determined after 24 h. There was no significant loss of human TNF- $\alpha$  immunoreactivity detected during storage at -20°C and 2-8°C.

A slight loss of human TNF- $\alpha$  immunoreactivity was detected during storage at RT and 37°C after 24 h, which therefore should be avoided.

## **14.6 Comparison of Serum and Plasma**

From several individuals, serum as well as EDTA, citrate and heparin plasma obtained at the same time, were evaluated. Human TNF- $\alpha$  concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

## **14.7 Specificity**

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human TNF- $\alpha$  positive serum.

There was no crossreactivity detected, notably not with TNF- $\beta$ .

## 14.8 Expected Values

A panel of 8 sera from apparently healthy blood donors (males and females) was tested for human TNF- $\alpha$ . The detected human TNF- $\alpha$  levels ranged between not detectable and 3.22 pg/ml with a mean level of 1.59 pg/ml. The normal levels measured may vary with the sample collective used.

## 14.9 Calibration

This immunoassay is calibrated with highly purified recombinant human TNF- $\alpha$ , which has been evaluated against the International Reference Standard NIBSC 87/650 and has been shown to be equivalent. NIBSC 87/650 is quantitated in International Units (IU), 1IU corresponding to 25 pg human TNF- $\alpha$ .

## 15 References

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## 17 Reagent Preparation Summary

### 17.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate 20x** (50 ml) to 950 ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

### 17.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate 20x** (5 ml) to 95 ml distilled water.

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

### 17.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in **Assay Buffer (1x)**:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

### 17.4 Streptavidin-HRP

Make a 1:400 dilution of **Streptavidin-HRP** in **Assay Buffer (1x)**:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.015	5.985
1 - 12	0.030	11.970

### 17.5 Human TNF- $\alpha$ Standard

Reconstitute lyophilized **human TNF- $\alpha$  standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

The concentrated **human TNF- $\alpha$  standard** must be diluted 1:25 with **Sample Diluent**.

## 17.6 Amplification Diluent (1x)

Preparation of **Amplification Diluent (1x)** has to be done **immediately prior to use**.

Number of Strips	Amplification Diluent (2x) (ml)	Distilled Water (ml)
1 - 6	3	3
1 - 12	6	6

## 17.7 Amplification Solution I

Centrifuge vial for a few seconds in a micro-centrifuge before opening to collect liquid trapped in the lid. Preparation of **Amplification Solution I** diluted in **Amplification Diluent (1x)** has to be done **immediately prior to application** on the plate.

Number of Strips	Amplification Reagent I (ml)	Amplification Diluent (1x) (ml)
1 - 6	0.02	5.98
1 - 12	0.04	11.96

## 17.8 Amplification Solution II

Centrifuge vial for a few seconds in a micro-centrifuge before opening to collect liquid trapped in the lid. Preparation of **Amplification Solution II** diluted in **Assay Buffer** has to be **done immediately prior to application** on the plate.

Number of Strips	Amplification Reagent II (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

## 17.9 Controls

Add 800 µl distilled water to lyophilized **controls**. Predilute reconstituted control 1:20 with **Sample Diluent**.

## 18 Test Protocol Summary

**Please note: Amplification Solutions have to be prepared immediately prior to application on the plate! It is extremely important to wash the wells properly to obtain a good test performance!**

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.  
Alternatively external standard dilution in tubes (see 10.5.1): Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Sample Diluent in duplicate, to the blank wells.
5. Add 50 µl Sample Diluent to sample wells.
6. Add 50 µl sample in duplicate, to designated sample wells.
7. Prepare Biotin-Conjugate.
8. Add 50 µl Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C). **(Shaking is absolutely necessary for an optimal test performance.)**
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 6 times with Wash Buffer.
12. Add 100 µl diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18°to 25°C). **(Shaking is absolutely necessary for an optimal test performance.)**
14. Prepare **Amplification Solution I** diluted in **Amplification Diluent (1x)** immediately prior to application on the plate.
15. Empty and wash microwell strips 6 times with Wash Buffer.
16. Add 100 µl Amplification Solution I to all wells.
17. Cover microwell strips and incubate for exactly 15 minutes at room temperature (18°to 25°C). **(Shaking is absolutely necessary for an optimal test performance.)**
18. Prepare **Amplification Solution II** diluted in **Assay buffer (1x)** immediately prior to application on the plate.
19. Empty and wash microwell strips 6 times with Wash Buffer
20. Add 100 µl Amplification Solution II to all wells.

21. Cover microwell strips and incubate for exactly 30 minutes at room temperature (18° to 25°C).
22. Empty and wash microwell strips 6 times with Wash Buffer.
23. Add 100 µl of TMB Substrate Solution to all wells.
24. Incubate the microwell strips for about 10-20 minutes at room temperature (18° to 25°C).
25. Add 100 µl Stop Solution to all wells.
26. Blank microwell reader and measure colour intensity at 450 nm.

**Note: If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent) and controls 1:40 (50 µl of 1:20 prediluted control + 50 µl Sample Diluent). Thus concentrations read from the standard curve must be multiplied by the dilution factor (x 2 for samples, x 40 for controls).**