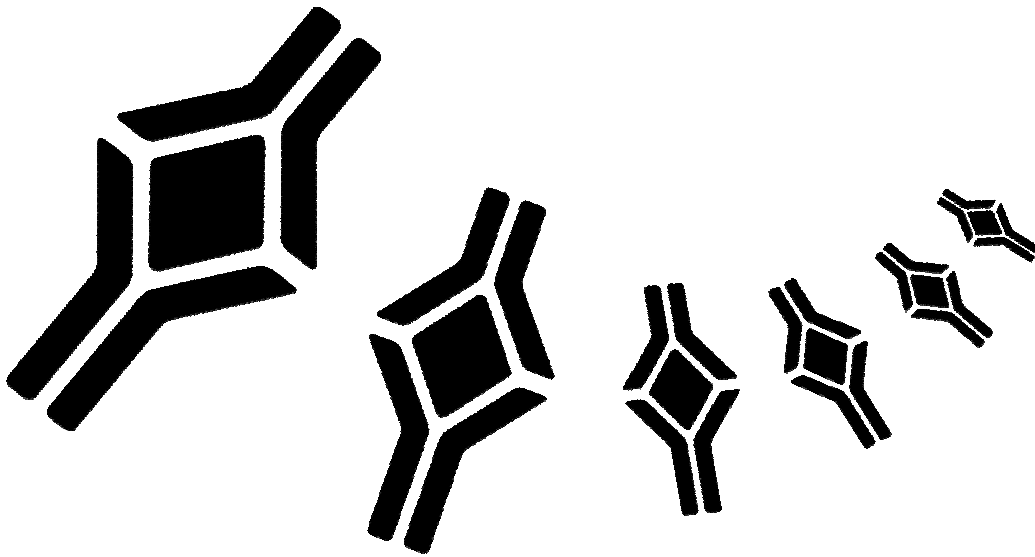


BioVendor

Research
and Diagnostic Products



HUMAN TGF- β 1 ELISA

Product Data Sheet

Cat. No.: RBMS249/2R

For Research Use Only

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**»» This kit is manufactured by:
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»» Use only the current version of Product Data Sheet enclosed with the kit!

1 INTENDED USE

The human TGF- β 1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human TGF- β 1. **The human TGF- β 1 ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

2 SUMMARY

Transforming growth factor- β (TGF- β) belongs to a family of dimeric 25 kDa polypeptides that are ubiquitously distributed in tissues and synthesized by many different cells (13). Three isoforms of transforming Growth Factor- β (TGF-beta1, beta-2 and beta-3) exist in mammals. They play critical roles in growth regulation and development. Each isoform is encoded by a unique gene on different chromosomes. All three of these growth factors are secreted by most cell types, generally in a latent form, requiring activation before they can exert biological activity. The TGF-betas possess three major activities: they inhibit proliferation of most cells, but can stimulate the growth of some mesenchymal cells; they exert immunosuppressive effects and they enhance the formation of extracellular matrix. Two types of membrane receptors possessing kinase activity are involved in signal transduction. The TGF-betas are involved in wound repair processes and in starting inflammatory reaction and then in the resolution through chemotactic attraction of inflammatory cells and fibroblast (15).

TGF- β 1 is the first recognized transforming growth factor (5), its subunits of each 12.5 kDa are bound via disulphide bridges. TGF- β 1 is inhibitive to T- and B cell proliferation as well as to maturation and activation of macrophages. It furthermore inhibits activity of natural killer cells and lymphokine activated killer cells and blocks production of cytokines.

Measurement of TGF- β 1 in blood has been advocated for diagnosis of various diseases. TGF- β 1 has been shown to be an organizer of responses to neurodegeneration (10).

In this context, it turned out to be interesting in monitoring Alzheimer's disease (18), Down's syndrome, AIDS and Parkinson's disease (11). Serum and cerebrospinal fluid levels of Multiple Sclerosis patients were shown to be of great value to monitor remission and acute phases (4, 21). TGF- β 1 is thought to play an important role in bone metabolism (22), it is considered a putative regulator of osteoclastic-osteoblastic interaction, thus it can be regarded as a marker for osteoporosis (14). TGF- β 1 is involved in the pathogenesis of glomerular diseases (3, 23) such as diabetic nephropathy and glomerulosclerosis (28). TGF- β 1 has been described to be functionally connected to major immune system abnormalities as in autoimmunity (SLE) (8). Serum levels have been shown to correlate with disease activity in autoimmune hepatitis (2). Elevated serum levels of TGF- β 1 are determined in Chronic fatigue syndrome patients (6) and in Guillain-Baire syndrome patients (24). An inverse correlation with disease activity was described for TGF- β 1 levels in Kawasaki disease (17) and patients with IgA deficiency (19).

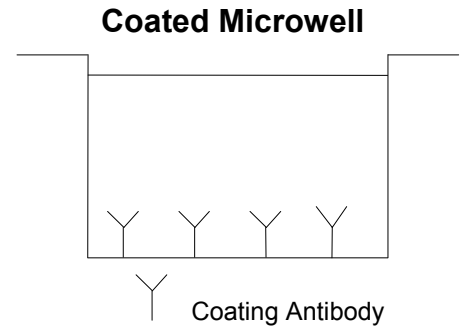
TGF- β 1 has been confirmed to promote fibrotic processes, thus it is implicated in the myelofibrosis with myeloid metaplasia (16). Increased serum levels of TGF- β 1 in patients affected by thrombotic thrombocytopenic purpura implicate its function on bone marrow haematopoiesis (29, 25). Determination of circulating TGF- β 1 turned out to reflect the various stages in solid tumors as has been shown for cervical cancer (7), elevations were furthermore found in prostatic cancer (27), bladder cancer (9), and liver cancer (20).

Decreased levels of TGF- β 1 in the serum of sepsis and acute stroke patients (1, 12) may reflect the changing immunological-inflammatory status of these patients. Decreased TGF- β 1 serum levels were described for patients with acute *Plasmodium falciparum* malaria (26).

3 PRINCIPLES OF THE TEST

An anti-human TGF- β 1 coating antibody is adsorbed onto microwells.

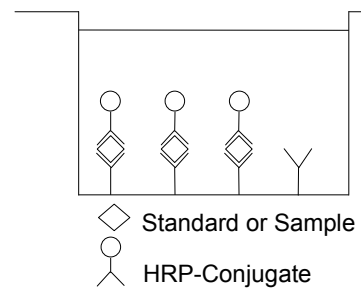
Figure 1



Human TGF- β 1 present in the sample or standard binds to antibodies adsorbed to the microwells. A HRP-conjugated anti-human TGF- β 1 antibody is added and binds to human TGF- β 1 captured by the first antibody.

Figure 2

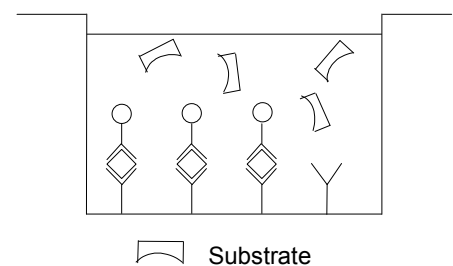
First Incubation



Following incubation unbound HRP-conjugated anti-human TGF- β 1 is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

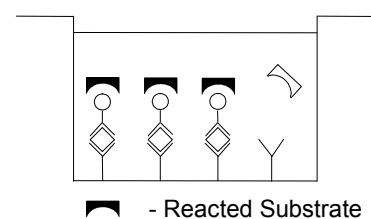
Figure 3

Second Incubation



A coloured product is formed in proportion to the amount of human TGF- β 1 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 TGF- β 1 standard dilutions and human TGF- β 1 concentration determined.

Figure 4



4 REAGENTS PROVIDED

- 1 aluminium pouch with a **Antibody Coated Microtiter Strips** with monoclonal antibody to human TGF- β 1
- 1 vial (100 μ l) **HRP-Conjugate** anti-human TGF- β 1 monoclonal antibody
- 2 vials human TGF- β 1 **Standard** concentrate, 60 ng/ml upon dilution
- 1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)
- 1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)
- 1 vial (3 ml) **1N HCl** (pretreatment of samples)
- 1 vial (3 ml) **1N NaOH** (pretreatment of samples)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)
- 1 vial (0.4 ml) **Blue-Dye**
- 1 vial (0.4 ml) **Green-Dye**
- 2 **Adhesive Films**

5 STORAGE INSTRUCTIONS – ELISA KIT

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). 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.

6 SPECIMEN COLLECTION AND STORAGE INSTRUCTIONS

Cell culture supernatant, serum and plasma (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.

Pay attention to a possible “**Hook Effect**” due to high sample concentrations (see chapter 11).

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 TGF-β1. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.

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

7 MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 5 μ l to 1000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ 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)
- 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

8 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 in vitro diagnostic use and are not for use in 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.

9 PREPARATION OF REAGENTS

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

9.1 Wash Buffer

If crystals have formed in the **Wash Buffer Concentrate**, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the Wash Buffer Concentrate 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. The pH of the final solution should adjust to 7.4.

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

Wash Buffer may also be prepared as needed according to the following table:

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

9.2 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 (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 HRP-Conjugate

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

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

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

9.4 Human TGF- β 1 Standard

Dilute **human TGF- β 1 standard** concentrate by addition of Assay Buffer (1x) as stated on the label and mix gently (concentration of diluted standard = 60 ng/ml).

Standard dilutions can be prepared directly on the microwell plate (see 10.d) or alternatively in tubes (see 9.4.1).

9.4.1 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 μ l of Assay Buffer (1x) into each tube.

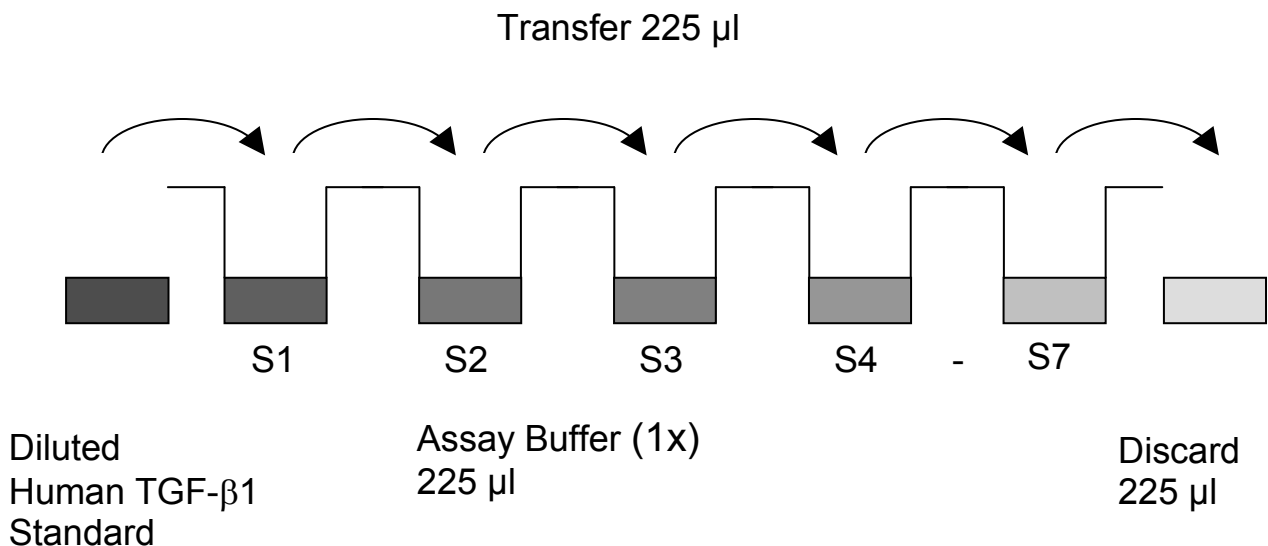
Pipette 225 μ l of diluted standard (concentration = 60 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 30 ng/ml).

Pipette 225 μ 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 5).

Assay Buffer (1x) serves as blank.

Figure 5



9.5 Addition of Colour-giving Reagents: Blue-Dye, Green-Dye

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (**Blue-Dye**, **Green-Dye**) can be added to the reagents according to the following guidelines:

1. Diluent:

Before standard and sample dilution add the **Blue-Dye** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of **Blue-Dye**, proceed according to the instruction booklet.

5 ml Assay Buffer (1x)	20 µl Blue-Dye
12 ml Assay Buffer (1x)	48 µl Blue-Dye
50 ml Assay Buffer (1x)	200 µl Blue-Dye

2. HRP-Conjugate:

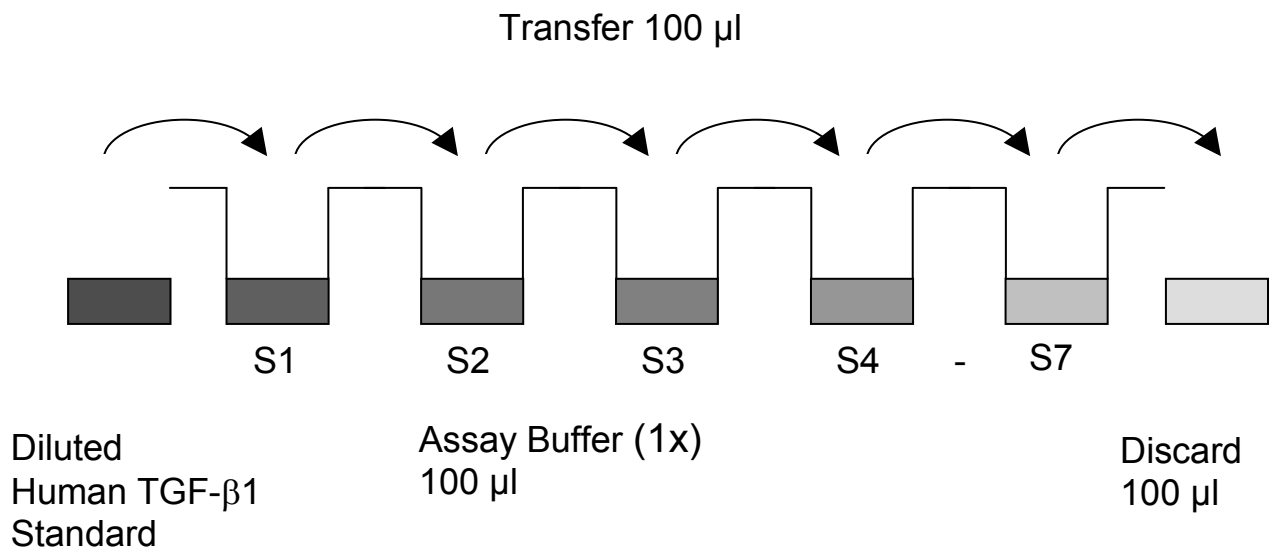
Before dilution of the concentrated HRP-Conjugate add the **Green-Dye** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of **Green-Dye** according to the instruction booklet: Preparation of HRP-Conjugate.

3 ml Assay Buffer (1x)	30 µl Green-Dye
6 ml Assay Buffer (1x)	60 µl Green-Dye

10 TEST PROTOCOL

- a. Prepare your samples before starting with the test procedure. Dilute serum, plasma and cell culture supernatant samples 1:10 with Assay Buffer (1x) according to the following scheme:
20 μ l sample + 180 μ l Assay Buffer (1x)
Add 20 μ l 1N HCl to 200 μ l of prediluted sample, mix and incubate for 1 hour at room temperature. Neutralize by addition of 20 μ l 1N NaOH.
- b. 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.
- c. Wash the microwell strips twice with approximately 400 μ l **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. 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. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**
- d. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.4.1): Add 100 μ l of Assay Buffer (1x) in duplicate to all **standard wells**. Pipette 100 μ l of diluted **standard** (see Preparation of Standard 9.4, concentration = 60 ng/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 = 30 ng/ml), and transfer 100 μ l to wells B1 and B2, respectively (see
- e. Figure 6). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human TGF- β 1 standard dilutions ranging from 30.00 to 0.47 ng/ml. Discard 100 μ l of the contents from the last microwells (G1, G2) used

Figure 6



In case of an **external standard dilution** (see 9.4.1), pipette 100 µl 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:

	1	2	3	4
A	Standard 1 (30.00 ng/ml)	Standard 1 (30.00 ng/ml)	Sample 1	Sample 1
B	Standard 2 (15.00 ng/ml)	Standard 2 (15.00 ng/ml)	Sample 2	Sample 2
C	Standard 3 (7.50 ng/ml)	Standard 3 (7.50 ng/ml)	Sample 3	Sample 3
D	Standard 4 (3.75 ng/ml)	Standard 4 (3.75 ng/ml)	Sample 4	Sample 4
E	Standard 5 (1.88 ng/ml)	Standard 5 (1.88 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.94 ng/ml)	Standard 6 (0.94 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.47 ng/ml)	Standard 7 (0.47 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- f. Add 100 µl of **Assay Buffer (1x)** in duplicate to the **blank wells**.
- g. Add 100 µl of each pretreated **sample** in duplicate to the **sample wells**.
- h. Prepare **HRP-Conjugate** (see Preparation of HRP-Conjugate 9.3).
- i. Add 50 µl of **HRP-Conjugate** to all wells.
- j. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 4 hours on a microplate shaker set at 100 rpm.
- k. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- l. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- m. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 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 0.6 – 0.65.

- n. Stop the enzyme reaction by quickly pipetting 100 µl 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.
- o. 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.

11 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 TGF- β 1 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 TGF- β 1 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 TGF- β 1 concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:12 (20 μ l sample + 180 μ l Assay Buffer (1x) + 20 μ l HCl + 20 μ l NaOH), the concentration read from the standard curve must be multiplied by the dilution factor (x 12).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human TGF- β 1 levels (Hook Effect). Such samples require further external predilution according to expected human TGF- β 1 values with Assay Buffer (1x) in order to precisely quantitate the actual human TGF- β 1 level.**
- It is suggested that each testing facility establishes a control sample of known human TGF- β 1 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 7. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 7

Representative standard curve for human TGF- β 1 ELISA. Human TGF- β 1 was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

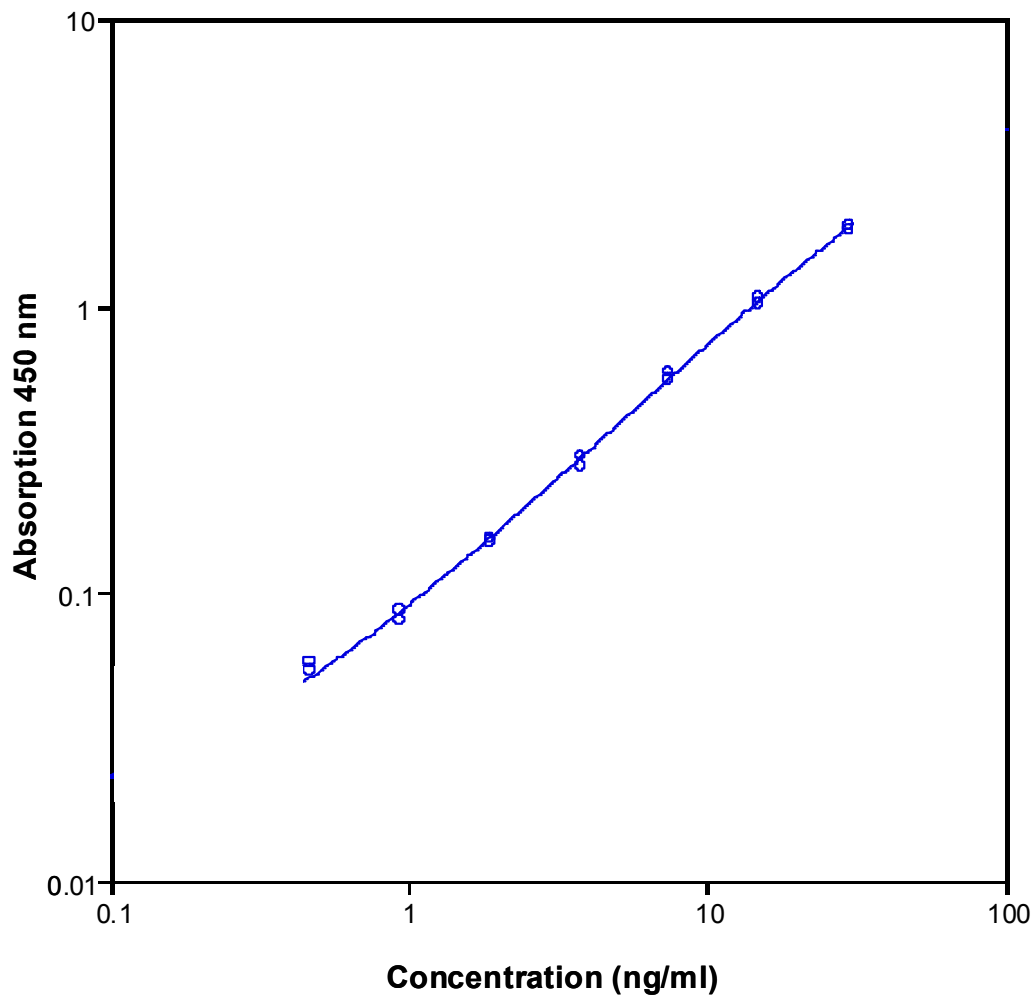


Table 2

Typical data using the human TGF- β 1 ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human TGF- β 1 Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	30.00	1.838	1.867	1.6
	30.00	1.896		
2	15.00	1.010	1.037	2.6
	15.00	1.064		
3	7.50	0.550	0.5665	2.9
	7.50	0.583		
4	3.75	0.275	0.287	4.2
	3.75	0.299		
5	1.88	0.150	0.152	1.3
	1.88	0.154		
6	0.94	0.080	0.0835	4.2
	0.94	0.087		
7	0.47	0.054	0.0555	2.7
	0.47	0.057		
Blank	0	0.007	0.008	
	0	0.009		

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.

12 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.

13 PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

The limit of detection of human TGF- β 1 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 0.02 ng/ml (mean of 3 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human TGF- β 1. 2 standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was 6.7%.

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human TGF- β 1. 2 standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was 8.5%.

13.3 Spiking Recovery

The spiking recovery was evaluated by spiking different levels of recombinant human TGF- β 1 into various samples. Recoveries were determined in 3 independent experiments with 4 replicates each. The unspiked sample was used as blank in these experiments. The overall mean recovery was 102%.

13.4 Dilution Linearity

4 samples with different levels of human TGF- β 1 were analysed at serial 2 fold dilutions with 4 replicates each.
The overall mean recovery was 110%.

13.5 Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human TGF- β 1 positive serum.
There was no crossreactivity detected, notably not with TGF- β 2 and TGF- β 3.

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15 REAGENT PREPARATION SUMMARY

15.1 Wash Buffer

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

15.2 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

15.3 HRP-Conjugate

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

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

15.4 Human TGF- β 1 Standard

Dilute **human TGF- β 1 standard** concentrate by addition of Assay Buffer (1x) as stated on the label and swirl or mix gently.

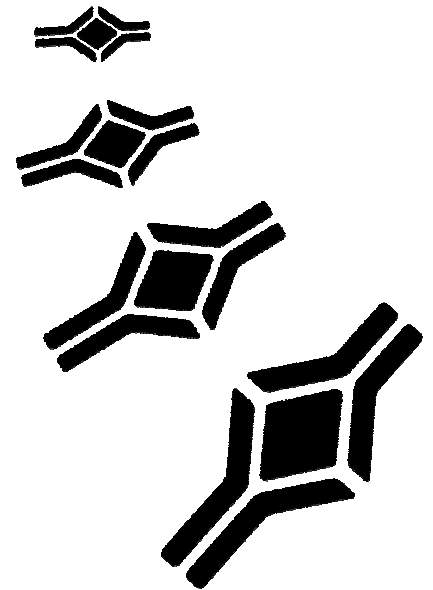
16 TEST PROTOCOL SUMMARY

1. Pretreat samples.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µl Assay Buffer (1x), 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 9.4.1): Pipette 100 µl of these standard dilutions in the microwell strips.
5. Add 100 µl Assay Buffer (1x), in duplicate, to the blank wells.
6. Add 100 µl pretreated sample in duplicate, to designated sample wells.
7. Prepare HRP-Conjugate.
8. Add 50 µl HRP-Conjugate to all wells.
9. Cover microwell strips and incubate 4 hours at room temperature (18° to 25°C) on a rotator set at 100 rpm.
10. Empty and wash microwell strips 3 times with Wash Buffer.
11. Add 100 µl of TMB Substrate Solution to all wells.
12. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
13. Add 100 µl Stop Solution to all wells.
14. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:12 (20 µl sample + 180 µl Assay Buffer (1x) + 20 µl HCl + 20 µl NaOH), the concentration read from the standard curve must be multiplied by the dilution factor (x 12).

NOTES





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